

## RADIATION-MEDIATED INDUCTION OF APOPTOTIC CELL DEATH IN RAT HIPPOCAMPUS

by

**Nataša A. VELIČKOVIĆ<sup>1\*</sup>, Ana D. DJORDJEVIĆ<sup>1</sup>, Dunja R. DRAKULIĆ<sup>2</sup>,  
Bojana Lj. ŠEĆEROV<sup>3</sup>, Ivana S. GRKOVIĆ<sup>2</sup>,  
Maja S. MILOŠEVIĆ<sup>2</sup>, and Anica I. HORVAT<sup>2</sup>**

<sup>1</sup>Department of Biochemistry, Institute for Biological Research "Siniša Stanković",  
University of Belgrade, Belgrade, Serbia

<sup>2</sup>Laboratory for Molecular Biology and Endocrinology, Vinča Institute of Nuclear Sciences,  
University of Belgrade, Belgrade, Serbia

<sup>3</sup>Laboratory for Radiation Chemistry and Physics, Vinča Institute of Nuclear Sciences,  
University of Belgrade, Belgrade, Serbia

Scientific paper

DOI: 10.2298/NTRP1302212V

Ionizing radiation is commonly used in the treatment of brain tumors but it can impair cognitive functions, such as learning and memory. Since cognitive dysfunctions are predominantly result of cell death by apoptosis in hippocampal cells, in this study we analyzed acute effects of cranial gamma-irradiation (10 Gy) on expression of proapoptotic molecules (p53, Bax) and antiapoptotic molecule Bcl-2, as well as caspase-3 activation and cytochrome c redistribution in the hippocampus of young rats. The selected regimen of irradiation resembles the established animal model for childhood prophylactic cranial radiotherapy.

Our results demonstrated that p53 mRNA expression was unchanged after irradiation, while induction of p53 protein was rapid. In parallel, Bax mRNA and protein levels were also increased following irradiation, whereas Bcl-2 expression was not changed during the examined post-irradiation period. These changes were accompanied with early hallmarks of apoptosis, such as increased cytochrome c release and stimulated activation of caspase-3.

Overall, this study demonstrates that cranial irradiation is associated with the augmented apoptotic pathway in the rat hippocampus, which could be related to the cognitive decline observed in patients after prophylactic cranial radiotherapy, but also opens perspective in finding radioprotectors that can mitigate radiation injury of normal brain tissue.

*Key words: cranial irradiation, apoptosis, hippocampus, rat*

### INTRODUCTION

The radiation therapy has been used in cancer treatment for many decades; it is used not only to treat patients with primary and secondary brain tumors, but also as prophylaxis, to prevent development of brain metastases and central nervous system (CNS) involvement in hematological malignancies [1]. For many years, the 24 Gy cranial radiotherapy has been part of the CNS prophylactic therapy for standard and high risk patients with acute lymphoblastic leukemia (ALL) [2]. However, in the course of the treatment, these patients have demonstrated different cognitive disturbances, such as diminished intellectual functioning, learning, memory, and spatial information processing abilities [3]. It is well known that hippocampal formation in the brain plays a central role in short-term

learning and memory – the functions most notably affected by radiation. The disturbances in hippocampal functions are most likely sequels of impaired neurogenesis of the progenitor cells, promoted through cell death by apoptotic mechanism. In favor of this assumption, Monje and Palmer [4] reported that radiation induces acute apoptosis of neural progenitors and vascular endothelial cells in the rat hippocampus, resulting in inhibition of neurogenesis.

The exposure of normal CNS tissues to clinically relevant low doses of ionizing radiation causes DNA damage and the generation of reactive oxygen species (ROS) [5], which can lead to a apoptotic death through a p53-dependent pathway [6]. The apoptosis is the process of programmed cell death with characteristic morphological changes as blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation and DNA fragmentation [7]. One of the central molecules involved in both apoptosis and DNA repair pathways after irradiation is

\* Corresponding author; e-mail: nvelickovic@ibiss.bg.ac.rs

the tumor suppressor protein p53 [8]. Under normal conditions p53 is a short-lived protein that is maintained at low level in the cell. In many tissues, irradiation results in the up-regulation of p53 protein by a post-translational stabilization mechanism, presumably not followed with change in the mRNA level [9, 10]. Active p53 protein induces the transcription of several downstream genes that, in turn, can trigger a variety of biological processes such as cell cycle arrest, apoptosis and DNA repair. The overall consensus is made that Bcl-2 family members are crucial in p53-mediated apoptosis [11]. This family includes two categories of proteins: those functioning as suppressors of apoptosis (Bcl-2, Bcl-x<sub>L</sub>, Bcl-w) and those that promote apoptosis (Bax, Bcl-x<sub>S</sub>, Bak, Bad, Bik, and Noxa). The pro-apoptotic members like Bax, Bak and Noxa are transcriptionally upregulated by p53, while expression of anti-apoptotic Bcl-2 is suppressed by p53 [12]. The relative ratios of these various pro- and anti-apoptotic members of the Bcl-2 family have been shown to determine the ultimate sensitivity or cell resistance in a response to the ionizing irradiation [13]. The predominance of apoptotic molecules promotes mitochondrial-dependent or "intrinsic" apoptotic pathway, by changing mitochondrial permeability that lead to the release of apoptotic proteins such as cytochrome c from mitochondria into cytoplasm [14]. The release of cytochrome c is followed by the activation of the caspase cascade, with activated caspase-3 as one of the key executioners of apoptosis [15]. The activation of caspase-3 requires proteolytic processing of its inactive zymogen into active p17 and p19 forms [16], which form enzymatically active dimer responsible for proteolytic cleavage of many key proteins [17]. Therefore, the release of cytochrome c into cytoplasm and cleavage of procaspase-3 are considered as early hallmarks of apoptosis [18, 19].

