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Investigation of the neuroprotective effect of Granisetron through SV2A and 5-HT3 modulation in a radiation-induced brain injury rat model

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► ***Original article***

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ABSTRACT

***Background*:** The development of neurotoxicity in healthy, non-targeted brain tissue exposed to radiation during cranial radiotherapy (RT) is the most frequent event of radiation-induced adverse effects. The 5-hydroxytryptamine-3 (5-HT3) receptor antagonists may also have a range of neuroprotective, anti-inflammatory, and antiphlogistic properties in addition to their anti-emetic effects. ***Materials and Methods*:** Study groups were formed in the following ways: Group 2: Irradiation (IR)-only (IR+Saline); Group 1: Normal control (orally fed control); Group 3: IR+Granisetron (IR+Granisetron): whole-brain IR and Granisetron 1 mg/kg/day (Merck) administered orally. 15 days of all therapies were given. The 15 days were completed with behavioral testing. In the entire brain IR-only (placebo) group, a substantial deterioration was seen in all studied marker levels and behavioral test results. ***Results*:** Compared to the IR-only group, all of these biochemical indicators significantly improved in the granisetron group (IR+Granisetron), and levels of the control group returned to normal. In behavioral test analyses, a substantial decline in the open field and passive avoidance learning social recognition tests was seen in the IR-only group compared to the healthy control group, whereas an improvement was seen in the IR+Granisetron group. In addition, the IR-only group showed a reduction in hippocampus neurons and Purkinje neurons as well as an increase in hippocampal gliosis, whereas the IR+Granisetron group showed an improvement and a return to the normal control group counts. ***Conclusion*:** In summary, we discovered that granisetron had neuroprotective properties in a rat model of radiation-induced brain damage.

INTRODUCTION

The most common sequence of radiation-induced side effects during cranial radiotherapy (RT) occurs due to neurotoxicity development in healthy non-targeted brain tissue exposed to radiation [(1)]. Granisetron, a 5-hydroxytryptamine-3 (5-HT3) receptor antagonist, is widely used to treat nausea and vomiting caused by chemotherapy, where the 5-HT3 receptor is a vital neurotransmitter engaged in the regulation of a broad spectrum of peripheral and central functions ranging from immunity to cognitive functions. Besides anti-emetic functions, it has been suggested that the 5-HT3 receptor antagonists possess a variety of neuroprotective, anti-inflammatory/antiphlogistic features.

The synaptic vesicle glycoprotein 2 (SV2) family may play various roles to promote vesicular function properly. A member of this family synaptic vesicle glycoprotein 2A (SV2A) plays a vital role in neuronal excitability. In addition, SV2A as a synaptic density marker has become the gold standard for the diagnosis of brain disorders. The aim of this study was to reveal the neuroprotective effect of granisetron in a radiation-induced brain injury developed rat model, through SV2A and 5-HT3 modulation and brain inflammatory markers.

Adult and pediatric neuro oncologic patients are often treated with radiation treatment for both primary and metastatic brain tumors [(1-3)]. RT employs high-energy ionizing radiation that damages DNA and kills cells [(4)]. Cellular stress is brought on by exposure to ionizing radiation either directly or indirectly. This stress is brought on by the production of reactive oxygen species, DNA damage, and damage to subcellular organelles including the endoplasmic reticulum and mitochondria [(5)].

Healthy brain tissue is inevitably exposed to

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radiation during routine RT applications including fractionated partial- and whole-brain radiation therapy (PBRT and WBRT) [1]. Even when the current radiation methods and fraction schemes are used to try and decrease the amount of irradiated healthy brain tissue, a sizeable amount of healthy brain tissue receives a low dose treatment. Depending on the intended RT dosage to the target areas, the dose to the normal brain that was off-target might be anywhere from 0.1 and 20 Gray (Gy). The therapeutic dosage for stereotactic radiosurgery (SRS) is typically 20 Gy in a single fraction and 2 Gy per fraction for standard fractionated RT [4].

Many patients experience side effects associated with damage to healthy brain tissue as a result of ionizing radiation exposure, including hippocampal-related learning and memory impairment [6], focal neurological deficits [7], increased intracranial pressure [8], and rarely secondary epilepsy [9] and progressive dementia [10]. Learning, processing speed, memory, executive function, and attention are among the cognitive areas that are impacted [1, 10].

There are three types of radiation-induced brain damage: acute, early-delayed, and late-delayed [11]. Acute and early-delayed damage often appear days to months after treatment and are temporary, but late-delayed injuries appear at least six months after radiation and are deemed permanent and progressive. Dexamethasone usually helps an acute injury, which is characterized by edema, headaches, and fatigue. Transient demyelination, sleepiness, inability to pay attention, and loss of short-term memory are the symptoms of the early-delayed reaction. White matter necrosis, vascular anomalies, more severe chronic demyelination, gliosis, and long-term cognitive impairment are all symptoms of late-delayed damage [1].

Granisetron, a 5-HT3 receptor antagonist, is frequently used to alleviate chemotherapy-related nausea and vomiting [12-13]. The mechanism of 5-HT3 antagonists' anti-emetic activity is mostly based on their capacity to inhibit 5-HT3 receptors, which are thought to be essential to the afferent portion of the emetic reflex [14]. An ion channel that is ligand-gated and raises intracellular cations like calcium (Ca+2), sodium, and potassium is the 5-HT3 receptor. Neurons are quickly and briefly depolarized and excited when the 5-HT3 receptor is stimulated, and 5-HT3 receptor antagonists like granisetron immediately counteract these effects by inhibiting the receptor [15].

A neurotransmitter and essential signaling molecule, 5-HT3 is both. It is present in the immunological-inflammatory axis, which affects animals' immune responses [16]. Additionally, the 5-HT3 receptor subtype, which is present in both pre- and post-synaptic areas, regulates the release of other neurotransmitters like as glutamate, dopamine, acetylcholine, gamma-aminobutyric acid (GABA), and 5-HT itself [17]. The 5-HT3 receptor antagonists were first created as quick-acting drugs to combat chemotherapy-induced emesis, but data from both human and animal research shows they also have a range of neuroprotective and antiphlogistic characteristics [18].

