Original Research Paper

Radiation-Induced Cognitive Dysfunction After Different Schemes of Fractionated Whole Brain Irradiation

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Abstract: Radiation-induced cognitive dysfunction is a serious complication of radiation therapy. In humans, numerous cases of radiation damage have been recorded after irradiation for therapeutic purposes. The degree of cognitive dysfunctions and its dependence on biological effective doses should be tested in animal preclinical models to minimize radiation side effects. Fractionated whole brain irradiation was performed according to the following schemes: 18 Fractions of 2 Gy each (36Gy/18), 9 fractions of 4 Gy each (36Gy/9), 6 fractions of 6 Gy each (36Gy/6), with the biological effective dose 36Gy, 50.4Gy, 64.8Gy correspondingly. Cognitive impairment was detected by a Novel object recognition test in 36Gy/18 and 36Gy/9 groups. Recognition memory impairment was increased in inverse proportion to the biological effective dose. In the 36Gy/6 group, increased anxiety and decreased research activity in Spontaneous alternation test in Y-shaped maze were observed. Reactions of astroglia and vascular reactions were evaluated using qPCR of Glial Fibrillar Acid Protein (GFAP) and Vascular Endothelial Growth Factor (VEGF) genes and immunohistochemical analyses of these proteins. Despite of various biological effective doses (and its subsequent increasing in chosen 3 fractionation schemes) a dose-depended effect in the expression of VEGF in the prefrontal cortex and GFAP in hippocampus and in cognitive impairment was not observed. Cognitive impairment after fractionated whole brain irradiation was clearly seen in our research. The described pathologic changes in healthy brain play an important role in development of radiation-induced cognitive dysfunction. Therefore, applicability of radiobiological models for evaluation of effects of fractionated whole brain irradiation on cognitive dysfunction put in question in our study. So we can suggest that it needs essential more researches about the choice an optimal fractionated scheme to minimize side effects of ionizing radiation.

Keywords: Fractionated Radiation, Cognitive Dysfunction, Spontaneous Alteration, Novel Object Recognition, Gene Expression

Introduction

Radiation-induced cognitive dysfunction is a common and serious complication after radiotherapy of brain tumors, however its mechanism is not clearly understood (Kangas *et al.*, 2012). Normal tissues are often irradiated during radiation therapy over the course of brain tumor treatment. Each year, over 200,000 patients with primary or metastatic tumors are subjected to Whole-Brain Irradiation (WBI). About 50% of long-

term survivors suffer cognitive deficiencies due to damage of healthy brain tissue (Johannesen *et al.*, 2003). Moreover, patients with certain locations of tumor, for example nasopharyngeal carcinoma, have to receive radiation treatment of neck and face regions, thus the procedure inevitably involves irradiating the inferior temporal lobes (Zhou *et al.*, 2011).

Radiation-induced cognitive dysfunction in certain series of fractionated WBI occurs in up to 50-90% of adult patients with brain tumors who survive more than 6 months after the treatment (Greene-Schloesser and Robbins, 2012). Decreased attention and impaired verbal and spatial memory are typical cognitive impairments, the frequency and degree of the impairments increasing over time. 1.9-5.1% of long-term survivors after WBI and rarely after focal radiation therapy, suffer from dementia, progressive memory loss, ataxia and enuresis. All of these consequences can be observed in the absence of radiographic or clinical signs of demyelination or white matter necrosis.

The degree of cognitive dysfunction should be considered as an important prognostic index and neuropsychological tests should be used during regular examinations (Shen *et al.*, 2012). Moreover, given that new chemotherapeutical agents emerge and surgery techniques of brain tumor treatment improve, the number of long-living patients will be constantly growing. Thus, study of such aftereffects of irradiation is becoming increasingly important.

The Linear-Quadratic (LQ) radiobiological model can be used to predict radiation effects after fractionated radiation. It is a mechanistic model that describes cell killing, both for tumor and normal tissue complications. The dose range where the LQ model is well supported by data is roughly 1-5 Gy per fraction. LQ model appeared 15 years ago and now it has a wide clinical use (Vetlova *et al.*, 2012). However, an importance of clinical and experimental radiation research is needed because of a growth number of new developments and technology advances (Joiner and Kogel, 2009).

To facilitate a more rapid assessment of toxicity to normal tissues in human, new approaches should be tested in preclinical animal models (Dilworth *et al.*, 2014). Moreover, studies of the cognitive and structural brain disorders in healthy rats, that occur only rarely due to the action of ionizing radiation and is not associated with brain tumor progression.