The primary focus in cranial radiotherapy is to increase the effectiveness in eradication of tumor cells, while preserving normal surrounding tissue. Therefore, animal models may be useful in assessing the effects of irradiation on the brain tissue. In this study an established animal model for CNS prophylactic therapy of childhood ALL was used [20]. The heads of young Wistar rats (18 days old) were subjected to a single dose of gamma irradiation (10 Gy), since this dose approximate clinical dose of 24 Gy given in 12-14 fractions in CNS prophylactic therapy of high risk patients with ALL [21].

The aim of the present study was to investigate the molecular mechanism of radiation-mediated apoptosis in normal brain tissue, by analyzing expression of tumor suppressor p53 in the hippocampus of head-irradiated rats at various time intervals after treatment, both at the level of mRNA and protein. Simultaneously, the expression of positive apoptosis regulator Bax and antiapoptotic molecule Bcl-2, as downstream p53-target genes, was explored in the same animal model. The early signs of apoptosis were

assessed on the level of cytochrome c, procaspase-3 and cleaved caspase-3 in the cytoplasmic fraction of rat hippocampus. Through analysis of these molecular markers we sought to determine whether hippocampus, as the key center for memory and learning, is affected by the apoptotic cell death after the prophylactic cranial irradiation.

## MATERIAL AND METHODS

### Animals

The experiments were conducted on 18-days-old male Wistar rats, weighing 32.45 ± 0.71 g, bred at the Vinča Institute of Nuclear Sciences, Belgrade, Serbia. The animals were maintained in the animal room on a 12:12 h light/dark cycle (lights on: 7:00 a. m.-7:00 p. m.), under constant temperature (22 °C) and humidity (55% ± 5%). The animals had free access to food-commercial rat pellets and water. All animal procedures were complied with the European Communities Council Directive (2010/63/EU) and approved by the Ethical Committee for the Use of Laboratory Animals of the Vinča Institute according to the guidelines of the EU FELASA-registered Serbian Laboratory Animal Science association (SLASA).

### Irradiation procedure

Previously established animal model for CNS prophylactic therapy of childhood ALL was used in this study [21]. At the age of 18 days (the day of birth taken as day 0), animals were divided into two groups (60 animals per group): the sham irradiated controls (C) and irradiated (IR) animals. Further, the groups of sham irradiated controls and irradiated animals were divided in four subgroups of 15 animals, according to the time of decapitation after treatment. Since the IR animals were anesthetized with the combination of ketamine (50 mg/kg) and xylazine (3 mg/kg) (Richter Pharma, Wels, Austria) for 1 h during irradiation procedure, the sham irradiated controls were treated equally, except the exposure to ionizing radiation. The heads of the IR rats were exposed to a single 10 Gy dose of  $\gamma$ -rays using <sup>60</sup>Co source (the Laboratory of Radiation Chemistry and Physics, Vinča Institute of Nuclear Sciences). Since the rat brain is more radiation resistant compared to the human, a single 10 Gy irradiation dose is well below the appearance threshold of frank vascular changes, demyelization or radionecrosis [22]. In order to minimize the radiation exposure of the abdomen and adrenal glands, the bodies of the animals were protected by a 5 cm lead blocks placed in front of the 2.5 cm thick polystyrene bar. A Fricke dosimeter was chosen (0.001 M Ferrous ions diluted in 0.4 mol/L sulphuric acid) for dose rates measurements because both the dosimeter

and the cells chemically are aqueous solutions where the radiation energy is essentially absorbed in water molecules [23]. The scattered radiation absorbed in shielded parts of the body was less than 5% of the dose delivered to the cranium. The radiation of four animals at the time was administered in the morning (9:00 to 10:00 a. m.) at a dose rate 0.32 Gy/min (source skin distance of 31 cm). The entire exposure lasted 32 min. After completing irradiation procedure, the irradiated animals and the appropriate sham irradiated controls were sacrificed by decapitation 1, 2, 4, and 8 h after irradiation. Each experimental group consisted of 15 animals.

### Tissue collection

After decapitation the brains were quickly removed and placed on ice for an immediate dissection of the hippocampus. For each group, the 3-5 hippocampi were pooled for subsequent Western blot and RT PCR, rapidly frozen in liquid nitrogen and stored at  $-70^{\circ}\text{C}$  for subsequent processing [24].

### Reverse transcription and semi-quantitative PCR (RT-PCR)

Total RNA was isolated from the hippocampi using TRIreagent® (Invitrogen, Carlsbad, USA). The RNA was quantified by reading the optical density at 260 nm and 280 nm. For the synthesis of cDNAs, the First Strand cDNA Synthesis Kit (#K1612, Fermentas, Lithuania) was used by manufacturer's instructions. The cDNAs were stored at  $70^{\circ}\text{C}$  until analysis.

The cDNA products were amplified using primers for p53, Bax and Bcl-2, together with the house-keeping gene glyceraldehyde 3 phosphate dehydrogenase (GAPDH) (the primers sequences are presented in tab. 1). The cDNAs were amplified in a Eppendorf thermocycler for 30 cycles (Bax, Bcl-2) or 28 cycles (p53, GAPDH) using the following conditions: denaturation  $94^{\circ}\text{C}/(1\text{ min})$ ; annealing  $62^{\circ}\text{C}$  (p53),  $58^{\circ}\text{C}$  (Bax) and  $55^{\circ}\text{C}$  (Bcl-2 and GAPDH), extension  $72^{\circ}\text{C}/(1\text{ min})$ ; final extension  $72^{\circ}\text{C}/(5\text{ min})$ . The PCR products were separated on 2% agarose gels and visualised by ethidium-bromide staining under UV light. The intensity of PCR products were measured with an image analysis system GelDoc 1000 (BioRad, Cal., USA) and expressed in arbitrary units (count). The arbitrary units related to the p53, Bax and

Bcl-2 amplification products were divided by that corresponding to GAPDH product obtained in the same samples.