Regardless of the presence of neurotransmitters, all synaptic terminals include the transmembrane protein known as SV2A [19]. Levetiracetam, an anti-epileptic medication, specifically binds to SV2A, and as a result, interest in SV2A rose [19]. This finding led to an upsurge in SV2A research in memory. Studies examine the regional densities of synaptophysin and SV2A in addition to synaptic density measurements. They find a linear association between the two proteins in regions of gray matter and recommend using SV2A as a synaptic density marker instead of synaptophysin [20]. Although SV2A is expressed everywhere, some brain areas showed higher connections between SV2A and GABAergic synapses than with glutamatergic synapses [19].

As far as we are aware, there has not been a previous study that investigated the neuroprotective effects of granisetron in a rat model of radiation-induced brain injury or clinically, and also evaluated the relationship between SV2A and 5-HT3 modulation and brain inflammatory markers mechanistically. This fact demonstrates the originality of our research. The aim of this study was to evaluate the therapeutic potential of granisetron against irradiation (IR)-induced neurotoxicity in a 20 Gy single dose whole brain irradiated rat model. For this purpose, granisetron treatment was applied to rats in addition to the irradiation (IR) process, and its mechanism tried to be explained by examining brain inflammatory markers and SV2A and 5-HT3 changes.

MATERIALS AND METHODS

Animals

21 female Wistar albino rats, 10–12 weeks old, weighing 150–200 g, were utilized in this investigation. The guidelines of the National Institutes of Health's Guide for the Care and Use of Laboratory Animals were followed when conducting the research for this study (U.S.A), having obtained approval from the Animal Ethics Committee (Science University, Ethical number: 28220713). The rats utilized in the experiment were from Science University's Experimental Animal Laboratory. Rats were kept singly in steel cages with a temperature-controlled environment (22±2 °C) and 12-hour light/dark cycles, and they were fed ad libitum.

Experimental procedures

21 female Wistar albino rats were taken to study. Whole-brain IR was performed on 14 rats to develop

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a radiation-induced brain injury model. Granisetron drug administration was started 7 days ago in the rats who underwent whole brain IR and continued for 15 days after IR. Rats were divided into 3 groups: a normal control group, a placebo group IR-only (IR+Saline), and a group IR+Granisetron administered.

Study groups were designed as follows: Group 1: Normal control (orally fed control, n=7); Group 2: IR-only (IR+Saline, n=7): Whole-brain IR and 1 ml/kg/day % 0.9 NaCl saline group via oral gavage, Group 3: IR+Granisetron (IR+Granisetron, n=7): Whole-brain and Granisetron 1 mg/kg/day (G3796, Merck, USA) via oral gavage. All treatments were administered for 15 days. The 15 days were completed with behavioral testing. The entire behavioral research was carried out between 10:00 AM and 3:00 PM. All rats were killed at the conclusion of the experiment via cervical dislocation under anesthesia (Ketasol, Richterpharma AG, Austria; 100 mg/kg; and xylazine, Rompun, Bayer, Germany; 50 mg/kg each). Rat brain tissue was taken out for histological analysis.

Irradiation procedure

Simulation of a rat was done with a 1 mm slice computerized tomography (CT) scan (CT simulator GE Bright Speed), and the images were transferred to Eclipse (Varian Medical Systems, Palo Alto, CA, USA) treatment planning system (TPS). The dose calculation was performed with the Anisotropic Analytical Algorithm (AAA) system version 13.7.20. The prescription dose of 20 Gy was chosen which corresponds to a biologically effective dose (BED) of 153 Gy for α/β =3, late responding tissue (for α/β =10, early responding tissue and tumor BED=60 Gy). On the CT images at TPS, the rat brain tissue was determined by the radiation oncologist, and the dose prescribed to the whole brain tissue was to be received 98% of the IR dose 20 Gy. For the IR plan source skin distance (SSD) was set as 100 cm and 6 megavoltage (MV) energy was chosen. An open treatment field is designed for whole brain IR while the body except the IR area is protected by the multi-leaf collimators (MLCs). Finally, the skin dose on the beam entrance was taken into consideration before approving the IR plan.

A plexiglass apparatus is designed to immobilize anesthetized rats properly under the IR field and to minimize the uncertainty in animal experiments (figure 1). Anesthetized (50 mg/kg ketamine and 10 mg/kg xylazine intraperitoneally) rats stabilized at the treatment couch in the prone position inside the IR apparatus (plexiglass 1,18g/cm^3) specially designed were subjected to whole-brain IR with a single dose of 20 Gy at a dose rate of 1 Gy/min, with 6MV photons using a linear accelerator (Clinac DHX, Varian Medical Systems, Palo Alto, CA, USA), while SDD is 100 cm on the surface of the body. Indoor laser and SSD techniques were used for the animal IR setup. Rats were returned to their home cages following the IR.

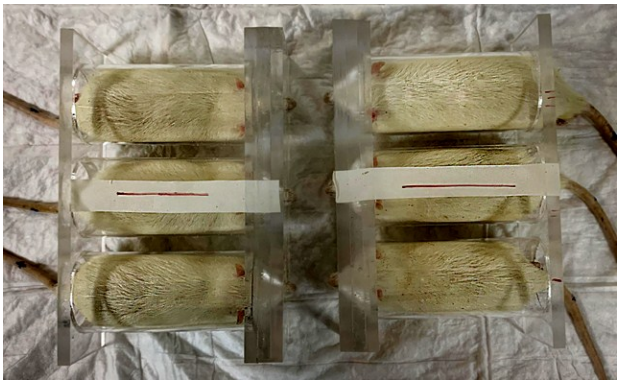


Figure 1. Representation of the set-up, positioning and stabilizing of the rats with the plexiglass apparatus on the treatment couch before IR.