Studies performed on rats suggest the existence of cognitive dysfunctions that arise due to radiation treatment. In rats after one time WBI there were found attention deficit disorder (Hienz *et al.*, 2008), motor dysfunction (Manda *et al.*, 2007) and spatial memory loss in the Morris maze (Akiyama *et al.*, 2001; Liu *et al.*, 2010). After fractionated irradiation of the whole brain, significant impairments of spatial memory, working memory and progressive deterioration of special

education in mice are also shown (Warrington *et al.*, 2012). Brown *et al.* (2007) showed a significant increase in working memory errors after fractionated gamma irradiation of the head after 6 and 9 months after exposure.

Cognitive dysfunctions are supposed to result from dynamic interaction between several types of brain cells such as astrocytes, endothelial cells, microglia, neurons and oligodendrocytes (Tofilon and Fike, 2000).

It has been shown that early and persistent radiation-induced micro vascular injury leads to cognitive deficits (Brown *et al.*, 2007). Increased vascular permeability and cognitive dysfunction associated with this pathology are also shown after WBI (Warrington *et al.*, 2011; 2012; Lee *et al.*, 2012). Vascular Endothelial Growth Factor (VEGF) plays an important role in angiogenesis in the developing central nervous system, but its expression is significantly suppressed in adults. Radiation-induced brain damage is primarily a consequence of vascular endothelium damage (Roth *et al.*, 2012). Therefore VEGF may play a significant role in the post-radiation damage (Kim *et al.*, 2004). A number of studies (Kim *et al.*, 2004; Wei *et al.*, 2012) show an increase in VEGF expression in response to different variants of gamma irradiation (from 10 to 72 Gy). However, Lee *et al.* (2011) showed a sharp decrease in the VEGF expression (up to 10 times) shortly after WBI (within 24 h).

Mechanism such as reactive astrogliosis are also involved in memory alteration (Zhou *et al.*, 2011; Akiyama *et al.*, 2001). Reactive astrogliosisis is typically characterized by astrocyte proliferation and hypertrophy (MacFabe *et al.*, 2008). Decreased or increased density of glial cells has been shown in many fundamental works on animal models that were aimed on research of irradiation consequences. Moreover, the expression of Glial Fibrillar Acid Protein (GFAP) changes irregularly in various brain structures; increased expression in one group and decreased in another (Nagayama *et al.*, 2001; Yuan *et al.*, 2006; Leshchinskaya *et al.*, 2000; Ma *et al.*, 2001; Balentova *et al.*, 2012). This indicates that various brain structures have different reactions to radiation injury. Starting on the 3rd day after a single dose irradiation (100 Gy), proliferation, hyperplasia and hypertrophy of astrocytes in the corpus callosum were observed; the peak of these changes was between 14 and 30 days (Yang *et al.*, 2000).

The search of the best strategy for irradiation of brain tumors, aiming to reduce such aftereffects as cognitive deficits, will greatly improve the quality of life for patients. To date, there has been no study in which cognitive impairment in rats and its biochemical component are examined in relation to different fractionation schemes. The aim of our work was to study radiation-induced cognitive dysfunction and its structure substrate in healthy rats under three different fractionation schemes with various Biological Effective Doses (BED).

Methods and Materials

Animals

Experiments were performed on 96 male non-pedigree albino rats (24 animals for each group), provided by Research Center of Biomedical Technologies, Russian Academy Medical Sciences (RAMS), where they are maintained as an inbred line. The experimental animals were divided into 4 groups. Prior to the experiments, the rats were housed for at least 2 weeks in 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 4 months of age and weighed 250±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).

Protocol of Irradiation

The different treatment schemes using a/b of 3 Gy (BED 3) were used. The prescribed dose was:

- 36Gy in 18 fractions of 2 Gy at the midline, five fractions per week; BED 3 = 36Gy; (36Gy/18)
- 36Gy in 9 fractions of 4 Gy at the midline, five fractions per week, BED 3 = 50.4 Gy; (36Gy/9)
- 36Gy in 6 fractions of 6 Gy at the midline, five fractions per week, BED 3 = 64.8 Gy. (36Gy/6)

Irradiation was carried out without anesthesia. The rats were placed in DecapiCones (BraintreeScientific's). Animals from the control group were subjected to the same stress, but were not irradiated (animals were restrained 18 times). Fractionated WBI was performed on a linear accelerator («PRIMUS», Siemens). 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 (Fig. 1), 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. All calculations were performed using the dosimeters protocols from the International Atomic Energy Agency and checked after the ionization chamber.

On the 4th, 8th and 12th week after completion of the irradiation, rats were sacrificed for tissue collection (8 animals from each group for every date).

Spontaneous alternation test was carried out in a Y-shaped maze with 30*18*9 cm arms. After introduction of the animal to the center of the maze, the number and order of arm entries were registered for 10 min. Index of spontaneous alternation was calculated using the formula:

$$(Nt/Ns)*100\%$$

where, Nt is the number of triads, Ns-the total number of arm entries.

