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Spatial memory and changes in expression of genes of neurotrophic factors in adult rat brain after fractionated whole brain irradiation

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ABSTRACT

Background: Ionizing radiation causes cognitive impairment in adult brain. However, the effects of various irradiation protocols with fractionated fixed total dose on hippocampal function have not yet been studied. ***Materials and Methods***: Fractionated whole brain irradiation with a total dose of 36 Gy was performed according to the following protocols: 2Gy-18 fractions (2Gy*18), 4Gy-9 fraction (4Gy*9) and 6Gy-6 fractions (6Gy*6). Changes in spatial memory were studied in Morris water maze tests at 12th and 17th day after irradiation with a hidden platform and at 38th day after irradiation without a platform. Levels of expression of brain derived neurotrophic factor (BDNF) and vascular endothelial growth factor (VEGF) genes were evaluated using qPCR. ***Results***: Expression of genes of neurotrophic factors (BDNF and VEGF) was decreased at 4th and 8th week after irradiation, the decrease depended on fractionation. The Morris water maze test with a hidden platform showed improvement in long-term spatial memory at 12th and 17th day after irradiation. In the Morris water maze test without platform cognitive deficit was detected only in 6Gy*6 group at 4th week after irradiation. ***Conclusion***: Our study shows that different fractionation protocols affect hippocampal functions differently, and that the greatest negative impact has the protocol with the maximum single dose. In addition, decrease in expression of genes of neurotrophic factors might play an important role in cognitive impairment.

Keywords: *Fractionated radiation, cognitive dysfunction, spatial memory, VEGF, BDNF.*

INTRODUCTION

Ionizing radiation can significantly affect integrative function of central nervous system in adults [1], and of course, on hippocampus-dependent learning and memory [2]. It relates to radiation-induced apoptosis of neural immature progenitor cells in the subventricular zone of the lateral ventricles and in the subgranular zone of the hippocampal dentate gyrus [3] and associated with inhibition of hippocampal neurogenesis [2].

Experiments indicate that even relatively small radiation doses are associated with complex changes in gene expression profile [4]. Multiple effects of ionizing radiation on the

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central nervous system, cognition and behavior depend on the resulting total brain radiation dose and the mode of exposure [5]. It was identified that cognitive functions changes strongly depend on the time elapsed after irradiation [6].

The link between cognitive impairment and changes in gene expression profile may be mediated by changes in neurotransmission, vascular and glial systems, impaired neurogenesis in the adult hippocampus, neuroinflammatory reactions in apoptotic cell death, etc. The most pronounced changes were observed in expression of genes responsible for protector and reparative functions as well as in genes involved in neurotransmission mechanisms [7]. However, the relationship between changes in expression profile of individual genes, irradiation time and dose has been poor investigated. This is especially true for late periods after irradiation. At the same time, such information is of great theoretical interest and practical importance.

Brain derived neurotrophic factor (BDNF) – is a member of the neurotrophin family of growth factors. BDNF stimulates the growth and differentiation of new neurons and synapses and supports the survival of existing neurons. BDNF is involved in neural plasticity and neurogenesis in the hippocampus and this function is important for learning and memory [8-11]. Changes in BDNF gene expression, its release and neuromodulator activity that triggered by epigenetic and post-translational mechanisms are observed in many pathologies and their experimental models on laboratory animals [12]. Decreased BDNF expression in the hippocampus is related to cognitive deficits [13]. It was shown that cranial irradiation induces depression-like behavior in mice, possibly via decreased hippocampal neurogenesis [14].

The first identified role of vascular endothelial growth factor (VEGF) is the regulation of vascular permeability system. Moreover, VEGF is a potent stimulator of endothelial cell proliferation and the formation of blood vessels [15]. VEGF is a critical player in neurodegeneration [16] and it may have therapeutic utility in promoting regeneration of neurons after injury or other brain pathologies [17]. Furthermore, VEGF is a multifunctional growth factor, as well a neurotrophic agent that regulates axonal growth and neuronal maturation during development and has an influence on learning and memory in mature brain [18].

In addition, VEGF activates neurogenesis by stimulating endothelial cells to release neorotrophic factors, such as BDNF, which improves neuronal survival and integration in the subventricular zone of the dentate gyrus of the hippocampus. Intracerebral administration of VEGF stimulated neurogenesis in the subventricular and subgranular zone of the dentate gyrus of hippocampus [19].

Therefore, the aim of our study was to identify the changes in spatial memory tested by water maze test, which used to evaluate the changes in hippocampus-dependent learning and memory in rodents [20], as well as the changes in genes expression of BDNF and VEGF in adult rat brain on the 4th and 8th week after different protocols of fractionated whole brain irradiation with fixed total dose of ionizing radiation of 36 Gy.