Behavioral tests

Three-chamber sociability examination

Minor adjustments to the previously reported sociability test were made [21] (figure 2). Briefly, three equal sections were created by dividing a 40 cm by 90 cm by 40 cm Plexiglas cage (40 cm, 30 cm, 40 cm). The rats were given five minutes to become used to being in the test cage on day one (pre-test session). A stranger rat (Stranger 1) was put into a tiny plastic cage with mesh-like apertures in one side chamber and an empty cage in the third compartment twenty-four hours later to evaluate friendliness. The test rat was then placed in the central chamber, and for 10 minutes, the time it spent in each area was recorded (Session I). When the test rat's head and two front paws entered the chamber, it was deemed to have entered. In order to get rid of any smells left over from the previous rat, the field floor was wiped with 70% alcohol and dried with a paper towel in between each test. The proportion of time spent with Stranger was computed.

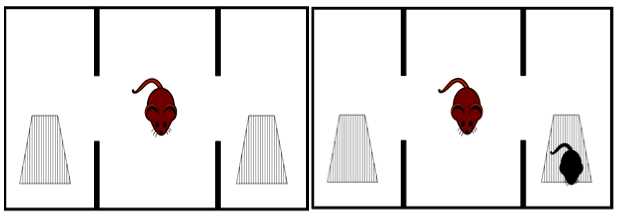


Figure 2. Demonstration of the three-chamber sociability test analysis system.

Open-field test

One of the most widely used behavioral assays to evaluate movement and exploration is the open-field (OF) paradigm. While it is very easy to spot altered OF behavior, it is more difficult to determine the causes of the changes. The behavior in this paradigm is often influenced by two factors: the first is a positive exploration drive resulting from rats' natural want to discover new settings (for food and refuge),

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and the second is the biological instinct to avoid open, highly lighted places (exposure to predators). The OF test is said to be helpful in identifying repetitive auto grooming, motor stereotyped behavior, and limitation of research activities in the autism paradigm [21]. An open-aired box with measurements of 50 cm, 50 cm, and 40 cm was used to conduct the OF test. Rats were put gently in the center of the box at the start of the experiment and given 5 minutes to freely explore the space. After then, each rat was monitored for 5 minutes to gauge its degree of spontaneous activity. It was noted how many floor divisions were traversed by all four paws during ambulation. In order to minimize olfactory signals, the field floor was then wiped with a 70% alcohol-water solution and dried with a paper towel between each animal.

Passive avoidance learning (PAL) analysis

Rats' learning and memory abilities were assessed using the passive avoidance learning (PAL) test [22]. The rat learns to avoid entering a door that appears to be safer but instead goes into a dark compartment with an electric grid system that shocks it through passive avoidance activities that are driven by fear. The PAL box included chambers that were both dark and lit and measured 20 cm 20 cm 20 cm. Rats normally chose to enter the dark room when they were placed in the illuminated compartment. The guillotine door between the light and dark chambers was opened after a 10-s habituation time in the lit compartment. The door dividing the light and dark chambers was shut when a rat entered the dark chamber. The rat was then taken out of the dark chamber and put back in its cage after receiving a 1.5 milliampere (mA) electric shock for a period of three seconds. The rats were put back into the PAL box after twenty-four hours. Although no shock was administered, the amount of time it took the rat to move from the light to the dark chamber was timed and recorded. It was possible to measure the delay for up to 300 s. The rat's memory was measured by the amount of time it took to decide against entering the shadowy chamber.

Histopathology of the hippocampus and the cerebellum

The target locations to check for hippocampal injury were the Cornu Ammonis (CA) 1 and CA 3 regions of the hippocampus and cerebellum. Rats were killed, their brains were taken, and they were preserved for three days in 10% formaldehyde in 0.1 M phosphate buffer saline. This was done after the behavioral testing (PBS; Gibco, Carlsbad, CA). They were after that transferred into 30% sucrose and kept at 4 °C until infiltration was finished. The brains were coronally sectioned at a 40 m depth using a sliding microtome, then placed on gelatinized glass slides. Brain slices were treated with H2O2 (10%) for 30 min to remove endogenous peroxidase activity before being blocked with 10% normal goat serum (Invitrogen, USA) for 1 h at room temperature for glial fibrillar acidic protein (GFAP) immunohistochemistry.

In the following step, slices were incubated with primary GFAP antibodies for 24 hours at 4°C (Abcam, Inc., MA, USA; 1/1000). Using the Histostain-Plus Bulk kit from Invitrogen (USA), antibodies against rabbit IgG were detected, and the finished result was visualized using 3,3′ diaminobenzidine (DAB). PBS was used to wash each segment before photos were taken using an Olympus C5050 digital camera attached to an Olympus BX51 microscope. In randomized sections (3–4) for each rat, GFAP-positive cells were counted at a magnification of 40 to determine the GFAP immunostaining index. The Nissl substance in the cytoplasm of neurons in paraformaldehyde or formalin-fixed tissue is stained with Cresyl Violet Acetate solution (Merck, USA). The neuropil will have a granular purple-blue stain on it. The same image analysis method used cresyl violet labeling to count the number of surviving neurons in six sections per study group. The same researcher, who was unaware of the study groups, conducted each histological investigation. As previously mentioned, this technique was carried out using an image analysis system (Image-Pro Express 1.4.5, Media Cybernetics, Inc. USA) in six portions for each group under study [21].

Biochemical evaluation of the brain samples

Brain tissues were quickly collected after decapitation and kept at 20 °C until biochemical examination. Whole brain tissues were homogenized using a glass homogenizer in 5 vol of PBS (pH 7.4) and centrifuged at 5000g for 15 min to prepare them for analysis. Following the collection of the supernatant, Bradford's technique was used to quantify the total protein content in the brain homogenates using bovine serum albumin (Merck, USA) as a reference [23].

Using rat enzyme-linked immunosorbent assay (ELISA) kits that are available for purchase, the concentrations of brain-derived neurotrophic factor (BDNF), nuclear factor kappa B (NF-κB), tumor necrosis factor- (TNF-α), SV2A, and 5-hydroxyindoleacetic acid (5-HIAA) biochemicals were determined in the brain supernatants (Sunred biological technology; Shangai, China). Each animal's samples were all measured in duplicate in accordance with the manufacturer's instructions. The absorbances were measured using a microplate reader (MultiscanGo, Thermo Fisher Scientific Laboratory Equipment, NH, US).

