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ORIGINAL ARTICLE

Acute radiation effect in the brain in infant rats: Neurocognitive and histopathological examination

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Summary

Purpose: *The aim of the present study was to evaluate the radiation-induced cognitive dysfunction and the radioprotective effect of amifostine (AMI) in the brain of infantile rats.*

Methods: *Thirty 2-week-old rats were randomly assigned into 3 groups of 10 rats each. Group 1: control (CONT), group 2: radiation alone (RT), and group 3: AMI before radiation (AMI+RT). The rats in the RT and AMI+RT groups were irradiated individually with a single dose of 20 Gy. All animals were evaluated by using the Morris water maze test to evaluate of their cognitive functions. Histopathological analyses of the hippocampus were also carried out after euthanasia.*

Results: *The study showed that the place navigational function and the spatial probe test were not significantly different between the groups.*

Conclusion: *It can be said that it is very important to determine when the radiation-induced brain injury is formed. From a clinical perspective, the patients can be intervened before irreversible functional deficits are formed and may be amenable to treatment.*

Key words: amifostine, cognitive dysfunction, irradiation, radioprotection

Introduction

RT is an important treatment modality in pediatric oncology. Brain injury occurring in the irradiation field in growing children is one of the most important dose-limiting factors of RT. The relationship of cranial irradiation (CRT) with late cognitive dysfunction is well recognized in children, but the treatment of this complication is unknown [1]. Prophylactic use of radioprotectants prior to RT is an important strategy for RT-induced brain injury in these patients.

The effect of ionizing radiation is primarily mediated through the action of free radicals, which can cause damage to DNA, proteins, and lipids [2]. Therefore, it can be stated that most of the radiation damage is caused by antioxidative defense mechanisms. Amifostine (S-2{3-aminopropylamino-ethylphosphorothioic acid; Ethyol; WR-2721) is a prodrug that is converted *in vivo* by alkaline phosphatase to an active sulfhydryl compound (WR-1065). Normal cells are selectively protected by this substance from antineoplastic drug toxicity by scavenging free radicals, by donating hydrogen ions to free radicals, by depleting oxygen, and by binding to active derivatives of antineoplastic agents [3,4].

In their previous work, Lamproglou et al. demonstrated the difference between the response to the radiation observed in young rats and that observed in old rats. It was found that young rats showed an earlier decrease in learning and memory than old rats, and this deficit was followed by partial recovery [5].

In this study, the radioprotective effect of amifostine on radiation-induced acute brain injury in 2-week-old rats was evaluated.

Methods

Animals and experimental design

All animal experiments adhered to the guidelines of the Institutional Animal Ethics Committee. Infant rats were housed with their mothers until 4 weeks-old, and then were housed in rat cages with *ad libitum* access

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to a standard rodent diet and tap water, with a 12:12-hr artificial light cycle, mean temperature 21±2° C, and mean humidity 55 ± 2%. When they reached 2 weeks of age, all animals were randomly assigned into 3 groups of 10 rats each, for the following treatments:

Group 1: Control (CONT), injected with normal saline (200 mg/kg) by intraperitoneal injection (i.p.) 30 min before a sham irradiation;

Group 2: Irradiation alone (RT), injected i.p. with normal saline (200 mg/kg) 30 min before irradiation;

Group 3: Amifostine before irradiation (AMI+RT), injected i.p. with amifostine (200 mg/kg) 30 min before irradiation [3].

All experimental procedures were performed on anesthetized rats. Anesthesia was maintained with ketamine and xylazine (35 mg/kg body weight [BW] and 3 mg/kg BW, i.m. for infant rats) during irradiation. The follow-up period was 45 days. During follow-up, all rats were monitored by the veterinary care staff.

Irradiation

The rats in AMI+RT and RT groups were irradiated individually with a single dose of 20 Gy. The rats were anesthetized and then fixed onto a 20×30 cm blue Styrofoam treatment couch (Med-Tec, Orange City, IA) in a lateral position. The entire brain was irradiated using two parallel-opposed, equally weighted lateral fields (5×5 cm at source-axis distance 80 cm). Individual lead blocks were used to shield the oral cavity, pharynx, larynx, nasal and paranasal cavities and eyes. Correct positioning of the fields was controlled for each individual rat using a therapy simulator (Mecaserto-Simics, Paris, France). A 1 cm thickness of equivalent tissue was positioned on the head of the rat to improve dose distribution in the brain. The dose distribution was calculated by the physics department. Special dosimetry was done for the irregular fields. The dose homogeneity across the field was ± 5%. Control rats were also anesthetized daily and received a sham CRT. After irradiation, the rats were housed under identical experimental conditions and examined every week.