MATERIALA AND METHODS

Animals

Experiments were performed on 64 male non-pedigree albino rats, provided by Research Center of Biomedical Technologies, Russian Academy Medical Sciences (RAMS), where they are maintained as inbred line. Experimental animals were divided into 4 groups. Before experiments, the rats were housed for at least 2 weeks under laboratory vivarium conditions with 8 animals in a cage on a 12/12-h light/dark cycle with food and water freely available. At the beginning of experiment, the rats were 2 months of age and weighed 200±20 grams. Housing conditions and all experimental procedures were in accordance with international rules of treatment of animals (European Council Directive 86/609/EEC of 24 November 1986).

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Protocol of irradiation

The following fractionation protocols were used for irradiation of rats (5 fractions per week):

18 fractions of 2 Gy each (2Gy*18), Biological effective dose (BED) using 3 as the alpha/beta ratio = 36Gy

9 fractions of 4 Gy each (4Gy*9), BED =50,4Гр;

6 fractions of 6 Gy each (6Gy*6), BED =64,8Гр.

Animals from the control group were restrained 18 times. Fractionated whole brain irradiation was performed on a linear accelerator («PRIMUS», Siemens) in the Burdenko Neurosurgery Institute. The dose rate was 2 Gy/min. The distance between the source of radiation and the treatment surface was 100 cm. Four rats were irradiated simultaneously. They were restrained inside a foam plastic mold and faced the center of the field. Rats were set against the wall of the mold so that the boundary of radiation field determined by a half- dose distribution was behind the brain with a margin of 5 mm ensuring the whole brain irradiation in all rats. Irradiation was carried out without anesthesia. Rats were placed in DecapiCones (Braintree Scientific's).

Morris Water Maze

Morris maze test was described previously [21], comprising a single training session composed of 8 exercises separated by 1 minute breaks.

The training for the first session of Morris water maze test (with platform) was performed on the 21th day after irradiation (3 weeks). Tests were carried on the 2th and 7th days after training (16 rats for each group).

The second session was performed on the 7th week. On the 49th day after irradiation rats (8 from each group) received one "reminder" session in which the rat was placed in a pool with 4 different points by the above described training protocol. After 48 hours the platform was removed and long-term memory was evaluated by measuring in a single 1 min session the time that the rat spent in the sector where the platform used to be.

During the whole experiment, the location of surrounding visual cues was the same.

Quantative real-time PCR

Rats were humanely killed for tissue collection on 4th (8 rats for each group) and 8th week after irradiation (8 rats for each group).

At the end of behavioral tests, on the 28th day (8 animals from each group) and on the 56th day after the end of irradiation (8 animals from each group) the animals were anesthetized deeply (5% solution of ketamine, 200 mg/kg, intraperitoneally). After decapitation, the brain was removed and part of hippocampus (30 mkg) was isolated on ice, quickly placed in RNAlater solution (Qiagen) and stored at +4 °C for several days, until used for extraction of total RNA. Isolation of total RNA was performed using TRI REAGENT (MRC) according to protocol provided by the manufacturer. After isolation of total RNA, remaining samples were frozen and stored at -70 °C. Concentration of total RNA in the obtained samples was measured using Nanovue Plus (GE Healthcare). Reverse transcription was performed using a set by "EUROGEN" on 2720 thermal cycler (Applied Biosystems). Total RNA (1000 ng) was treated with deoxyribonuclease I (Fermentas) and used for reverse transcription. Real-time PCR was performed on Step One Plus thermal cycler (Applied Biosystems) using SYBR Green probes. GAPDH gene was selected as reference gene for the analysis. The following genes were analyzed: VEGF, GFAP. Before setting real-time PCR, cDNA was diluted 5-fold to a concentration of 200 ng/µl. For each of the obtained cDNA libraries, PCR efficiency was measured and was shown to be in 96 – 99% range. Then, expression of the genes of interest was measured.

Real-time PCR was performed using a ready-to-use PCR mix (qPCRmix-HS ROX) in the following conditions: initial denaturation step (95 °C, 4 min) followed by 40 cycles of denaturation at 95 °C for 20 seconds, annealing at 54 °C for 90 seconds. Reactions were performed in 10 µl volume using 1 µl of analyzed cDNA. Each experimental sample was measured in triplicate. The experiment was performed in

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duplicate. Threshold cycle (Ct) values correspond to a relative amount of target RNA.