### Preparation of whole cell extracts and cytoplasmic extracts for western blot analysis

For the preparation of whole cell extracts, frozen hippocampal tissue was homogenized (1:4 w/v) in ice-cold 50 mM Tris-HCl (pH 8) buffer containing 150 mM NaCl, 1 mM EDTA, 1 mM MEGTA, 1 mM DTT, 1 mM  $\text{Na}_3\text{VO}_4$ , 1% Nonidet P-40 (NP-40), 0.1% SDS, 0.5% Na-deoxycholate and protease inhibitors (0.5 mM PMSF, 5  $\mu\text{g}/\text{ml}$  antipain, 5  $\text{g}/\text{ml}$  leupeptin, 5  $\text{g}/\text{ml}$  aprotinin). After 30 min of incubation on ice, homogenates were centrifuged on 13000 g, 20 min,  $4^{\circ}\text{C}$ , and resulting supernatant represents whole cell extracts.

For cytoplasmic extracts, frozen tissue was weighed and homogenized (1:2 w/v) in ice-cold 20 mM Tris-HCl (pH 7.2) buffer containing 10% glycerol, 50 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2 mM DTT and several protease inhibitors (20 mM  $\text{Na}_2\text{M}_2\text{O}_4$ , 0.15 mM spermine, 0.15 mM spermidine, 0.1 mM PMSF, 5  $\text{g}/\text{ml}$  antipain, 5  $\text{g}/\text{ml}$  leupeptin, 5  $\mu\text{g}/\text{ml}$  aprotinin) as well phosphatase inhibitors (20 mM glycerophosphate, 5 mM  $\text{Na}_4\text{P}_2\text{O}_7 \times 10\text{ H}_2\text{O}$ , 2 mM  $\text{Na}_3\text{VO}_4$ , 25 mM NaF) with 20 strokes of teflon-glass homogenizer. Samples were centrifuged at 2000 g, 10 min,  $4^{\circ}\text{C}$  to obtain the supernatant and pellet (containing nuclear and cell remains). The supernatant was ultracentrifuged at 105000 g, 60 min,  $4^{\circ}\text{C}$  and the resulting supernatant is used as cytoplasmic fraction. The protein concentration of the samples was measured by the modified method of Lowry [25] using bovine serum albumin (BSA) as a standard.

### Western blot analysis

The samples containing 30  $\mu\text{g}$  protein were mixed with Laemmli's sample buffer, boiled for 5 min and loaded on 10% or 12% polyacrylamide gels. For Western blot analysis, the samples intended to be directly compared were always run on the same gel; p53, Bax and Bcl-2 were detected in the whole cell extracts, while procaspase-3, cleaved caspase-3 and cytochrome c were analyzed in the cytoplasmic fraction. The primary antibodies used in this study were: anti-p53 (KAM-CC002, Stressgen, USA, 1:2000), anti-procaspase 3 (H-277,

**Table 1. Primer sequences for RT-PCR**

| Primers | Sense                         | Antisense                    |
|---------|-------------------------------|------------------------------|
| Bcl-2   | 5'-GGAGATCGTGATGAAGTAC-3'     | 5'-TCAGGTACTIONCAGTCATCCA-3' |
| p53     | 5'-TTCCCTCAATAAGCTGTTCTGCC-3' | 5'-TGCYCTCTTTCACCTCCCTGG-3'  |
| Bax     | 5'-GGCGAATTGGAGATGAACTG-3'    | 5'-TTCTCCAGATGGTGAGCGA-3'    |
| GAPDH   | 5'-TTCATTGACCTCAACTACATG-3'   | 5'-GTGGCAGTGATGGCATGGAC-3'   |

Santa Cruz Biotechnology Inc, USA, 1:2000), anti-cleaved caspase 3 (Asp175, Cell Signaling Inc, USA, 1:500), anti-Bcl-2 (#2876, Cell Signaling Inc, USA, 1:1000), anti-Bax (B-9, Santa Cruz Biotechnology Inc, USA, 1:1000) and anti-cytochrome c (6H2, Santa Cruz Biotechnology Inc, USA, 1:1000). The goat polyclonal anti- $\beta$ -actin (C-11, Santa Cruz Biotechnology Inc, USA, 1:5000) antibody was used as a loading control. The secondary antibodies were: peroxidase-conjugated anti-rabbit, anti-mouse and anti-goat IgG antibodies (Santa Cruz Biotechnology, 1:10000). The immunopositive bands were visualized by the enhanced chemiluminescence (ECL) method (Cell Signaling, USA). The optical density (OD) of bands visible on light-sensitive films (Fuji, Japan) was measured using the image analysis system (ImageJ). The background OD levels were subtracted from the OD of each individual immunoreactive band.

### Statistics

All data were expressed as the mean  $\pm$  standard error of the mean (SEM) ( $n = 15$ ). The statistical analysis was performed by using one way ANOVA followed by *post hoc* Tukey test; the comparisons were performed between sham-irradiated controls and corresponding irradiated animals. Differences were considered significant at  $p < 0.05$ .