Procedure

Morris water maze consisted of a circular pool (diameter 150 cm, height 60 cm) filled with water 50 cm depth. The water (22° C) was made opaque with a black dye. The pool was located in a room having some cues on its walls, and was virtually divided into 4 equal quadrants as northwest (NW), northeast (NE), southeast (SE) and southwest (SW). A platform (10×10 cm) was placed into the pool in the SW quadrant 2 cm below the water surface. Animals were trained for 5 days with 6 daily trials (acquisition phase). On day 6, the platform was removed, and the animals swam for 45 sec (retention phase). The swimming sessions were recorded and analyzed by a video-tracking system (Noldus, Ethovision XT, The Netherlands).

Acquisition phase

During the first 5 test days, the platform was located in the SW quadrant, and the rats were given a series of 6 daily trials (intertrial interval 10 min). The animals were then released facing the wall from one of the starting points (NW, NE, SE) in a randomized order. When a rat reached to the platform, it was allowed to remain for 10 sec to explore the environment before it was taken back to its cage. If the rat failed to find the platform within 120 sec, it was placed on it by the experimenter. The following parameters were examined to evaluate the acquisition phase: latency to reach to platform, distance moved to reach to platform, mean distance to platform.

Retention phase

On day 6, the platform was removed, and the animals swam for 45 sec (probe test). The following parameters were examined to evaluate the retention phase: latency to reach to platform area, distance moved to reach to platform area, mean distance to platform area, duration in target quadrant, latency to reach target quadrant.

Euthanasia

The rats were euthanasised 45 days after RT. Prior to euthanasia, the rats were anesthesised using ketamine and xylazine combination. Euthanasia was performed by decapitation.

Histopathological analysis

The brains were dissected and fixed in paraformaldehyde solution for 2 days before being embedded in paraffin. Tissue sections (6 μm thick) were cut sequentially along with a coronal profile and stained with standard H&E procedures.

Statistical analysis

Intra- and inter-group comparisons for the acquisition and retention phase analysis were made using 2-way ANOVA and *post hoc* Bonferroni test. Statistical analyses were made by Graphpad Prism 5 for MacOSX. Differences were considered significant when the probability was less than 0.05.

Results

All rats showed normal daily activities, including feeding and drinking. No paralysis and convulsions were observed, and BW gain was no different among the groups. The study showed that the place navigational function and the spatial probe test were not significantly different between the groups.

Latency to reach to platform

In the acquisition phase, latency to reach to the platform was continuously decreased, and reached an asymptote on day 4 in all groups (Figure 1A). On day 5, the mean time latency to reach to the platform was 14.04±1.06 sec in the RT group, 14.71±1.33 sec in the CONT group and 10.93±0.89 sec in the RT+AMI group. No statistically significant difference was observed among groups.

Distance moved to reach the platform

In the acquisition phase, the distance moved to reach to the platform was also continuously decreased, and reached an asymptote on day 4 in all groups (Figure 1B). On day 5, the mean distance moved to reach the platform was 324.02±34.25 cm in the RT group, 308.92±24.34 cm in the CONT group and 215.60±23.62 cm in the RT+AMI group. No statistically significant difference was observed among groups.

Mean distance to platform

In the acquisition phase, the mean distance to the platform was continuously decreased, and reached an asymptote on day 4 in the RT and CONT groups (Figure 1C). On day 5, the mean distance to platform was 28.01±1.53 cm in the RT group, 30.45±1.56 cm in the CONT group and 24.40±1.93 cm in the RT+AMI group. No statistically significant difference was observed among groups.

Latency to reach the platform area

In the retention phase, latency to reach the platform area was 12.98±4.67 sec in the CONT group, 11.82±3.99 sec in the RT group, and 9.68±1.82 sec in the RT+AMO (Figure 2A). No statistically significant difference was observed among groups.

Distance moved to reach the platform area

In the retention phase, the distance moved to reach the platform area was 46779±3248 cm for the CONT group, 53491±4660 cm for the RT group, and 46853±3586 cm for the RT+AMI group (Figure 2B). No statistically significant difference was observed among groups.