Primer sequences were obtained using Beacon Designer 7 software (Premier Biosoft International). Nucleotide sequences of primers used: BDNFrat –forward AGCCTCCTCTGCTCTTTCTG; BDNFrat –reverse CGCCGAACCCTCATAGACAT; GAPDH –forward AAGTTCAACGGCACAGTTCAA; GAPDH –reverse CTCCTGGAAGATGGTGATGG. VEGFrat –forward AAGACCGATTAACCATGTCA; VEGFrat –reverse ATGTCAGGCTTTCTGGATTA. GAPDH gene serves as the reference. The relative expression level of target genes was calculated using the following formula $2^{-\Delta\Delta Ct} \pm SD$ [22].

Statistical analysis

Gene expression data was analyzed using the Duncan`s test of multiple comparisons. To analyze the trend Jounkheere`s trend test was used. Morris water maze data (with a hidden platform) were analyzed using non-parametric methods of Mann-Whitney and Kruskal-Wallis tests and Dunnett's test to compare each group with the control because of non-parametric distribution. The data are presented as median±quartile. Morris maze data (without platform) were analyzed using ANOVA (F-test) because of the Gauss distribution in obtained data. The data are presented as mean±SD. Differences were considered reliable at $p < 0.05$.

RESULTS

Whole brain irradiation with various protocols led to changes in expression of VEGF and BDNF mRNA in hippocampus.

Changes in expression of BDNF mRNA on the 4th week after irradiation did not reach statistical significance, and there was no difference between the experimental and control groups (Kruskal-Wallis test: H=6.38 p =0.09). On the 8th week after irradiation expression of BDNF mRNA was decreased in all experimental groups ($p<0.01$ each of the groups compared to control, Dunnett's test). A statistically significant difference of gene expression was revealed between 2Gy*18 and 4Gy*9 groups ($p=0.01$, Duncan`s test), 4Gy*9 and 6Gy*6 groups ($p<0.05$, Duncan's test). The Jounkheere`s trend test showed the statistically significant increase the level of expression of BDNF mRNA in the order 2Gy*18, 4Gy*9, 6Gy*6 on the 8th week after irradiation ($p<0.05$) figure 1A.

In the rat hippocampus we observed the statistically significant decrease of expression of VEGF mRNA. The lowest level was in 6Gy*6 group and it increased in the row 6Gy*6 – 4Gy*9 – 2Gy*18, that was shown by the Jounkheere`s trend test ($p<0.05$). A statistically significant difference was revealed between each group 2Gy*18, 4Gy*9, 6Gy*6 compared to control

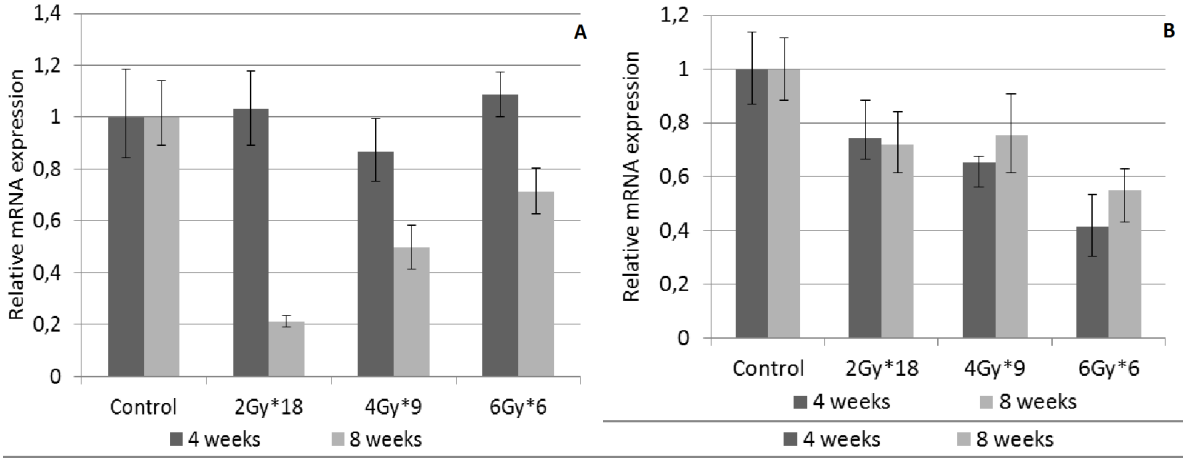

Figure 1. A. Relative expression of BDNF mRNA in hippocampus after irradiation with different protocols. $2^{-\Delta\Delta Ct} \pm SD$ * $p<0.05$ compared to 2Gy*18 and 6Gy*6 (8 weeks). ** $p<0.05$ each experimental group compared to control (8 weeks).
B. Relative expression of VEGF mRNA in hippocampus after irradiation with different protocols. $2^{-\Delta\Delta Ct} \pm SD$. **$p<0.05$ each experimental group compared to control for all dates. # $p<0.05$ compared to 6Gy*6 (4 weeks). *$p<0.05$ compared to 2Gy*18 and 4Gy*9 (8 weeks).