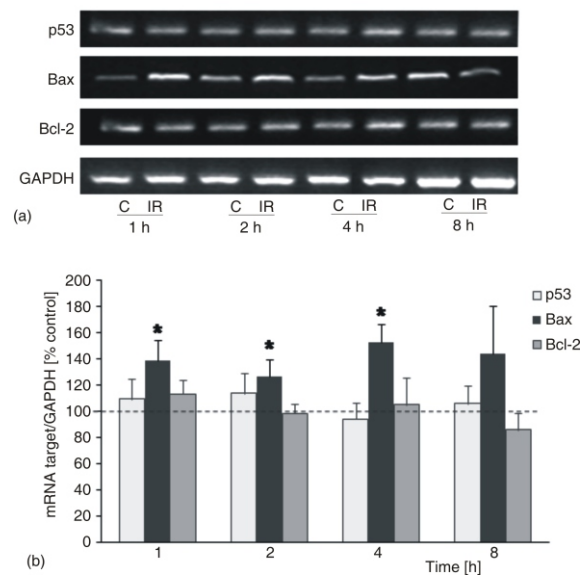
## RESULTS

### The effects of cranial irradiation on gene expression of apoptotic molecules p53, Bax and Bcl-2 in the hippocampus

In the first set of experiments, the effect of irradiation on gene expression of proapoptotic molecules p53 and Bax and antiapoptotic molecule Bcl-2 was evaluated. As shown in fig. 1, the p53 mRNA level was unchanged in the hippocampus of irradiated animals, whereas cranial irradiation led to the time-dependent increase in Bax mRNA level, compared to the sham-irradiated controls, with maximum achieved 4 h after the radiation treatment, fig. 1(b),  $F = 3.93$ ,  $p < 0.01$ . On the other hand, the Bcl-2 mRNA level was not significantly different between the irradiated animals and the appropriate controls, fig. 1(b),  $F = 0.212$ ,  $p = 0.93$ .

### The effects of cranial irradiation on apoptotic molecules protein expression in hippocampal whole cell and cytoplasmic extracts

The acute effects of irradiation on the p53, Bax and Bcl-2 protein levels were examined in the whole

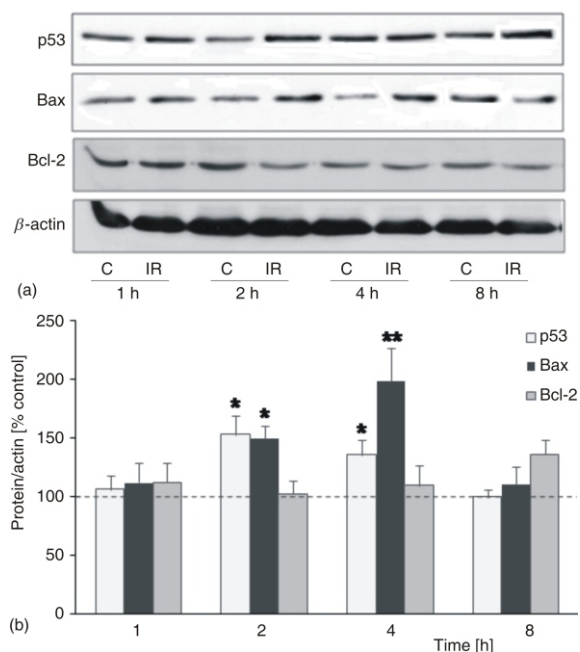


**Figure 1. The effect of cranial irradiation on p53, Bax and Bcl-2 mRNA in the rat hippocampus. Representative PCR of p53, Bax and Bcl-2 mRNA in the hippocampi of sham irradiated controls (C) and irradiated (IR) animals in different time intervals after treatment, as indicated (a). The relative abundance of target mRNA is quantified by densitometry, normalized against GAPDH in the same sample and expressed as the percent of appropriate control (b). Data represent the mean  $\pm$  SEM ( $n = 15$ ). Asterisk indicate significant differences between irradiated animals and sham irradiated controls;  $*p < 0.05$**

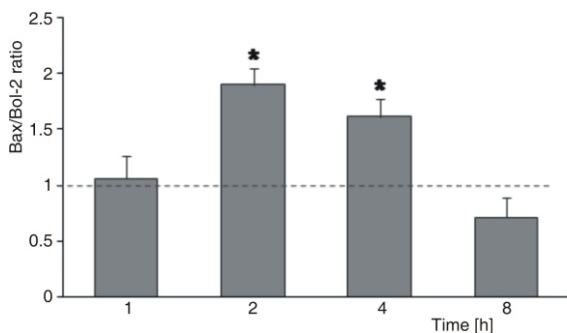
cell extracts of the 18-days-old animal hippocampus. As the time-response experiments revealed, the p53 and Bax protein were increased in similar manner, with maximum observed 2 h and 4 h after the treatment – fig. 2(b) (p53:  $F = 4.94$ ,  $p < 0.01$ ; Bax:  $F = 4.82$ ,  $p = 0.01$ ). On the contrary, the Bcl-2 protein was not changed during the examined time interval, fig. 2(b),  $F = 2.06$ ,  $p = 0.11$ .

The direct comparison between the Bax and the Bcl-2 protein levels in the hippocampus revealed radiation-induced increase in Bax/Bcl-2 ratio (fig. 3). The increment of Bax protein above Bcl-2 protein was observed in 2 h and 4 h post-irradiation time due to the Bax increase, while the Bcl-2 expression was unaltered (fig. 3).

It is known that the up regulated Bax promotes the apoptosis through insertion into the mitochondrial membrane, thus facilitating the cytochrome c release from the mitochondria to the cytoplasm [26]. Therefore, the cytochrome c level was analyzed in the cytoplasmic fraction, in order to estimate its possible leaking from mitochondria. As demonstrated in fig. 4, the cytochrome c protein level was increased in 2 h and 4 h post-irradiation time ( $F = 3.18$ ,  $p < 0.05$ ), which coincides with the induction of Bax protein in whole cell extracts.