Malondialdehyde levels were measured as a sign of brain lipid peroxidation

Malondialdehyde (MDA) levels as thiobarbituric acid reactive compounds were measured to assess the presence of lipid peroxidation in brain tissue samples

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(TBARS). The brain tissue samples were briefly treated with trichloroacetic acid and TBARS reagent (Merck, USA), combined, and incubated at 100 °C for 60 min. The samples were centrifuged at 3000 rpm for 20 min after chilling on ice, and the absorbance of the supernatant was measured at 535 nm. Tetraethoxypropane was used to compute malondialdehyde levels from the standard calibration curve, which were then represented as nmol/gr protein.

Measuring the amounts of brain proteins

Using bovine serum albumin (Merck, USA) as the reference material, Bradford's technique was used to quantify the total protein content in brain samples [23].

Statistic evaluation

For the statistical analysis, SPSS version 15.0 for Windows was used (SPSS Inc., Chicago, IL, USA). The normality and homogeneity of variance were examined using Shapiro-W Wilk's and Levene's tests, respectively. Mean and standard error of the mean are used to represent the findings (SEM). It was agreed that the value of $p < 0.05$ was statistically significant.

RESULTS

A significant degeneration was observed in all of the investigated marker levels and behavioral test scores in the whole brain IR-only (placebo) group. Compared to the normal control group, the IR-only placebo group had a significant increase in MDA, TNF -α, and NF-κB levels as brain oxidative stress and inflammation markers; and a decrease in BDNF levels as the brain's growth factor, and also a decrease in the SV2A levels as synaptic density protein and 5-HIAA levels as the serotonin degradation protein.

For the granisetron-applied in addition to the IR group (IR+Granisetron) compared to the IR-only group, significant improvement in all these biochemical markers and a return to the normal control group levels were observed. In behavioral test analyses, a significant decrease was observed in the social recognition tests, which are OF and PAL tests in the IR-only group compared to the normal control group; and an improvement was observed in the IR+Granisetron group. In addition, a decrease in the number of hippocampal neurons and Purkinje neurons and an increase in hippocampal gliosis were observed in the IR-only group, while improvement and return to the normal control group counts were observed in the IR+Granisetron group.

Levels of brain biochemical markers

As a result of radiation exposure in the IR-only group arm, the levels of inflammation and oxidative stress markers examined in the study increased significantly. In the IR+Granisetron group, the levels of all brain biochemical markers reached the normal control group levels.

Malondialdehyde levels increased significantly ($p<0.01$) in the IR-only group compared to the control group, and MDA levels decreased significantly ($p<0.001$) in the IR+Granisetron group compared to the IR-only group (table 1).

Also, TNF-α levels were significantly increased in the IR-only placebo group compared to the control group ($p<0.001$), and TNF-α levels were decreased in the IR+Granisetron group compared to the IR-only group ($p<0.001$) (table 1).

When NF-κB was examined, a significant increase ($p<0.001$) was observed in the IR-only group compared to the control group, and a significant decrease was observed in the IR+Granisetron group compared to the IR-only ($p<0.001$) (table 1).

The growth factor BDNF levels in the examined brain tissue were increased in the IR+Granisetron group compared to the IR-only group ($p<0.01$), whereas there was a significant decrease in the IR-only group compared to the control group ($p<0.01$) (table 1).

When the synaptic density protein marker SV2A was examined, a decrease was observed in the IR-only placebo group compared to the control group ($p<0.01$), while there was a significant increase in the IR+Granisetron group compared to the IR-only group ($p<0.01$) (table 1).

Serotonin degradation protein 5-HIAA levels decreased in the IR-only placebo group compared to the control group ($p<0.01$), whereas a significant increase was observed in the IR+Granisetron group compared to the IR-only group ($p<0.01$) (table 1).

Table 1. Biochemical Results.

	Normal Control	Brain Irradiation + Saline	Brain Irradiation + Granisetron
Brain MDA level (nmol/gr protein)	39.7 ± 3.3	94.7 ± 2.9 *	48.2 ± 4.5 ##
Brain TNF-α level (pg/mg protein)	21.6 ± 1.5	101.9 ± 7.3 **	48.2 ± 6.7 ##
Brain NF-κB level (pg/mg protein)	18.3 ± 0.8	96.7 ± 7.3 **	65.5 ± 3.7 ##
Brain BDNF level (pg/mg protein)	2.1 ± 0.3	0.8 ± 0.1 *	1.79 ± 0.2 #
Brain SV2A level (pg/mg protein)	1.45 ± 0.09	0.53 ± 0.04*	0.94 ± 0.02 #
5-HIAA (µmol/mg protein)	12.08 ± 1.1	5.5 ± 0.8*	8.3 ± 0.5 #

Results were presented as mean ± SEM. Statistical analyses were performed by one-way ANOVA. * $p < 0.01$, ** $p < 0.001$ different from normal groups; # $p < 0.01$, ## $p < 0.001$ different from IR-only group. MDA: Malondialdehyde; TNF-alfa: Tumor necrosis factor-alfa; NF-κB: Nuclear Factor kappa B; BDNF: Brain Derived Neurotrophic Factor; SV2A: Synaptic vesicle glycoprotein 2A; 5-HIAA: 5-Hydroxyindoleacetic acid; SEM: standard error of the mean

Behavioral analysis

For all behavioral tests, granisetron treatment increased the behavioral scores of the animals to the levels of the control group, when compared to the

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IR-only placebo group.

The sociability test scores of the IR-only group were significantly lower when compared to the control group ($p<0.001$), while the IR+Granisetron group had significantly higher scores when compared to the IR-only group ($p<0.001$) (table 2).

Likewise, learning scores of the IR+Granisetron group in the open field and PAL tests increased significantly compared to the IR-only placebo group ($p<0.01$ and $p<0.01$), while a significant decrease in the scores in the IR-only group when compared to the control group ($p<0.01$ and $p<0.001$), respectively (table 2).

Table 2. Behavioral Analysis Scores.