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(p<0.05, p<0.01 and p<0.001 correspondently, Dunnett's test), and between 2Gy*18 and 6Gy*6 groups (p<0.05 Duncan's test).

Changes in expression of VEGF mRNA on the 8th week after irradiation reached statistical significance for 2Gy*18, 4Gy*9 and 6Gy*6 groups compared to control (p<0.05, <0.05 and p<0.001 correspondently, Dunnett's test). A statistically significant difference was revealed between 6Gy*6 and each group 2Gy*18, 4Gy*9 (p<0.05 for each group, Duncan's test) The figure 2B.

The long-term spatial memory was estimated after training in the Morris water maze tests.

The first session of Morris water maze test (with platform) was begun on the 3th week after irradiation. On the 2th day after training irradiated rats found the hidden underwater platform significantly faster (p<0.05 for each of the experimental groups compared to the control, Dunnett's test). However, there was no difference between experimental groups (Kruskal-Wallis test: H=2.036 p =0.36). With repetition of the test at the 7th day after the training, the test showed the same results (p = 0.02 for each of the experimental groups compared to the control, Dunnett's test), the differences between the experimental groups were not found (Kruskal-Wallis test: H=1.73 p =0.42). On 2th and 7th day after training the time in finding of the platform did not reach statistical significance (Mann-Whitney test for 2Gy*18 group p = 0.18 for 4Gy*9 group p = 0.19, for 6Gy*6 group p = 0.85; control group p = 0.31) (See figure 2A).

On the 29th day after the first training the "reminder" session was performed. 48 hours after the "reminder" session (52th day after irradiation) Morris water maze test without platform showed the statistically significant reducing the residence time in the target quadrant only for 6Gy*6 group compared to the control (p<0.05, Fisher's exact test) and we also observed differences between 4Gy*9 and 6Gy*6 groups (p<0.05) (figure 2B).

The short-term spatial memory that measured during training sessions in all groups was not different. The probability value for the first training, composed of 8 exercises, are: 0.32, 0.22, 0.31, 0.07, 0.98, 0.17, 0.48, 0.11; for the "reminder" session: 0.24, 0.3, 0.06, 0.45 (Kruskal -Wallis test).

DISCUSSION

The study showed that various irradiation protocols with fractionated fixed total dose of 36 Gy excite a decrease in BDNF and VEGF gene expression.

In our study, decreased expression of BDNF mRNA was observed on the 8th week after irradiation, and the level of mRNA was increased in direct proportion to BED (in the row 2Gy*18 – 4Gy*9 – 6Gy*6). Reduced BDNF after gamma irradiation is consistent with the

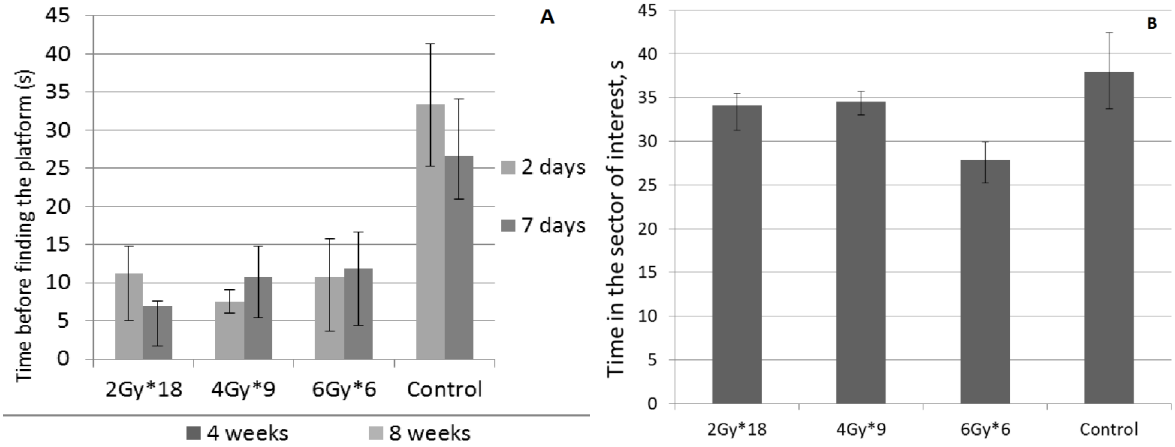

Figure 2. A. Morris water maze test with a platform. Median ± quartile . * p<0.05 each experimental group compared to control. **B.** Morris water maze test without a platform. Mean±SE * p<0.05 compared to control and 4Gy*9.