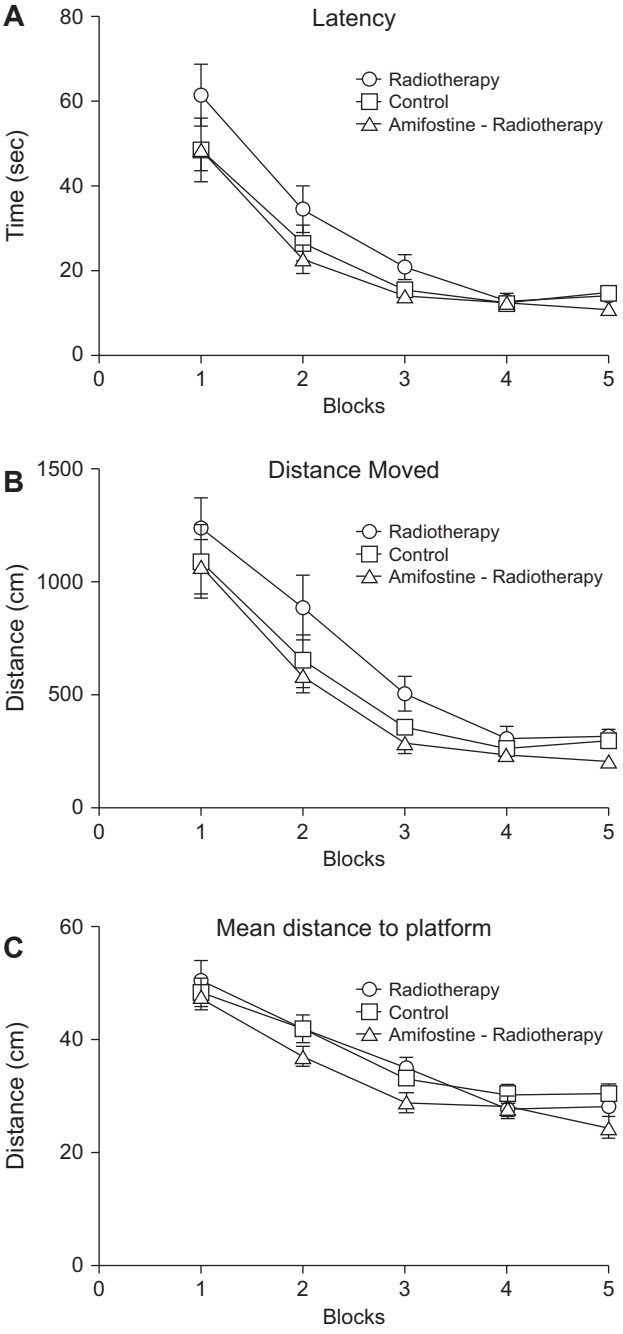

Figure 1. A: Latency to reach the platform. Each block represents an average of 6 trials (n=10). Vertical bars indicate standard error of the mean. **B:** Distance moved to reach the platform. Each block represents the average of 6 trials (n=10). Vertical bars indicate standard error of the mean. **C:** Mean distance to platform. Each block represents the average of 6 trials (n=10). Vertical bars indicate standard error of the mean.

Mean distance to reach the platform area

In the retention phase, the mean distance to reach

the platform area was 41.54±2.89 cm for the CONT group, 47.51±4.14 cm for the RT group, and 41.61±3.19 cm for the RT+AMI group (Figure 2C). No statistically significant difference was observed among groups.

Duration in target quadrant

In the retention phase, the duration in target quadrant was 19.30±1.83 sec for the CONT group, 15.39±2.33 sec for the RT group, and 19.46±2.16 sec for the RT+AMI group (Figure 2D). No statistically significant difference was observed among groups.

Latency to reach the target quadrant

In the retention phase, latency to reach the target quadrant was 4.92±1.24 sec for the CONT group, 3.44±0.85 sec for the RT group, and 4.85±0.77 sec for the RT+AMI group (Figure 2E). No statistically significant difference was observed among groups.