	Normal Control	Brain Irradiation + Saline	Brain Irradiation + Granisetron
Sociability test: The spend of time with stranger rat percent (%)	64.8 ± 7.9	33.6 ± 8.5 **	61.2 ± 4.5 #
Sociability test: The spend of time with stranger/ The spend of time with empty (ratio)	2.5 ± 0.2	0.85 ± 0.1 **	2.3± 0.2 ##
Open Field Test: Number of ambulation	17.1 ± 1.9	6.5 ± 0.6 *	8.9 ± 0.5 #
Passive avoidance learning (PAL) Latency (Sec.)	248.5 ± 29.4	14.7 ± 7.8 **	194.5 ± 55.07 #

Results were presented as mean ± SEM. Statistical analyses were performed by one-way ANOVA. * $p < 0.01$, ** $p < 0.001$ different from normal groups; # $p < 0.05$, ## $p < 0.001$ different from Brain Irradiation and saline group. PAL: Passive avoidance learning; SEM: standard error of the mean.

Histopathological evaluation

Changes in total neuron counts in the hippocampus CA1 and CA3 regions were examined, and a significant decrease ($p<0.01$) was observed in the IR-only group compared to the control group. With granisetron treatment, total neuron counts for both CA1 and CA3 significantly increase observed in the IR+Granisetron group compared to the IR-only group ($p<0.01$) (figure 3, table 3).

In the immunostaining examination of the CA1 and CA3 regions of the hippocampus; the GFAP (+) index was significantly higher in the IR-only group compared to the control group ($p<0.01$ and $p<0.01$, respectively). The GFAP (+) index decreased in both CA1 and CA3 regions in the IR+Granisetron group compared to the IR-only group ($p<0.01$ and $p<0.01$, respectively) (figure 4, table 3).

A significant decrease ($p<0.01$) was observed in the Purkinje count of the cerebellum in the IR-only group compared to the control group, while a significant increase in the IR+Granisetron group compared to the IR-only group ($p<0.01$) (figure 5, table 4). Examination of the GFAP (+) index in the cerebellum is significantly higher in the IR-only group compared to the control group ($p<0.01$), and it is significantly lower in the IR+Granisetron group compared to the IR-only group ($p<0.01$) (figure 6, table 4).

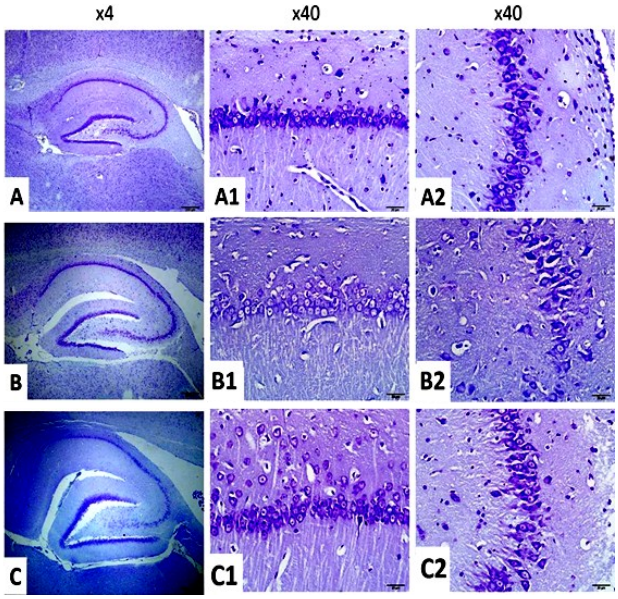


Figure 3. Cresyl violet staining images of CA1 and CA3 regions of the hippocampus at x 4 and x 40 magnification. A-A1-A2: CA1 and CA3 regions of normal control group female rats. Normal pyramidal neuron. B-B1-B2: Brain Irradiation and saline group female rats have neural body degeneration & decreased neural count and dysmorphological changes in CA1 and CA3 (arrow). C-C1-C2: Brain Irradiation and Granisetron group female rats have increased neural count and improved neural morphological changes in CA1 and CA3 (Scale bars for 1 cm = 50 µm). CA: Cornu Ammonis.

Table 3. Hippocampal Histopathological Evaluation.

	Normal Control	Brain Irradiation + Saline	Brain Irradiation + Granisetron
Neuronal Count CA1	78.6 ± 1.7	49.1 ± 4.5 *	66.8 ± 2.2 #
Neuronal Count CA3	45.5 ± 1.1	30.2 ± 0.9 *	41.3 ± 1.5 #
GFAP immunostaining index (CA1)	25.1 ± 3.4	38.9 ± 1. 5 *	27.4 ± 2.1 #
GFAP immunostaining index (CA3)	24.5 ± 0.9	38.4 ± 1.8 *	29.9 ± 2.5 #

Results were presented as mean ± SEM. Statistical analyses were performed by one- way ANOVA. * $p < 0.05$, different from normal groups; # $p < 0.05$, different from Brain Irradiation and saline group. CA1: Cornu Ammonis 1; CA3: Cornu Ammonis 3; GFAP: Glial fibrillary acidic protein; SEM: standard error of the mean.

Table 4. Cerebellar Histopathological Evaluation.

	Normal Control	Brain Irradiation + Saline	Brain Irradiation + Granisetron
Purkinje Count Cerebellum	27.4 ± 1.3	13.4 ± 0.8 *	25.3 ± 0.6 #
GFAP immunostaining index (Cerebellum)	18.9 ± 1.1	30.3 ± 2.7 *	21.1 ± 0.9 #

Results were presented as mean ± SEM. Statistical analyses were performed by one- way ANOVA. * $p < 0.05$, # $p < 0.05$, different from Brain Irradiation and saline group. GFAP: Glial fibrillary acidic protein; SEM: standard error of the mean.