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data of other authors [14, 23].

Expression of VEGF mRNA on the 4th week after irradiation did not differ from the level of expression on the 8th week. On the 4th week we observed a correlation between the degree of gene suppression and BED (the level was decreased in the row 2Gy*18–4Gy*9– 6Gy*6).

It should be noted that Lee *et.al.* [24] also observed the significant decrease of this gene expression after one single dose of 10 Gy. In our study, level of VEGF gene expression decreased in all groups on the 4th and 8th week after irradiation. The lowest level of VEGF mRNA was observed in 6Gy*6 group. It is interesting that reproduction of spatial skill only in this group was broken on the 51th day after irradiation, i.e. when we observed also a decrease in expression of BDNF mRNA with the lowest level of VEGF mRNA.

Analyzing the effect of different fractionated protocols on spatial memory, it should be noted that we used a model of a single intensive training, beginning at the 21th day after irradiation with testing at 2, 7, 28 (relearning) and 30 days after the formation of skill (respectively 23, 28, 49 and 51 days after irradiation). We obtained paradoxical findings on the keeping of memory trace in irradiated rats at 2th and 7th day after the formation of spatial skill that are contrary to available literature data on the development of cognitive deficits after irradiation in human [25] and animals [26]. Nevertheless, there was similar of such paradoxical radiation influence. So, Saxe *et al.* [27] used X-ray irradiation to suppress neurogenesis in mice. After this, they observed improvement in hippocampal-dependent behavior compared to non-hippocampal task. Obviously, the mechanism of this effect requires further study. Based on data from literature it should be noted that the formation and preservation of memory trace in a model with massive training is a much less depend on the formation of new neurons in adult hippocampus than in classical model of spaced learning [28]. Furthermore, it was shown that the formation of spatial skills leads to reduction of neurons survival 11-15 day old cells) [29]. These cells can compete with younger cells and inhibit the formation of memory trace [30]. Thus, elimination of 11-15 days old neurons after irradiation may cause transient stimulant effect on spatial learning. However, the improvement of skill reproduction, which was observed on 2th and 7th day, was no longer maintained at a later date – at the 30th day after training. Moreover, animals treated with high single dose (6Gy) show a decrease in the stability of the skill in the test without a platform compared to the control. Although some authors described the violation of hippocampal functions, that depend on the dose and the age of animals [31,32], but changes in neurogenesis after different protocols of fractionated radiation with fixed total dose have not yet been described. Thus, the question needs further study.

Along with mechanisms of neurogenesis/ apoptosis at the basis of observed improving cognitive functions by ionizing radiation may lie also other mechanisms, such as stress, which promotes spatial memory [33], compensatory mechanisms, etc. It is interesting, that BDNF and VEGF play an important role as components of cascade of changes in the pathomechanism of stress-induced affective diseases. Stress-induced suppression of these factors evokes in animals behaviors characteristic of depression, which are reversed by antidepressant drugs or exercise and environmental enrichment that increase expression of these factors [34]. Also it was shown, that cranial irradiation induces depression-like behavior in mice [14].

Also it can be noted, that cognitive dysfunction, particularly changes in hippocampus-dependent learning and memory, is a common and serious complication after radiotherapy in humans. The search of the best strategy for irradiation of brain tumors by a selecting optimal fractionation that can minimize side effects of ionizing radiation.

CONCLUSION

It can be concluded about the relationship between undoubted effects of radiation exposure on hippocampus functions and the protocol of fractionation. Particularly, the

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protocol with the maximum BED has the greatest negative impact on the gene expression of neurotrophic factors in the hippocampus and cognitive processes associated with the activity of this structure. Nevertheless, expression of BDNF mRNA after fractionated WBI was not decreased in direct proportion to the BED. Also we observed paradoxical improvement in hippocampal-dependent behaviour after fractionated whole brain irradiation and it was not depended from BED.

Further investigations with testing on various dates after irradiation, as well as introducing to the study other growth and neurotrophic factors, and a wider range of behavior tests will make more definitive conclusions about the mechanisms of effects of different fractionated protocols on the integrative activity of the central nervous system.

Conflicts of interest: *none to declare.*

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