Swim velocity

While swim velocity was not a parameter of learning and retention, it was used in order to understand whether there was a difference between the swimming

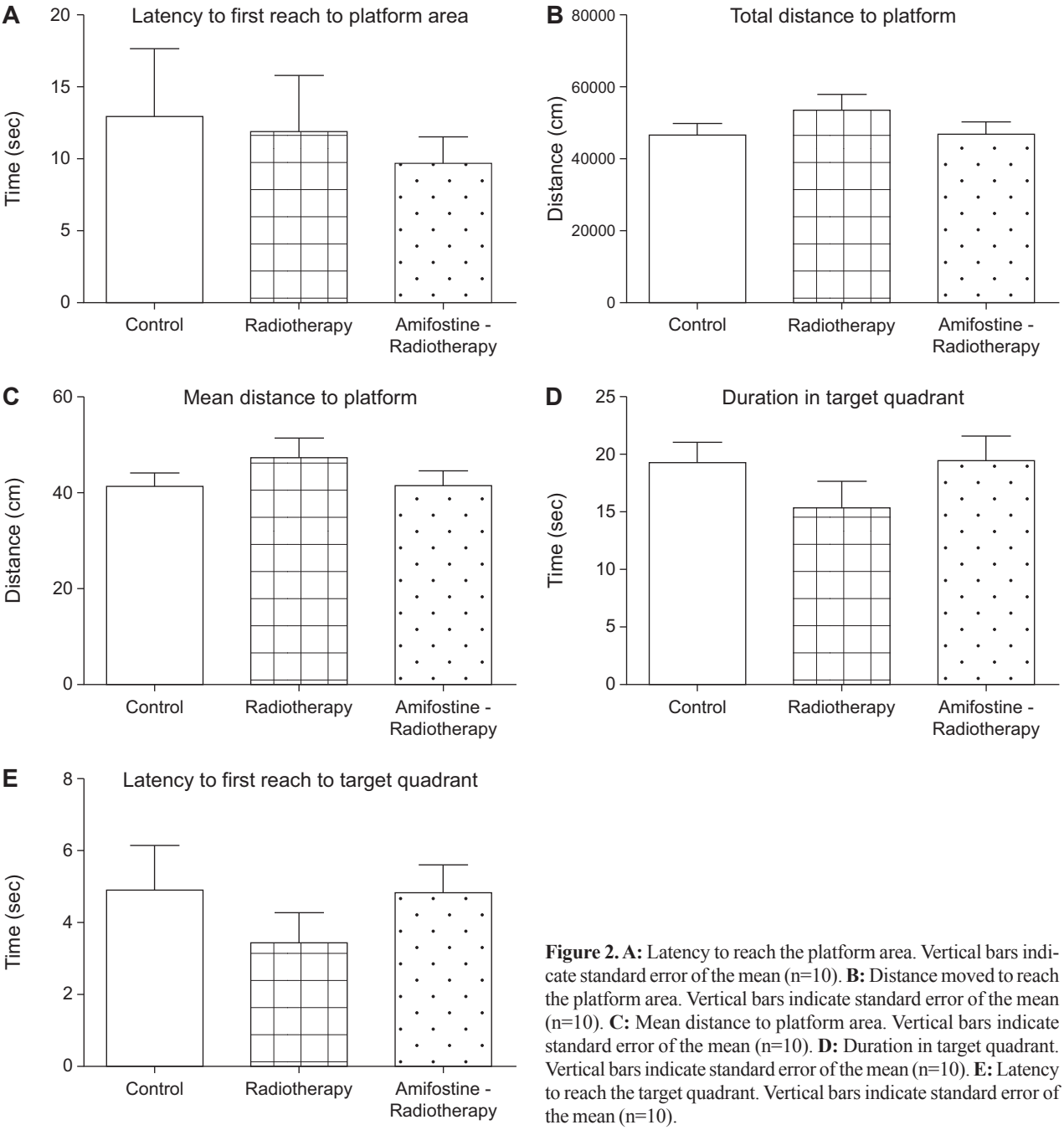

Figure 2. A: Latency to reach the platform area. Vertical bars indicate standard error of the mean (n=10). **B:** Distance moved to reach the platform area. Vertical bars indicate standard error of the mean (n=10). **C:** Mean distance to platform area. Vertical bars indicate standard error of the mean (n=10). **D:** Duration in target quadrant. Vertical bars indicate standard error of the mean (n=10). **E:** Latency to reach the target quadrant. Vertical bars indicate standard error of the mean (n=10).

rates of the animals and in order to prevent the difference in swimming rates from affecting the test performance. Swim velocities of rats showed no statistically significant difference among the groups during the acquisition phase and in probe tests (Figure 3, A and B, respectively).

Pathologic results

After light microscopy studies no changes could be observed in the hippocampal region, such as partial loose and irregular arrangement of neurons and vascular degeneration, in the parietal white matter near the cortex in the CONT, RT, and AMI+RT groups during 30 days.