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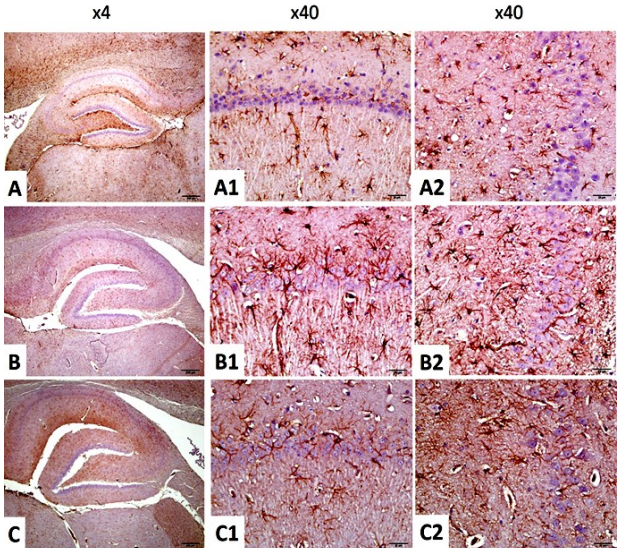


Figure 4. GFAP immunostaining images of CA1 and CA3 regions of the hippocampus at x 4 and x 40 magnification. Astrogliosis was characterized by GFAP immunostaining (Brown staining). A-A1-A2: CA1 and CA3 regions of normal control group female rats, B-B1-B2: Brain Irradiation and saline group female rats have increased glial activity in CA1 and CA3. C-C1-C2: Brain Irradiation and Granisetron group female rats have decreased glial activity in CA1 and CA3 (Scale bars for 1 cm = 50 µm). CA: Cornu Ammonis; GFAP: Glial fibrillary acidic protein.

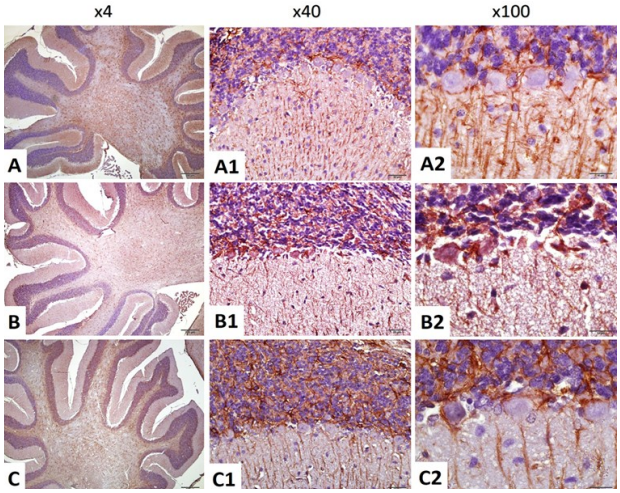


Figure 6. GFAP immunostaining images of cerebellum at x 4, x 40 and x 100 magnification. Astrogliosis was characterized by GFAP immunostaining (Brown staining). A-A1-A2: Cerebellum of normal control group female rats; B-B1-B2: Brain Irradiation and saline group female rats have increased glial activity; C-C1 -C2: Brain Irradiation and Granisetron group female rats have decreased glial activity. (Scale bars for 1 cm = 50 µm). GFAP: Glial fibrillary acidic protein.

DISCUSSION

The results of the present study demonstrated the curative effect of granisetron on radiation-induced neurotoxicity in a rat model through biochemical marker analysis, histopathological staining, and behavioral tests. Biochemical analysis of the irradiated brain tissue has demonstrated that

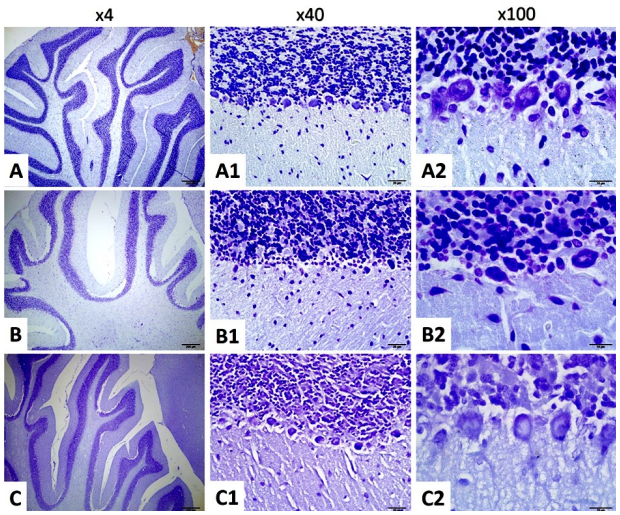


Figure 5. Cresyl violet staining images of cerebellum at x 4 and x 40 magnification. A-A1-A2, Cerebellum of normal control group female rats, normal Purkinje Neuron; B-B1-B2: Brain Irradiation and saline group female rats have decreased neural count and dysmorphological changes for purkinje neurons at cerebellum level. C-C1-C2: Brain Irradiation and Granisetron group female rats have increased neural count and improved Purkinje neural morphology and changes at cerebellum level (Scale bars for 1 cm = 50 µm).

granisetron treatment increases synaptic function and reduces inflammation, as well as its neuroprotective effects that reduce neuron apoptosis and lead to improvement in neuron functions.

Reactive oxygen species (ROS) toxicity underpins radiation therapy, which can harm macromolecules including DNA, RNA, microRNA, and proteins through a variety of pathways including lipid peroxidation and enzyme oxidation [24-29]. Malondialdehyde is a biological marker for both oxidative stress and lipid peroxidation and is a sign of cellular damage [30, 31]. Several studies assessing MDA levels in individuals after head and neck radiation found an increase [24, 29, 32]. MDA values, a measure of lipid peroxidation and inflammation in our investigation, rose only in the IR-applicated group while considerably falling with the granisetron therapy. The analgesic, anti-inflammatory, and even immunosuppressive activities of 5-HT3 receptor antagonists are the most intriguing results from a therapeutic perspective [33].