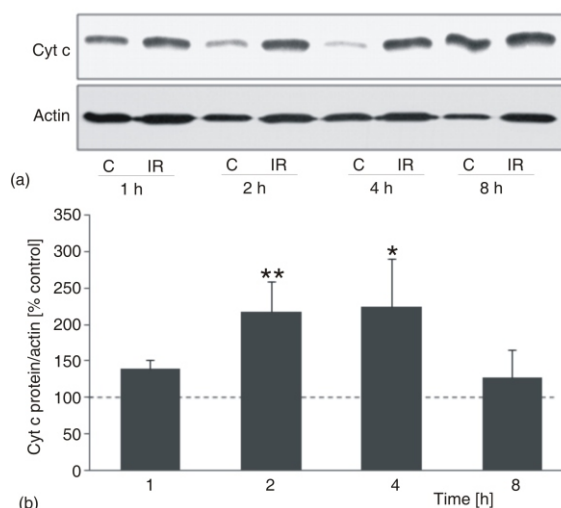


**Figure 2.** p53, Bax and Bcl-2 protein levels in the whole cell extracts of rat hippocampus after cranial irradiation. Representative Western blots of p53, Bax and Bcl-2 proteins in the hippocampi of sham irradiated (C) and irradiated (IR) animals at different time intervals after treatment, as indicated. The lower part of each blot was probed with anti- $\beta$ -actin antibody as a loading control (a). The relative abundance of target proteins in the whole cell extracts is quantified by densitometry, normalized against  $\beta$ -actin in the same lane and expressed as percent of appropriate control (b). Data represent the mean  $\pm$  SEM ( $n = 15$ ). Asterisk indicate significant differences between irradiated animals and sham irradiated controls; \* $p < 0.05$ , \*\* $p < 0.01$



**Figure 3.** Relative ratio of Bax protein to Bcl-2 protein in the rat hippocampus after cranial irradiation. Ratio values above 1 indicate the dominance of Bax, while values below 1 imply the prevalence of Bcl-2 in the whole cell extracts. The results are expressed as the mean  $\pm$  SEM. Asterisk indicate significant differences between irradiated animals and sham irradiated controls; \* $p < 0.05$

The cytochrome c release from mitochondria triggers a cascade of caspase activation, generally resulting in proteolytic cleavage of procaspase-3 into ac-

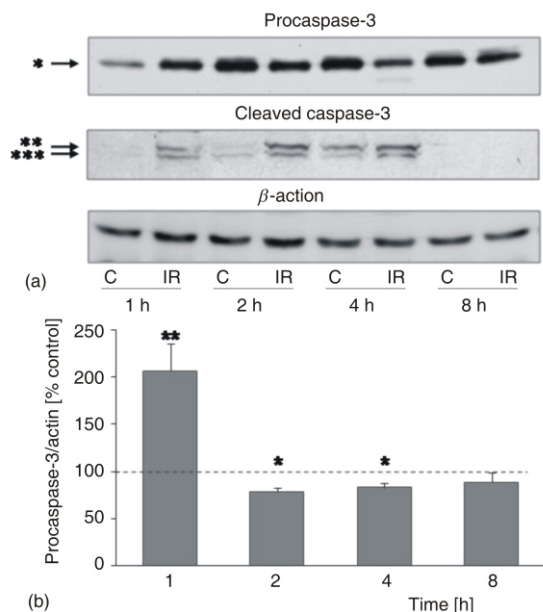


**Figure 4.** The effect of cranial irradiation on cytochrome c (cyt c) protein level in the cytoplasmic fraction of rat hippocampus. Representative Western blot of cyt c protein in the hippocampi of sham irradiated controls (C) and irradiated (IR) animals in different time intervals after treatment, as indicated. The lower part of the blot was probed with anti- $\beta$ -actin antibody as a loading control (a). The relative abundance of cyt c protein is quantified by densitometry, normalized against  $\beta$ -actin in the same sample and expressed as the percent of appropriate control (b). Data represent the mean  $\pm$  SEM ( $n = 15$ ). Asterisk indicate significant differences between irradiated animals and sham irradiated controls; \* $p < 0.05$ , \*\* $p < 0.01$

tive p17 and p19 fragments. In our study the procaspase-3 protein level in cytoplasmic fraction was rapidly increased after irradiation, fig. 5(a), upper panel, IR<sub>1h</sub> vs. C<sub>1h</sub>,  $p < 0.01$ , however, the increment observed in 1 h post-irradiation time declined, leading to the decreased protein level after 2 h and 4 h ( $p < 0.05$ ). This decrement of procaspase-3 is associated with increase of its cleaved form, which gives bands of 17 and 19 kDa, fig. 5(a), middle panel.

## DISCUSSION

Our previous results showed that the acute radiation phase is featured by enhanced hypothalamic-pituitary-adrenal (HPA) axis activity [27] and increased inflammation in the brain [28]. In the present study we demonstrated that cranial irradiation, in terms of prophylactic therapy, is associated with the induction of proapoptotic molecules p53 and Bax, and with the shift in Bax/Bcl-2 balance. This is accompanied with early hallmarks of apoptosis, such as an increased cytochrome c level in the cytoplasm and caspase-3 activation. The activation of the apoptotic pathway in the rat hippocampus could be related to the cognitive decline observed in patients after the prophylactic cranial radiotherapy.



**Figure 5.** The effect of cranial irradiation on caspase-3 protein level in the cytoplasmic fraction of rat hippocampus. Representative Western blot of procaspase-3 (32 kDa) (upper panel) and cleaved caspase-3 (19 and 17 kDa fragments) (middle panel) in the hippocampi of sham irradiated (C) and irradiated (IR) animals in different time intervals after treatment, as indicated. Asterisk indicates proteins of different molecular weight (\*, 32 kDa; \*\*, 19 kDa; \*\*\*, 17 kDa). The lower part of the blot was probed with anti- $\beta$ -actin antibody as a loading control (a). The relative abundance of procaspase-3 protein is quantified by densitometry, normalized against  $\beta$ -actin in the same sample and expressed as percent of appropriate control (b). Data represent the mean  $\pm$  SEM ( $n = 15$ ). Asterisk indicate significant differences between irradiated animals and sham irradiated controls; \* $p < 0.05$ , \*\* $p < 0.01$

In response to the ionizing radiation, the apoptosis signaling is triggered by the tumor suppressor p53, which is a critical regulator of apoptotic pathway and DNA repair [7]. In this study, the cranial irradiation rapidly stimulated p53 protein expression in a time-dependent manner, whereas the level of p53 mRNA did not show statistically significant changes. These results are in agreement with previous report, demonstrating that the gamma-irradiation induces apoptosis [29] as well as the induction of p53 protein in fetal rat brain, without a major up-regulation of p53 mRNA [30]. Moreover, the p53 pathway may be a mechanism through which radiation induces apoptosis in the adult CNS [10]. It should be noted, however, that not all types of radiation-induced apoptosis involve p53 induction [31].