Novel Object Recognition Test (NORT) was performed under indoor lighting in a chamber of grey plastic (45*45*40 cm). The animal's behavior was recorded with a digital camera and analyzed using Any-maze computer program (Stoelting Co.). The test consisted of three 5 min trials separated by 24 h intervals. During the 1st trial, there were no objects in the chamber and locomotive activity of rats was registered. The 2nd trial was with two identical objects. In the 3rd trial, one of the objects was replaced with a new object of different shape. Recognition index was calculated according to the formula:

$$(Tn\text{-}Tf / Tn + Tf) * 100\%$$

where, Tn is the time spent on the study of the "new" object and Tf is the time spent on the study of "familiar" object during the third trial.
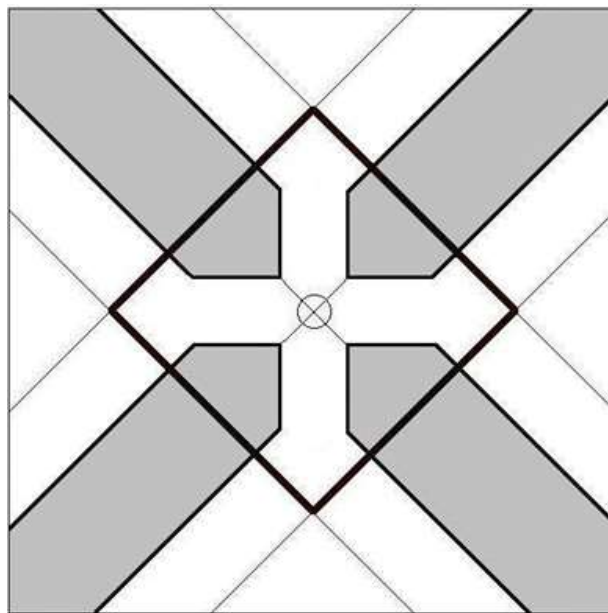

Fig. 1. Plan of plastic mold for rats' irradiation (animals were placed in grey areas). Heads of animals were located within bold black square

Passive avoidance test was performed in a chamber produced by "Neurobotics" (Moscow, Russia). The chamber was 40*30*36 cm in size and was divided into two equal compartments by a partition with a hole 8×8 cm. The floor of the chamber was covered with metal bars. One of the compartments was brightly lit, the other was dim lit. During the training trial, a rat was placed into the bright compartment and the time of entry into the dim compartment was registered. Once all four paws of the rat were in the dark compartment, the animal was subjected to a mild electrical shock through the floor bars (3 mA, 3 sec), after which the test was terminated and the rat was returned to the cage. The testing trial was performed 48 h and 7 days after the training. During the testing trial, the rat was placed into the bright compartment and the time of entry into the dim compartment was registered. Training and testing trials were limited to 5 min.

Elevated Cross-Maze (ECM) was a cross-shaped maze with two closed (29 cm walls) and two open (0.5 cm rims) arms 52 cm long and 14 cm wide elevated 100 cm off the floor. The junction of the arms formed an open area 14×14 cm. The open arms were lit by a lamp (80 watts). Rats were placed at the junction of the arms, facing the close arm. The time spent in closed/open arms, the number of arm entries and the number of "lean out" from open arms were registered for 5 min.

The tests were performed in the following order: Passive avoidance test (9th and 11th day after irradiation), spontaneous alternation test in a Y-shaped maze (13th day after irradiation), ECM (16th day). NORT (18th, 19th, 20th day after irradiation).

Quantitate Real-Time PCR

At the end of behavioral tests, on the 30th day after the end of irradiation the animals were anesthetized (5% solution of ketamine, 200 mg kg^{-1}, intraperitoneally). After decapitation, the brain was removed and part of prefrontal cortex and hippocampus (30 mkg) were isolated on ice, quickly placed in RNAlater solution (Qiagen) and stored at +4°C. Isolation of the total RNA was performed using TRI REAGENT (MRC) according to protocol provided by the manufacturer. 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. Nucleotide sequences of primers used: GFAPrat-forward TAAGCGTCCATCCTCCTGTTTGAAAG; GFAPrat-reverse ACACTAATCGAAGGCACTCCA. GAPDH-forward AAGTTCAACGGCACAGTTCAA; GAPDH-reverse CTCCTGGAAGATGGTGATGG. VEGFrat-forward AAGACCGATTAACCATGTCA; VEGFrat-

reverse ATGTCAGGCTTTCTGGATTA. Primer sequences were obtained using Beacon Designer 7 software (Premier Biosoft International). Before setting real-time PCR, cDNA was diluted 5-fold to a concentration of 200 ng μL^{-1}. For each of the obtained cDNA libraries, PCR efficiency was measured and was shown to be in the 96-99% range. Then, expression of the genes of interest was measured. The relative expression level of target genes was calculated using the following formula $2^{-\Delta\Delta Ct} \pm SD$ (Livak and Schmittgen, 2001).