Since the granisetron application offers a reduction in IR-induced inflammation TNF-α pro-inflammatory marker was also examined in our study. So, after granisetron administration it has been observed positive improvement in TNF-α levels, as well as MDA levels. In a study, it has been revealed that the administration of tropisetron, which is also a 5HT-t3 receptor antagonist, inhibits the secretion of TNF-α which is an inflammatory substance [33, 34]. The most plausible regulatory mechanism for this inhibition must be taken into consideration. It involves the effect of 5-HT3 receptor antagonists on p38 mitogen-activated protein (MAP) kinase. Data from a planned investigation suggested that the

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5-HT3 receptor antagonist tropisetron's anti-inflammatory actions may be caused by a selective suppression of pro-inflammatory cytokines at the post-transcriptional level [33, 35]. In reality, blocking this receptor with a range of highly affine specific competitive antagonists, including ondansetron, tropisetron, granisetron, dolasetron, and others, has a number of advantageous therapeutic outcomes [33].

Over 200 genes, including those involved in the immunological response [36-37], inflammation [38], differentiation, proliferation, cell survival, and apoptosis, are regulated by the transcription factor nuclear factor (NF)-κB [39, 40]. This transcription factor's activation has been linked to the consequences of ionizing radiation exposure, and NF-κB is abnormally activated in many human disorders, including cancer and inflammatory conditions [36]. In our study, NF-κB levels, as the inflammation and apoptosis indicator, decreased in the granisetron treatment applicated group whereas increased in only IR applicated group.

As a 5-HT3 receptor antagonist, granisetron has a clear anti-inflammatory action. Additionally, by directly reducing depolarization and raising intracellular Ca+2, 5-HT3 receptor antagonists diminish these effects by inhibiting the receptor [15]. Additionally, due to decreased expressions of the inositol trisphosphate receptor (IP3R), granisetron modifies the IP3-receptor, which is in charge of Ca+2 efflux from endoplasmic reticulum storage, Ca+2 flow balances, and maintains Ca+2 homeostasis [12]. Reduced apoptosis and higher levels of cAMP response element-binding protein (CREB) and BDNF result from this alteration [12]. In our investigation, granisetron therapy was linked to a reduction in apoptosis-related NF-kB factor levels via this mechanism.

BDNF plays important role in learning and memory processes, as well as in neurogenesis, neuronal survival, neuronal differentiation, and synaptic plasticity [41]. Irradiation application may also cause disruption of synapse homeostasis, an animal study demonstrated that ultraviolet IR to the skin down-regulated BDNF expression and extracellular signal-regulated kinases signaling in the hippocampus, which is known to modulate neurogenesis and synaptic plasticity [42]. In our study BDNF levels, as the neurotrophic factor, decrease only with IR administration, where significantly increase in the granisetron applicated group. Granisetron treatment regulates Ca+2 homeostasis as indicated previously. Ca+2 dyshomeostasis has been linked to protein kinase A (PKA)/CREB pathway downregulation, according to reports [43]. For synaptic reorganization and memory formation, CREB phosphorylation by Ca2+/calmodulin-dependent protein kinase II (CaMKII) and PKA is necessary [44, 45]. Here, CaMKII and PKA activation phosphorylates CREB at Ser133 to start BDNF transcription. CREB is a transcriptional regulator of BDNF [45-47]. A drop in serum BDNF levels during the initial stages of SSRI treatment may be related to a later SSRI response in adolescents with severe depressive disorders, according to a different investigation on the relationship between BDNF levels and SSRIs [48]. There is a correlation between BDNF levels and depression in adolescents. Healthy controls had higher serum BDNF levels than depressive adolescents, regardless of gender [49]. Constipation and vomiting are common digestive adverse effects of selective serotonin reuptake inhibitors, and these side effects are linked to an increase in serotonin availability at 5-HT3 receptors. Granisetron, a serotonin 5-HT3 receptor antagonist, counteracts the negative effects of SSRIs on the digestive system [50]. The granisetron-applied group compared to the IR-only group, decreased BDNF levels upregulated significantly. Where inhibition of the SSRIs, regulates serotonin levels and BDNF levels, as a result, protected memory and neural functions.

Regardless of the presence of neurotransmitters, all synaptic terminals include the transmembrane protein known as synaptic vesicle glycoprotein 2A [17]. The SV2A is a universal biomarker of synaptic density that is present in glutamatergic and GABAergic neurons [51]. Numerous neuropsychiatric illnesses have been found to disrupt synaptic homeostasis [42,52]. SV2s are expressed in the mitochondrial compartment in addition to vesicles, and a damaged mitochondrial structure may have an impact on the downregulation of SV2A signaling [53,54]. It has also been suggested that levetiracetam may have additional therapeutic benefits for treating late-onset Alzheimer's disease (AD), where the SV2A protein is a specific target for the drug. These other benefits may include enhancing mitochondrial function [53]. Various human and animal investigations have shown that reduced SV2A levels are a sign of epileptic diseases [55-58]. As a result of radiation-induced damage to energy-producing mitochondria, which in turn causes additional damage to cellular nucleic acids, proteins, and lipids, exposure to radiation may also affect the homeostasis of synapses [59]. Our findings demonstrated that SV2A levels, as the synaptic density and neurotransmitter regulator, significantly decrease in only IR application.

However, after granisetron application SV2A levels significantly increase. Exposure to radiation manifests reduced SV2A levels by means of damaged synaptic and mitochondrial homeostasis. Granisetron treatment modulates SV2A levels and blocks Ca+2 flux. This ameliorative pathway of granisetron is observed in the study based on AD. Granisetron's explanation of the pathway that improved blood-brain barrier (BBB) integrity, significantly decreased amyloid-induced Ca+2 influx, and corrected Ca+2 dyshomeostasis by restoring the calmodulin-dependent protein kinase II/cAMP-

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response element binding protein pathway was linked to an improvement in cognition [12].

One of the most important signaling molecules is the neurotransmitter 5-HT3 [16]. Another biomarker that might be used is serotonin, a neurotransmitter that is produced in the brain's raphe nuclei. Because monoamine oxidase (MAO), a mitochondrial enzyme, converts it into 5-hydroxy indole acetic acid rapidly in this case, it has a limited effect (5-HIAA). Analyzing the brain's serotonin levels using this subsequent metabolite is possible [60]. In a rat model of cholestatic hypertrophy, research on animals found that the hypothalamus 5-HT3 receptor was expressed less, and the fatigue-like behavior was improved more by 5-HT3 receptor antagonists. They have discovered biochemical proof of enhanced hypothalamic serotonin turnover in cholestatic compared to sham rats, as seen by higher hypothalamic 5-HIAA to 5-HT ratios [61,62].