It is known that the p53 transcriptionally activates Bax in some types of cells after treatment with ionizing radiation, chemotherapeutic drugs and other genotoxic agents [30]. We found that the Bax protein level was higher in irradiated rats than in the control ones; the maximum level was attained at 4 h, and then subsequently re-

duced again to the low level. This induction temporally coincides with the elevation of p53 protein, as expected for a cause-and-effect relationship [32]. Moreover, the cranial irradiation moderately increased the level of Bax mRNA, as revealed by RT-PCR. These results are in accordance with the previous studies reporting Bax up-regulation after ionizing irradiation [33, 34]. On the other hand, neither Bcl-2 mRNA level nor Bcl-2 protein were changed in the hippocampus of head-irradiated animals. The disbalance in Bax/Bcl-2 ratio in hippocampal neurons appears critical for regulation of apoptosis and function as a rheostat that determines cell susceptibility to apoptosis [35]. In our study, a shift in Bax/Bcl-2 balance results in the predominance of death-promoting molecules, which creates a critical checkpoint in the apoptotic program execution. Namely, Bax promotes apoptosis by impairing integrity of mitochondrial membrane and facilitating the release of cytochrome c from the mitochondria, thus triggering a cascade of caspase activation. In our study, the cytochrome c protein level was gradually increased in the cytoplasm, presuming that it was released from the mitochondria due to the altered permeability of mitochondrial membrane. This event triggers distinctive caspase cascade characteristics of apoptotic pathway, in which the caspase-3 activation plays a dominant role. In the present study, procaspase-3 (a zymogen form of caspase-3) protein, which is enzymatically inactive, was rapidly increased at 1 h after irradiation in the hippocampus of irradiated animals, but subsequently its level was decreased. The observed decrease of procaspase-3 protein in the latter time points (2 h and 4 h) markedly coincides with the elevation of cleaved caspase-3, characterized with two fragments of 17 kDa and 19 kDa which form dimers that have enzymatic activity. Therefore, the cleavage of procaspase-3, which is equated with its activation, is considered as an early hallmark of apoptosis [36]. On the basis of these results, we can conclude that cranial irradiation led to the activation of apoptotic pathway in the normal brain tissue. This is in agreement with previous animal studies, using the same dose of radiation, which have demonstrated apoptosis of hippocampal oligodendrocytes [37] or proliferating stem cells [38]. Those changes most likely lead to the radiation-diminished neurogenesis in the hippocampus [4, 39] that may result in progressive learning and memory deficits in the long-term cancer survivors [40]. It is noteworthy that these cognitive disturbances are closely related to the radiation dose [41], but even the prophylactic dose, like the one used in our study, can profoundly disturb the hippocampal-dependent behavior, learning and memory in rodents [42, 43].

The significant late sequelae associated with cranial radiotherapy have evoked the interest in finding radioprotectors that can diminish radiation injury of normal tissue without sacrificing clinical outcomes. One of them is the angiotensin converting enzyme (ACE) inhibitor, ramipril, which reduces apoptosis in the rat hippocampus after 10 Gy irradiation [44]. The

protection of normal brain cells from  $\gamma$ -irradiation induced apoptosis could also be achieved by a mitochondria-targeted triphenyl-phosphonium-nitroxide [45] or with amifostine and tempol [46]. Therefore, the future directions of our investigations will be focused on effects of different radioprotectors in the present animal model of prophylactic cranial radiotherapy.

## CONCLUSIONS

Our results exemplify the activation of p53-dependent apoptotic pathway by irradiation in the animal model system. This is followed by the induction of positive apoptosis regulator Bax, whereas antiapoptotic molecule Bcl-2 was not changed. The disbalance in Bax/Bcl-2 ratio results in early signs of apoptotic cell death, like cytochrome *c* release and caspase-3 activation. The observed changes in the hippocampus of head-irradiated animals may offer a compelling explanation for delayed cognitive dysfunctions, such as impaired learning, memory, and academic achievement, in the long-term cancer survivors.

## ACKNOWLEDGMENTS

We gratefully acknowledge Dr. Miroslav Demajo for helpful advices on the present study. This work was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia, project No. 173044.

## AUTHOR CONTRIBUTIONS

Animal handling and collecting of samples were carried out by N. A. Veličković, A. D. Djordjević, and D. R. Drakulić. The theoretical analysis was carried out by N. A. Veličković. Experiments were carried out by N. A. Veličković, A. D. Djordjević, D. R. Drakulić, I. S. Grković, and M. S. Milošević. The manipulation of irradiation facility was performed by B. Lj. Šećerov. All authors analyzed and discussed the results. The manuscript was written by N. A. Veličković and A. I. Horvat. All authors read the manuscript and agreed with its content.

## REFERENCES

[1] Gibbs, I. C., Tuamokumo, N., Yock, T. I., Role of Radiation Therapy in Pediatric Cancer, *Hematol. Oncol. Clin. North. Am.*, 20 (2006), 2, pp. 455-470  
[2] Goldsby, R. E., et al., Late-Occurring Neurologic Sequelae in Adult Survivors of Childhood Acute Lymphoblastic Leukemia: A Report from the Child-