Immunohistochemical Analysis

After the completion of irradiation and functional tests, all rats were anesthetized intraperitoneally with 5% solution of ketamine and diazepam with pre-perfusion of 4% paraformaldehyde and sacrificed. After 2 days of post-fixation in 4% paraformaldehyde solution, 30 µm sections of hippocampus and frontal cortex were prepared. Immunohistochemical analysis was conducted using the method of double immunofluorescence staining with monoclonal antibodies to VEGF and polyclonal antibodies to GFAP. Primary antibodies were obtained in the laboratory of immunochemistry of the Serbsky State Scientific Center for Social and Forensic Psychiatry. Secondary antibodies labeled by Alexa Fluor 488 and 594 were purchased from Invitrogen. Analysis of sections was performed on a confocal microscope (Nikon, Japan).

Statistical Analysis

The obtained data showed as median ± quartile. Non-parametric methods of Mann-Whitney and Kruskal-Wallis were used for processing statistical data due to non-parametric distribution. Differences were considered reliable at $p < 0.05$.

Results

Radiation-Induced Cognitive Dysfunction

In passive avoidance test, no difference between groups was found (Kruskal-Wallis test: H = 0.65 p = 0.88). Time in the light part was 205 (158; 273) s for the control group, 221 (170; 257) s for the 36Gy/18 group, 260 (150; 278) s for the 36Gy/9 group and 239 (173; 256) sec for the 36Gy/6 group (Median (quartiles)).

Spontaneous alternation test in Y-shaped maze (Fig. 2), showed no difference between the experimental and control groups (Kruskal-Wallis H = 5.06, p = 0.17), but about half of the animals from the group 36Gy/6 did not enter the other arms of the maze (0.14 (0; 0.26) Median (quartiles) for 36Gy/6 group). The number of arm entries differed significantly between the 36Gy/6 group and the control group (p = 0.02). Taking this finding into account, the 36Gy/6 group was subjected to a test in ECM test (Fig. 3), which showed differences compared with the control animals (p<0.01).

In NORT differences were observed between the control group and both 36Gy/18 and 4Gy*6 groups (p<0.01). Both experimental groups showed negative index of recognition. When comparing the experimental groups, differences between 36Gy/18 and both 36Gy/9 and 36Gy/6 groups (p<0.05 and 0.01 correspondingly) as well as between 36Gy/9 and 36Gy/6 groups (p<0.01) were found (Fig. 4). The lateralization (preference for any installation corner) was not revealed in any of the groups. Initial research activity (total distance traveled during habituation) did not differ between the groups (Kruskal-Wallis test: H = 1.18 p = 0.76).

Structural Changes

In the hippocampus, the level of VEGF mRNA was significantly lower in all experimental groups on all dates than in the control group (p<0.05 for 36Gy/18 group (all dates), 36Gy/9 on the 8th week and 36Gy/6 on the 12th week, p<0.01 for 36Gy/9 on the 4th and 12th weeks, p<0.001 for 36Gy/6 on the 4th and 8th weeks). The level of VEGF mRNA was equal in 36 Gy/18 and 36Gy/9 groups on the 4th, 8th and 12th weeks after irradiation (no statistical significance was found between 36Gy/18 and 36Gy/9 groups on all dates). But in 36Gy/6 group VEGF mRNA level on the 4th week after irradiation was lower than in 36Gy/18 and 36Gy/9 groups (p<0.01). VEGF mRNA level in 36Gy/6 group on the 12th week and in 36Gy/18 and 36Gy/9 groups were the same. The statistical significance was also found in 36Gy/6 group on the 4th week and 12th week (p<0.001) Table 1.

In the prefrontal cortex, the level of VEGF mRNA was significantly lower in all experimental groups on the 4th and 8th weeks compared to the control (p<0.05 for 36Gy/18 group on the 4th and 8th weeks, p<0.01 for 36Gy/9 group on the 4th week and p<0.05 on the 8th week, p<0.001 for 36Gy/6 group on the 4th and 8th weeks). However, there was no difference between experimental groups Table 2.

In the hippocampus, the level of GFAP mRNA was significantly lower in all experimental groups only on the 12th week than in the control group. On the 12th week GFAP mRNA level was 2 times lower than in the control group (p<0.01 for each of experimental group), but there was no difference between experimental groups Table 3.

On the 4th week, the level of GFAP mRNA in prefrontal cortex of the rats with increased levels of GFAP mRNA was most pronounced in 36Gy/6 group (2 times higher than in the control group) and for 36Gy/9 group 1.6 times higher. Statistically significant differences were found between the control and 36Gy/9 group (p = 0.02) and 36Gy/6 (p = 0.01), as well as between the group 36Gy/18 compared to 36Gy/9 (p<0.01) and 36Gy/6 (p = 0.01), between 36Gy/9 and 36Gy/6 (p = 0.03). On the 8th week the level of GFAP expression was decreased only in 36Gy/6 group (p<0.01). There was no difference between the experimental group and control on the 12th week Table 4.