Discussion

It was found that RT-induced acute brain damage occurred during or a few days after RT and represents a very important problem for patients who receive brain RT. These patients often show a deterioration of almost all domains of memory. To better understand the pathogenesis of early changes after brain RT, it is very important to establish an animal model that allows detailed examinations of cognitive dysfunction along with the accompanying histopathologic changes.

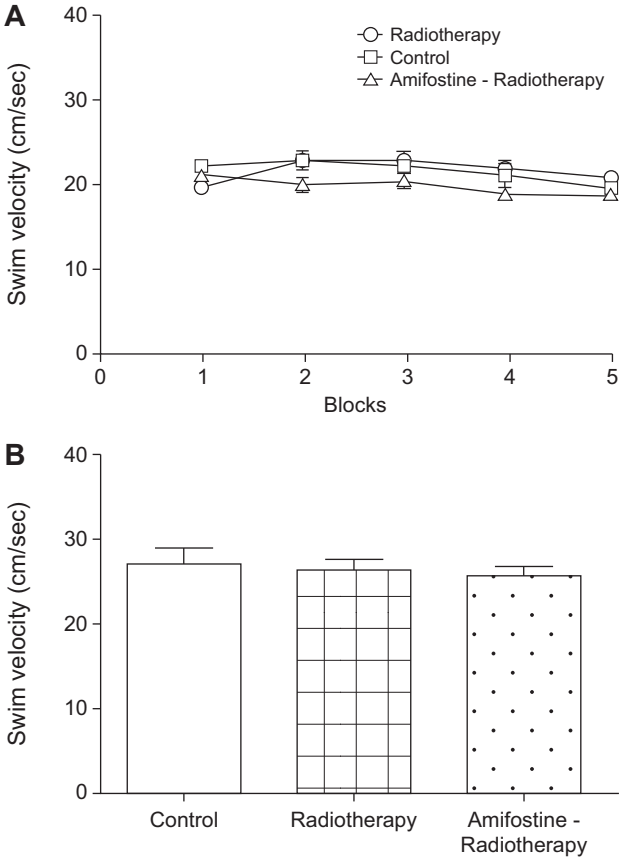

Figure 3. A: Swim velocity during the acquisition phase. **B:** Swim velocity in probe test. Vertical bars indicate standard error of the mean (n=10).

The mechanisms underlying acute brain damage after cranial RT may involve a treatment-induced brain edema or an interruption of the blood-brain barrier. Capillaries and arterioles are the most radiosensitive components of the vasculature, and endothelial cells are regarded as the most radiosensitive cells of the vessel wall [6]. Two hypotheses of late radiation injury in the CNS have been proposed. The vascular hypothesis indicates RT-induced vascular injury, accelerated atherosclerosis and mineralizing microangiopathy, which result in vascular insufficiency and infarction. The glial hypothesis suggests RT-induced ablation of glial precursors and resultant demyelinative necrosis. However, both hypotheses fail to adequately explain the fact that most patients with significant cognitive deterioration exhibit no signs of overt vasculopathy or demyelination [7].

Hippocampal dysfunction is a prominent feature of RT-induced neuropsychological sequel. Indicators of the pathogenesis of RT-induced cognitive decline can be obtained by a careful examination of physiologies unique to the hippocampus. One such attribute is neurogenesis. Throughout life, neural progenitors provide new neurons to the adult hippocampus and irradiation interferes with neurogenesis [8,9]. The Morris water navigation task is a behavioral procedure widely used in behavioral neuroscience to study spatial learning and memory. It was developed by the neuroscientist Morris in 1981 [10], who used it to demonstrate that lesions of the hippocampus impaired spatial learning [11].

Located in the medial temporal lobes, the hippocampus is central to short-term declarative memory and spatial information processing [9,12]. Neural stem cells, which are self-renewing cells that generate neurons, astrocytes and oligodendrocytes, reside in the hippocampus [13,14], and produce new dentate granule neurons in all vertebrates studied, including humans [15]. Compelling evidence in animal models supports the importance of hippocampal neurogenesis to normal cognitive function. Manipulations that decrease neurogenesis, such as chemotherapy [16] or glucocorticoid exposure [17], impair an animal's performance in hippocampal-dependent behavioral tasks. Conversely, factors that increase neurogenesis, such as running, improve hippocampal performance [18].