hood Cancer Survivor Study, *J. Clin. Oncol.*, 28 (2010), 2, pp. 324-331  
[3] Conklin, H. M., et al., Cognitive Outcomes Following Contemporary Treatment without Cranial Irradiation for Childhood Acute Lymphoblastic Leukemia, *J. Natl. Cancer Inst.*, 104 (2012), 18, pp. 1386-1395  
[4] Monje, M. L., Palmer, T., Radiation Injury and Neurogenesis, *Curr. Opin. Neurol.*, 16 (2003), 2, pp. 129-134  
[5] Short, S. C., et al., DNA Repair after Irradiation in Glioma Cells and Normal Human Astrocytes, *Neuro. Oncol.*, 9 (2007), 4, pp. 404-411  
[6] Valerie, K., et al., Radiation-Induced Cell Signaling: Inside-Out and Outside-in, *Mol. Cancer Ther.*, 6 (2007), 3, pp. 789-801  
[7] Elmore, S., Apoptosis: A Review of Programmed Cell Death, *Toxicol Pathol.*, 35 (2007), 4, pp. 495-516  
[8] Donehower, L. A., Bradley, A., The Tumor Suppressor p53, *Biochim Biophys Acta*, 1155 (1993), 2, pp. 181-205  
[9] Arai, T., et al., Comparative Alterations in p53 Expression and Apoptosis in the Irradiated Rat Small and Large Intestine, *Br. J. Cancer*, 74 (1996), 3, pp. 406-412  
[10] Chow, B. M., Li, Y. Q., Wong, C. S., Radiation-Induced Apoptosis in the Adult Central Nervous System is p53-Dependent, *Cell Death Differ.*, 7 (2000), 8, pp. 712-720  
[11] Haupt, S., et al., Apoptosis – the p53 Network, *J. Cell. Sci.*, 116 (2003), Pt 20, pp. 4077-4085  
[12] Ko, L. J., Prives, C., p53: Puzzle and Paradigm, *Genes Dev.*, 10 (1996), 9, pp. 1054-1072  
[13] Scopa, C. D., et al., Bcl-2/Bax Ratio as a Predictive Marker for Therapeutic Response to Radiotherapy in Patients with Rectal Cancer, *Appl. Immunohistochem. Mol. Morphol.*, 9 (2001), 4, pp. 329-334  
[14] Finucane, D. M., et al., Bax-Induced Caspase Activation and Apoptosis Via Cytochrome *c* Release from Mitochondria is Inhibitably by Bcl-xL, *J. Biol. Chem.*, 274 (1999), 4, pp. 2225-2233  
[15] Zimmermann, K. C., Bonzon, C., Green, D. R., The Machinery of Programmed Cell Death, *Pharmacol Ther.*, 92 (2001), 1, pp. 57-70  
[16] Nicholson, D. W., et al., Identification and Inhibition of the ICE/CED-3 Protease Necessary for Mammalian Apoptosis, *Nature*, 376 (1995), 6535, pp. 37-43  
[17] Fernandes-Alnemri, T., Litwack, G., Alnemri, E. S., CPP32, a Novel Human Apoptotic Protein with Homology to Caenorhabditis Elegans Cell Death Protein Ced-3 and Mammalian Interleukin-1 Beta-Converting Enzyme, *J. Biol. Chem.*, 269 (1994), 49, pp. 30761-30764  
[18] Green, D. R., Reed, J. C., Mitochondria and Apoptosis, *Science*, 281 (1998), 5381, pp. 1309-1312  
[19] Eldadah, B. A., Faden, A. I., Caspase Pathways, Neuronal Apoptosis, and CNS Injury, *J. Neurotrauma*, 17 (2000), 10, pp. 811-829  
[20] Schunior, A., et al., An Animal Model to Study Toxicity of Central Nervous System Therapy for Childhood Acute Lymphoblastic Leukemia: Effects on Growth and Craniofacial Proportion, *Cancer Res.*, 50 (1990), 20, pp. 6455-6460  
[21] Mullenix, P. J., et al., An Animal Model to Study Toxicity of Central Nervous System Therapy for Childhood Acute Lymphoblastic Leukemia: Effects on Behavior, *Cancer Res.*, 50 (1990), 20, pp. 6461-6465  
[22] Monje, M. L., et al., Irradiation Induces Neural Precursor-Cell Dysfunction, *Nat. Med.*, 8 (2002), 9, pp. 955-962  
[23] Wang, W. H., Matthews II, K. L., Teague, R. E., Dose Rates from a Cobalt-60 Pool Irradiator Measured with