Immunohistochemical analysis of VEGF expression in the prefrontal cortex on the 4th week revealed decreased quantity of VEGF-positive blood vessels compared with control samples of brain tissue sections. Nonetheless, the decrease in expression was not observed to be dose-dependent. Dependence on the variation of the fractional dose was observed in the GFAP expression. In the sections of frontal cortex, quantity of GFAP-positive astrocytes increases in the series 36Gy/18, 36Gy/9 and 36Gy/6 (Fig. 5). On the 12th week there was no difference in GFAP and VEGF expression between all experimental and control groups (data not shown).

Immunohistochemical analysis of the hippocampus of irradiated rats on the 4th week showed a decrease in VEGF expression in all experimental groups compared with healthy animals not exposed to irradiation. According to the immunohistochemical analysis, differences in the expression of GFAP on the 4th week were not identified (data not shown). However, a decreased fluorescence of GFAP-positive astrocytes occurred on the 12th week after irradiation and the quantity of VEGF-positive blood vessels was still low (Fig. 6).
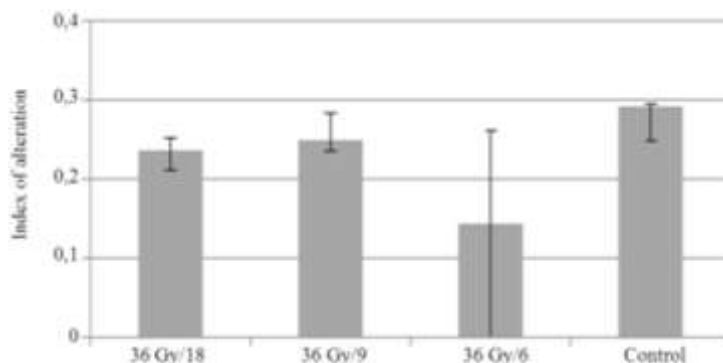

Fig. 2. Spontaneous alternation test in Y-shaped maze. Index of spontaneous alternation was calculated using the formula: (Nt/Ns) *100%, where Nt is the number of triads, Ns-the total number of arm entries. Median ± quartile. Differences between groups were not found, p>0.05
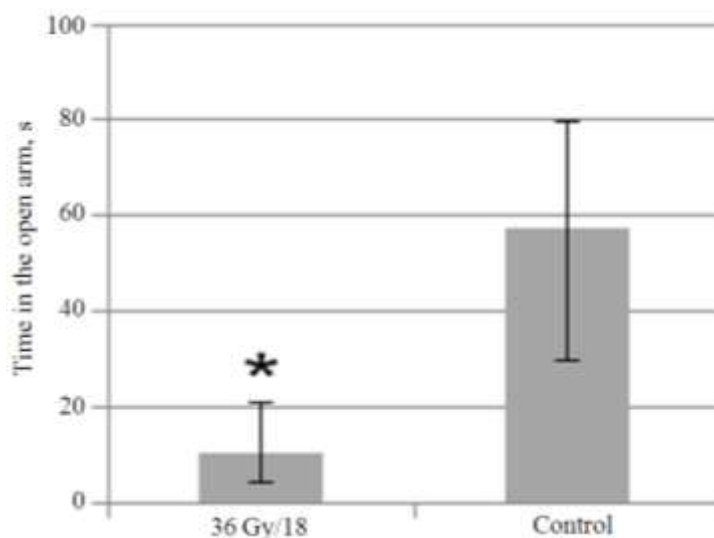
Fig. 3. Elevated cross-maze test. Time in open arms was registered during 5 min. Median ± quartile. * p<0.01 compared with control
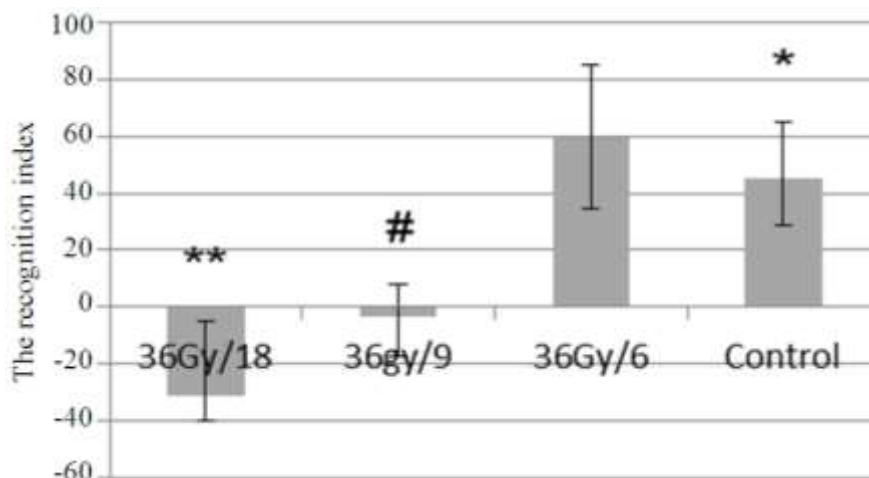