Work in animal models has demonstrated that exposure to therapeutic doses of irradiation results in an increase in apoptosis, a decrease in cell proliferation and a decrease in stem/precursor cell differentiation into neurons within the neurogenic region of the hippocampus [19,20]. The additional concern is a massive microglial

inflammatory response in the neurogenic region of the hippocampus related to irradiation. Monje et al. showed that rats treated with a single dose cranial radiation of 10 Gy only made 3% of the new hippocampal neurons that would be produced in a normal animal. The deficit in cell proliferation could simply be the result of ablation of the precursor pool. Alternatively, the precursors may survive irradiation but may not divide because of intrinsic damage or lack of extrinsic mitogenic signals. The direct isolation and culture of hippocampal stem/precursor cells from rat brain 1 month after irradiation with 2 or 10 Gy clearly showed that an equivalent number of stem/precursor cells could be isolated from irradiated and non-irradiated hippocampi (regardless of dose), suggesting that acute ablation of the stem/precursor cell population does not occur. However, stem/precursor cells from 2 Gy-irradiated animals were delayed in their *in vitro* growth, and cells of 10 Gy-irradiated hippocampi failed to expand at all in culture, possibly because of RT-induced DNA damage and subsequent mitotic catastrophe. Therefore, the profound decrease in proliferative cells *in vivo* in the months after irradiation probably results from a combination of acute cell death and decreased proliferative potential of the remaining cells [8].

Behavioral studies on the acute effects of cranial irradiation are relatively rare. To our knowledge, only few reports have established cognitive dysfunction in rats exposed to RT, but have mainly focused on late effects of RT. Hodges et al. reported RT-induced deficits in a T-maze forced choice alteration and a subsequent dose-dependent water maze deficit during a period of 44 weeks. They indicated that local cranial RT with a 20 Gy dose of x-rays could produce a cognitive deficit in adult rats without evidence of pathologic changes. However, they could not find acute behavioral and neuropathologic effects [21].

Lamproglou et al. reported that a memory deficit was seen in 4-month-old Wistar rats one month after 30 Gy RT. They found that the response to RT observed in young rats differed from that observed in old rats. Young rats showed an earlier decrease in learning and memory than old rats, and this deficit was followed by partial recovery [5].

In another study which demonstrated the recovery of learning and memory after failing in the acute phase following cranial RT in young rats, Liu et al. showed that 20 Gy irradiation led to a temporary impairment of the place navigation function as evidenced by their significantly increased latency ($p<0.05$) and swimming distance ($p<0.05$) compared with the sham control on the 7th and 20th days, but not on the 0th and 60th days. Moreover, spatial probe test results were found to be in line with the place navigation results. Irradiation with 20 Gy was determined to cause a transient impairment on the 7th and 20th days ($p< 0.05$). The duration of the deficit was at least 2 more weeks in the 20 Gy group. In the histopathological evaluation carried out using H&E staining, no pathologies were found [22]. In our study, no statistically significant impairment of memory and learning was determined in the rats after 30 days following the 20 Gy of irradiation.

In the study of Naylor et al., 6 Gy of cranial RT was applied to rats in the 9th postnatal day. Progenitor proliferation was analysed at 36 h after RT and a severe loss (85%) of proliferating cells was observed throughout the entire hippocampal formation in the developing brain. While a statistically significant loss was determined in rats after 8 weeks in the precursor cell pool and in neurogenesis in the whole hippocampal formation, no difference was found in running wheel activities. They reported that in the P9 mouse, the hippocampus was still undergoing development. It may be assumed that the young developing brain, with a higher level of neurogenesis, is more plastic and could more easily compensate for morphological damage [23]. This explanation may support our results. So we think that a few reasons such as inflammation, apoptosis, and decrease in the progenitor proliferation may deteriorate the hippocampal function in acute phase. However, precursor cell pool immediately activates but it is enough for just a period of time. Therefore, late effects were observed in hippocampal disfunction.

As a prospective study, our laboratory aims to examine the neurocognitive changes in the 3rd, 6th, 9th, and 12th months postirradiation. In this way, it is aimed to determine the times when interventions, such as environmental enrichment or physical exercise, can be more effective.

In conclusion, it can be said that it is very important to determine when the RT-induced brain injury is formed. From a clinical perspective, the patients can be intervened before irreversible functional deficits are formed and may be amenable to treatment. We believe it is important that a small number of stem cells survive RT to participate in the restructuring process.

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