- Fricke Dosimeters, *Health Phys.*, 94 (2008), 5, Suppl 2, S44-50
- [24] Nichols, N. R., et al., Rapid Corticosterone-Induced Changes in Gene Expression in Rat Hippocampus Display Type II Glucocorticoid Receptor Specificity, *Mol. Endocrinol.*, 2 (1988), 3, pp. 284-290
- [25] Markwell, M. A., et al., A Modification of the Lowry Procedure to Simplify Protein Determination in Membrane and Lipoprotein Samples, *Anal. Biochem.*, 87 (1978), 1, pp. 206-210
- [26] Suen, D. F., Norris, K. L., Youle, R. J., Mitochondrial Dynamics and Apoptosis, *Genes Dev.*, 22 (2008), 12, pp. 1577-1590
- [27] Veličković, N., et al., Cranial Irradiation Modulates Hypothalamic-Pituitary-Adrenal Axis Activity and Corticosteroid Receptor Expression in the Hippocampus of Juvenile Rat, *Gen. Physiol. Biophys.*, 28 (2009), pp. 219-227
- [28] Veličković, N., et al., Time-Course of Hypothalamic-Pituitary-Adrenal Axis Activity and Inflammation in Juvenile Rat Brain after Cranial Irradiation, *Cell. Mol. Neurobiol.*, 32 (2012), 7, pp. 1175-1185
- [29] Qian, L., et al., Hydrogen-Rich PBS Protects Cultured Human Cells from Ionizing Radiation-Induced Cellular Damage, *Nucl Technol Radiat*, 25 (2010), 1, pp. 23-29
- [30] Borovitskaya, A. E., Evtushenko, V. I., Sabol, S. L., Gamma-Radiation-Induced Cell Death in the Fetal Rat Brain Possesses Molecular Characteristics of Apoptosis and is Associated with Specific Messenger RNA Elevations, *Brain Res. Mol. Brain Res.*, 35 (1996), 1-2, pp. 19-30
- [31] Strasser, A., et al., DNA Damage Can Induce Apoptosis in Proliferating Lymphoid Cells Via p53-Independent Mechanisms Inhibitable by Bcl-2, *Cell*, 79 (1994), 2, pp. 329-339
- [32] Basu, A., Haldar, S., The Relationship between Bcl2, Bax and p53: Consequences for Cell Cycle Progression and Cell Death, *Mol. Hum. Reprod.*, 4 (1998), 12, pp. 1099-1109
- [33] Gong, B., et al., Ionizing Radiation-Induced, Bax-Mediated Cell Death is Dependent on Activation of Cysteine and Serine Proteases, *Cell Growth Differ.*, 10 (1999), 7, pp. 491-502
- [34] Chong, M. J., et al., Atm and Bax Cooperate in Ionizing Radiation-Induced Apoptosis in the Central Nervous System, *Proc. Natl. Acad. Sci. USA*, 97 (2000), 2, pp. 889-894
- [35] Almeida, O. F., et al., Subtle Shifts in the Ratio Between Pro- and Antiapoptotic Molecules after Activation of Corticosteroid Receptors Decide Neuronal Fate, *FASEB J.*, 14 (2000), 5, pp. 779-790
- [36] Clark, R. S., et al., Increases in Bcl-2 and Cleavage of Caspase-1 and Caspase-3 in Human Brain after Head Injury, *FASEB J.*, 13 (1999), 8, pp. 813-821
- [37] Kurita, H., et al., Radiation-Induced Apoptosis of Oligodendrocytes in the Adult Rat Brain, *Neurol. Res.*, 23 (2001), 8, pp. 869-874
- [38] Peissner, W., et al., Ionizing Radiation-Induced Apoptosis of Proliferating Stem Cells in the Dentate Gyrus of the Adult Rat Hippocampus, *Brain. Res. Mol. Brain Res.*, 71 (1999), 1, pp. 61-68
- [39] Jahanshahi, M., et al., Radiation-Induced Lowered Neurogenesis Associated with Shortened Latency of Inhibitory Avoidance Memory Response, *Folia Neuropathol*, 49 (2011), 2, pp. 103-108
- [40] Monje, M., Cranial Radiation Therapy and Damage to Hippocampal Neurogenesis, *Dev. Disabil. Res. Rev.*, 14 (2008), 3, pp. 238-242
- [41] Moore, I. M., et al., Cognitive Function in Children with Leukemia, Effect of Radiation Dose and Time Since Irradiation, *Cancer*, 68 (1991), 9, pp. 1913-1917
- [42] Rola, R., et al., Radiation-Induced Impairment of Hippocampal Neurogenesis is Associated with Cognitive Deficits in Young Mice, *Exp Neurol.*, 188 (2004), 2, pp. 316-330
- [43] Raber, J., et al., Radiation-Induced Cognitive Impairments are Associated with Changes in Indicators of Hippocampal Neurogenesis, *Radiat. Res.*, 162 (2004), 1, pp. 39-47
- [44] Jenrow, K. A., et al., Ramipril Mitigates Radiation-Induced Impairment of Neurogenesis in the Rat Dentate Gyrus, *Radiat. Oncol.*, 5 (2010), 6
- [45] Huang, Z., et al., Protection of Normal Brain Cells from Gamma-Irradiation-Induced Apoptosis by a Mitochondria-Targeted Triphenyl-Phosphonium-Nitroxide: A Possible Utility in Glioblastoma Therapy, *J. Neurooncol.*, 100 (2010), 1, pp. 1-8
- [46] Citrin, D., et al., Radioprotectors and Mitigators of Radiation-Induced Normal Tissue Injury, *Oncologist*, 15 (2010), 4, pp. 360-371

Received on January 30, 2013

Accepted on April 29, 2013



**Наташа А. ВЕЛИЧКОВИЋ, Ана Д. ЂОРЂЕВИЋ, Дуња Р. ДРАКУЛИЋ,  
Бојана Љ. ШЕЂЕРОВ, Ивана С. ГРКОВИЋ, Маја С. МИЛОШЕВИЋ, Аница И. ХОРВАТ**

**ДЕЛОВАЊЕ ЗРАЧЕЊА НА ИНДУКЦИЈУ АПОПТОТСКЕ ЋЕЛИЈСКЕ  
СМРТИ У ХИПОКАМПУСУ ПАЦОВА**

Јонизујуће зрачење се често користи у третману тумора мозга али оно може да оштети когнитивне функције, као што су учење и памћење. Пошто је смањење когнитивних функција већином резултат ћелијске смрти путем апоптозе у хипокампалним ћелијама у овој студији су анализирани ефекти кранијалног гама-зрачења (10 Gy) на експресију проапоптотских (p53, Вах) и антиапоптотских молекула (Bcl-2), као и на активацију каспазе-3 и редистрибуцију цитохрома ц у хипокампусу младих пацова. Одабрани режим зрачења одговара успостављеном животињском моделу за дечију профилактичку кранијалну радиотерапију.

Наши резултати показују да је експресија p53 и РНК непромењена након зрачења, док је индукција p53 протеина веома брза. Истовремено Вах и РНК и протеин су повећани након зрачења, док је експресија Bcl-2 непромењена током испитиваног периода. Ове промене су праћене раним знацима апоптозе, као што су повећано ослобађање цитохрома ц и активација каспазе-3.

Генерално, ова студија показује да је кранијално зрачење повезано са стимулираним апоптотским путем у хипокампусу пацова, што се може повезати са когнитивним оштећењима уочених код пацијената након профилактичке кранијалне радиотерапије, али такође отвара перспективе у проналажењу радиопротектора који смањују оштећење нормалног нервног ткива након зрачења.

*Кључне речи: кранијално зрачење, айџиџоза, хипокампус, пацов*

---