Fig. 4. Novel object recognition test. Recognition index was calculated according to the formula: (Tn-Tf / Tn + Tf) *100%, where Tn is the time spent on the study of the "new" object and Tf is the time spent on the study of "familiar" object during the third trial. Test was lasted 5 min. Median ± quartile. * p<0.01 compared with 36Gy/18 and 36Gy/9, **p<0.01 compared with 36Gy/9 and 36Gy/6 groups. #p<0.01 compared with 36Gy/6 group

Table 1. The level of VEGF mRNA in hippocampus. $2\text{-}\Delta\Delta Ct \pm SD$

Control	36Gy/18	36Gy/9	36Gy/6	Control	36Gy/18	36Gy/9	36Gy/6	Control	36Gy/18	36Gy/9	36Gy/6
4 weeks	4 weeks	4 weeks	4 weeks	4 weeks	8 weeks	8 weeks	8 weeks	12 weeks	12 weeks	12 weeks	12 weeks
1	0.75	0.65	0.41	1	0.72	0.75	0.55	1	0.69	0.686	0.7
SD (plus and minus)											
0.24	0.235	0.021	0.122	0.14	0.15	0.154	0.179	0.2	0.14	0.134	0.122
0.234	0.15	0.192	0.116	0.17	0.105	0.139	0.15	0.165	0.128	0.121	0.118

Table 2. The level of VEGF mRNA in prefrontal cortex. $2\text{-}\Delta\Delta Ct \pm SD$

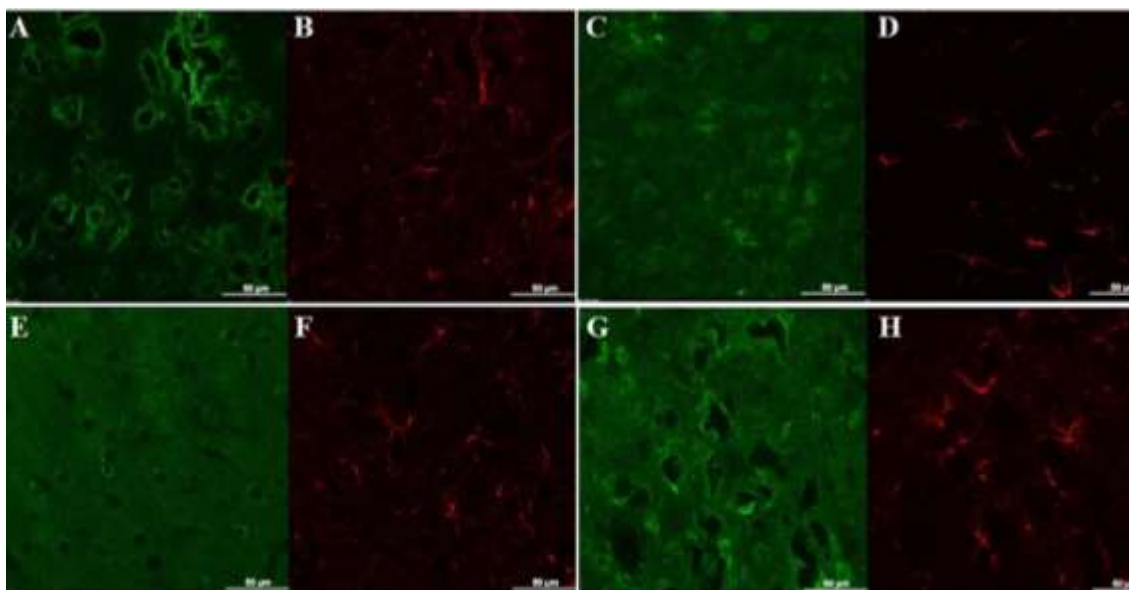| Control	36Gy/18	36Gy/9	36Gy/6	Control	36Gy/18	36Gy/9	36Gy/6	Control	36Gy/18	36Gy/9	36Gy/6
4 weeks	4 weeks	4 weeks	4 weeks	8 weeks	8 weeks	8 weeks	8 weeks	12 weeks	12 weeks	12 weeks	12 weeks
1	0.47	0.46	0.42	1	0.70	0.67	0.68	1	1.05	1.1	0.96
SD (plus and minus)											
0.225	0.180	0.160	0.091	0.12	0.082	0.102	0.107	0.193	0.186	0.204	0.213
0.145	0.071	0.149	0.087	0.095	0.081	0.099	0.106	0.187	0.180	0.146	0.207