Since the relation between the 5-HT3 receptor and 5-HIAA was mentioned previously, we examine a serotonin degradation protein 5-HIAA as the indirect indicator of 5-HT3. Our findings demonstrated that 5-HIAA levels, as the serotonin degradation protein, significantly decrease in only IR application. However, after granisetron treatment application 5-HIAA levels significantly increase. These individuals have lower levels of 5-HIAA, however this might be because serotonin synthesis is declining or its absorption from neural synapses is increasing. For instance, due to the position of the raphe nuclei inside areas of memory and cognition, a patient with depression has a significant fall in the levels of 5-HIAA [60]. Our study examined the treatment of granisetron, the 5-HT3 receptor antagonist, which blocks the 5-HT3 receptor, and increases 5-HT3 levels in the pro-synaptic area. As a result of the metabolization of 5-HT3 to 5-HIAA, 5-HIAA levels also increase.

Many clinical studies demonstrated that radiation therapy leads to depression, memory loss and impairment in cognitive functions by means of radiation-induced brain injury [1, 4, 59]. The most severe effects of childhood brain irradiation, including double-digit IQ decreases, vocational restrictions, and an increased frequency of mental disorders, are experienced by long-term survivors in particular [63-65]. Our social test results demonstrated that granisetron treatment significantly improves cognitive function, memory and learning skills by means of its neuroprotective, synaptic and mitochondrial homeostasis regulation.

Granisetron appears to have an antidepressant effect per se in animal research, at least at modest dosages. Whereas granisetron, a serotonin 5-HT3 receptor antagonist, counteracts the side effects of SSRIs on the digestive system [48]. Tricyclic antidepressants like imipramine, desipramine, and doxepin, monoamine oxidase inhibitors (MAOIs) like phenelzine, and SSRIs like fluoxetine have been demonstrated to uncompetitively decrease 5-HT3 transreceptor currents in previous investigations. Antidepressants may act like 5-HT3 receptor antagonists by inhibiting the 5-HT3 receptor. Another example of granisetron's potential antidepressant impact [48, 66-69].

The loss of hippocampus's neural progenitor cells has received attention in studies of radiation's effects on cognition [70, 71]. It has been studied and is stressed in numerous theories of cognitive function that the direct monosynaptic route, which originates in the hippocampus CA1/subicular region and projects to the medial orbital and prelimbic sections of the prefrontal cortex (PFC), is important for cognitive performance [72-74]. In a study using slices of the CA1 region of the rat hippocampal brain, it was found that activating 5-HT3 receptors worsened the effects of ischemia on the reduction of the CA1 field potential while inhibiting these receptors produced dose-dependent neuroprotection from the damage to the neurons brought on by ischemia [75]. An earlier study investigated the possibility that neuroinflammation was the mechanism through which radiation may cause brain damage. The loss of neural progenitor cells (NPCs), suppression of hippocampal neurogenesis, and direct activation of glia that results in the senescence-associated secretory phenotype (SASP) are all hypothesized to occur concurrently with factors that cause a neuroinflammatory cascade [1].

In our investigation, total neuron counts in the CA1 and CA3 areas of the hippocampus were investigated, and a substantial decrease with only IR treatment and an increase with granisetron application were found.

It has previously been established that granisetron possesses anti-inflammatory properties against chronic inflammatory disorders in animal models [76, 77]. Additionally, 5-HT3 receptor antagonists have been shown in the literature to have neuroprotective effects in elderly mice, as well as in animals who have been exposed to neurotoxin and had memory loss [78-81]. An extended form and dense branches of GFAP are one of the initial signs of astrocyte inflammatory activity. Granisetron was shown in research to decrease astrocyte activation and improve astrocyte morphology when compared to the control group [12]. Increased brain inflammation was seen as a result of IR treatment when the CA1 and CA3 areas of the hippocampus were immunostained with GFAP. Granisetron treatment resulted in a considerable decrease in GFAP index. Granisetron decreased astrogliosis and neuronal death, according to the hippocampus CA1-CA3 regions' histological analyses.

Purkinje cells serve a crucial role as cerebellar neurons in the processes of memory and learning, as well as in other cerebellar activities [82, 83]. A study

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focused on cerebellum doses of children undergoing radiation therapy for brain tumors, observed cognitive decline as an effect of IR [84]. Significant doses taken into the cerebellum cause neural damage and inflammation. As in our study, a decrease in Purkinje cell numbers and an increase in neural inflammation markers were observed after only IR application. However, subsequent to granisetron application histopathological evaluations of the cerebellum reveal that neuronal loss was reduced by means of Purkinje counts, as well as neural inflammation.

CONCLUSION

In this work, we used a rat model of radiation-induced brain damage to observe how granisetron protects neurons. As a 5-HT3 receptor antagonist, granisetron modulates neuronal functions via the 5-HT3 receptor, SV2A protein, brain growth factor BDNF, and different inflammation markers to reduce neurodegeneration induced by radiation exposure. Many beneficial effects of 5-HT3 receptor antagonists are under consideration where including neuroprotection and neurodegenerative age-related disorders.

Declarations

***Ethical Approval*:** The Animal Ethics Committee of Demiroglu Science University authorized the experimental procedures used in this study (Approval no: 28220713).

***Competing interests*:** The authors declare that they have no conflicts of interest.

***Authors' contributions*:** Conceptualization NC, MAE, OE; Methodology NC, OA, MAE, GY, EE, CS, YU, OE; Investigation NC, OA, MAE, GY, EE, CS, YU, OE; Data Curation NC, OA, MAE, EE, CS, YU; Writing - Original Draft NC, MAE, OE; Writing - Review & Editing MAE, OE; Project administration NC, OE.

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***Availability of data and materials*:** The datasets generated during and/or analysed during the current study are not publicly available due to [REASON(S) WHY DATA ARE NOT PUBLIC] but are available from the corresponding author on reasonable request.

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