Fig. 5. Immunohistochemical analysis of VEGF and GFAP expression in prefrontal cortex of rats on the 4th week after different schemes of fractionated irradiation. Green-Mouse Anti-VEGF monoclonal antibody + Anti-mouse Alexa 488, Red-Rabbit Anti-GFAP polyclonal antibody + Anti-rabbit Alexa 594; A, B-control; C,D-36Gy/18; E,F-36Gy/9; G,H-36Gy/6. Bars = 50 µm
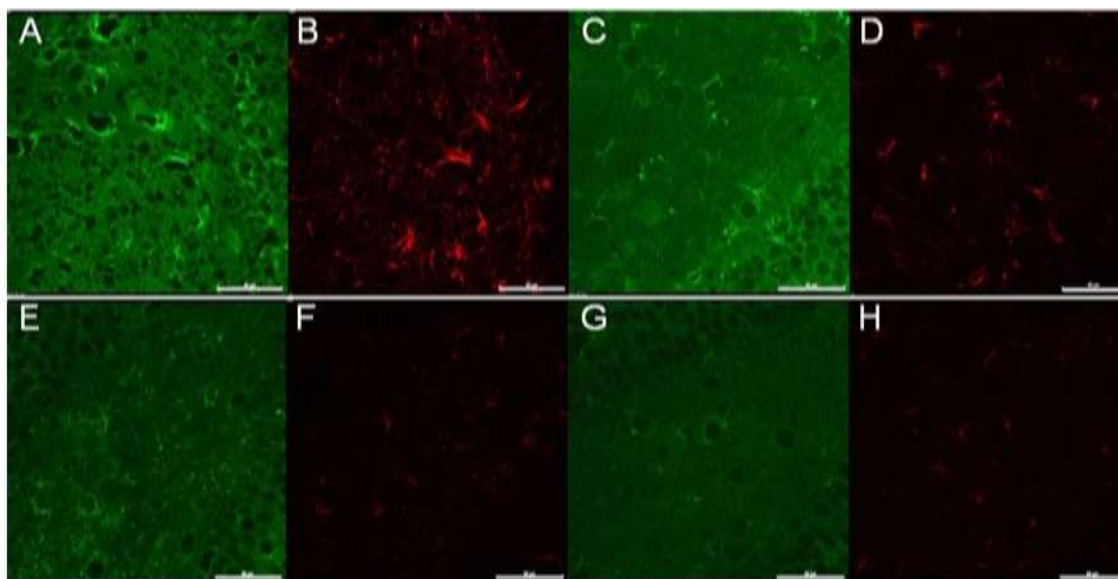

Fig. 6. Immunohistochemical analysis of VEGF and GFAP expression in hippocampus of rats on the 12th week after different schemes of fractionated irradiation. Green-Mouse Anti-VEGF monoclonal antibody + Anti-mouse Alexa 488, Red-Rabbit Anti-GFAP polyclonal antibody + Anti-rabbit Alexa 594; A, B-control; C,D-36Gy/18; E,F-36Gy/9; G,H-36Gy/6. Bars = 50 µm

Table 3. The level of GFAP mRNA in hippocampus. $2-\Delta\Delta Ct \pm SD$

Control	36Gy/18	36Gy/9	36Gy/6	Control	36Gy/18	36Gy/9	36Gy/6	Control	36Gy/18	36Gy/9	36Gy/6
4 weeks	4 weeks	4 weeks	4 weeks	8 weeks	8 weeks	8 weeks	8 weeks	12 weeks	12 weeks	12 weeks	12 weeks
1	0.84	0.85	0.91	1	0.96	1.19	1.15	1	0.445	0.487	0.517
SD (plus and minus)											
0.260	0.221	0.249	0.301	0.210	0.213	0.112	0.165	0.09	0.057	0.239	0.255
0.207	0.276	0.193	0.240	0.198	0.154	0.252	0.254	0.154	0.132	0.215	0.232

Table 4. The level of GFAP mRNA in prefrontal cortex. 2-$\Delta\Delta$Ct ± SD

Control 4 weeks 1	36Gy/18 4 weeks 1.257	36Gy/9 4 weeks 1.65	36Gy/6 4 weeks 2.04	Control 8 weeks 1	36Gy/18 8 weeks 0.89	36Gy/9 8 weeks 0.94	36Gy/6 8 weeks 1.67	Control 12 weeks 1	36Gy/18 12 weeks 0.935	36Gy/9 12 weeks 1.09	36Gy/6 12 weeks 0.99
SD (plus and minus)											
0.220	0.091	0.305	0.267	0.179	0.191	0.188	0.220	0.209	0.1274	0.240	0.221
0.187	0.095	0.193	0.263	0.185	0.180	0.187	0.212	0.256	0.1271	0.223	0.206

Discussion

It is becoming apparent that irradiation affects VEGF expression and therefore, angiogenesis. Our research indicates that while there is a decrease in VEGF expression both in prefrontal cortex and in hippocampus, no dose-dependence in prefrontal cortex was observed. In the hippocampus the lowest level of VEGF was in 36Gy/6 group, which received the maximum BED. In available literature both increase and decrease of the VEGF expression are reported (Leshchinskaya *et al.*, 2000; Ma *et al.*, 2001; Balentova *et al.*, 2012), however, the dose of radiation, the method of fractionation and the timing of material sampling are different, so, comparing the data is difficult. It is possible that increase or decrease of the VEGF expression depends on the time elapsed after radiation and on the received dose. Low radiation dose does not cause necrosis and hypoxia in extent to provoke increased expression of VEGF.

Reactive astrogliosis with both a decrease of GFAP-positive glial cells and GFAP mRNA level was observed in the prefrontal cortex of irradiated rats, the degree depending on a variant of fractionation and increasing in a series 36 Gy/18, 36Gy/9, 36Gy/6, which is consistent with the increase of BED, that is in accordance with the general notions. The reaction of astroglia accompanied by an increased GFAP expression corresponds with the data obtained by other researchers (Yang *et al.*, 2000). Absence of a difference in GFAP mRNA levels in hippocampus on the 4th and 8th week is probably due to initially higher expression of GFAP in this structure as compared with the frontal cortex. However, Zhou *et al.* (2011) showed an increased expression of GFAP in hippocampus after fractionated gamma irradiation using immunohistochemical analysis. The latter result may be due to the higher dose of fractionation and a different fractionation mode. Balentova *et al.* (2012) were observed the decrease of number of GFAP-positive cells in the hippocampus after fractionated whole body irradiation with a total dose of 4 Gy on the 90th day after exposure. We observed the same differences with the control group on the same date (12 week), but BED-dependence was not observed.

The obtained data allow us to conclude that the used irradiation schemes have selective influence on various cognitive processes.

Recognition memory in 36Gy/18 group was impaired more than in the other groups. At the same time, the decrease in VEGF levels in this group is similar to one in the other groups and GFAP levels do not differ from control. Therefore, it is not clear what structural changes that followed larger number of fractions with 2 Gy single dose (the lowest BED) resulted in a greater impairment of cognitive function in NORT. It is also significant that in this test, recognition memory was in inverse proportion to the BED.

About 36Gy/9 group showed a decreased discrimination index in the NORT, as compared with the control. Previously it was shown that behavior in this test depends on functions of prefrontal cortex (Nagai *et al.*, 2007), however the obtained data does not allow us to state that recognition memory impairment in this group was due to changes in frontal cortex. GFAP level is considered as an indicator of tissue damage after irradiation. GFAP levels were the highest in the 36Gy/6group. Nonetheless, animals of this group did not differ from the control in the NORT.

About 36Gy/6 group showed increased anxiety. Such anxiety was also observed upon whole-body irradiation with a single dose of 6 Gy (Tomasova *et al.*, 2011). This may have led to decrease in the number of arm entries in Y-maze test. This assumption is supported by published data (Park *et al.*, 2010), although some studies showed an inverse relationship between avoidance and long-term spatial memory (He *et al.*, 2011). The paradoxical stimulating effect of the irradiation on object recognition in 36Gy/6 group can also be associated with increased anxiety in this group. Data consistent with such a hypothesis were obtained by Goepfrich *et al.* (2013) who found that Wistar rats with a longer time in dark arms of ECM had a higher percentage of discrimination in the NORT. In our experiment, increased anxiety after irradiation in 36Gy/6 group could lead to increased attention to the new object. Although anxiety can be increased by a variety of factors, it is most likely that in our experiment, it was prefrontal cortex damage which induced anxiety and which is marked by the highest level of GFAP expression in the 36Gy/6 group.

Conclusion

The described pathologic changes in prefrontal cortex and hippocampus may play an important role in development of radiation-induced cognitive dysfunction. Expression of VEGF in prefrontal cortex and GFAP in hippocampus was not dose-depended. BED-dependence was observed only in expression of GFAP in prefrontal cortex and VEGF in hippocampus. With the obtained

data, we can confirm, that cognitive impairment after fractionated WBI was not increased in direct proportion to the BED. Further research using different fractionated schemes with same or different BED may be useful for study of radiation-induced cognitive dysfunction and its structural substrates and for selecting optimal fractionation in order to minimize side effects of ionizing radiation.

The authors declare that they have no competing interests.

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Author's Contributions

All authors equally contributed in this work.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and no ethical issues involved.

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