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Study on Injuries to Hippocampus, Amygdala, Cerebral Cortex and Their Effects on Conditioned Taste Aversion in Total Body ^{60}Co - γ -irradiated Rats.

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ABSTRACT

The objective was to study the effects of one time exposure to small clinically relevant dose of ^{60}Co -gamma (^{60}Co - γ) radiation on histological changes in brain and consequent effects on conditioned taste aversion (CTA) in experimental rats. Rats were total body ^{60}Co - γ -irradiated in GammaCell; CTA to saccharin was assessed in terms of saccharin preference ratio (SPR) using a standardized two bottle regime; brain histology was performed as per standard procedures. Total body exposure to ^{60}Co - γ -irradiation (2Gy) caused nuclear degeneration, increase in intercellular spaces, enucleation in amygdala as well as in cerebral cortex, neuronal loss and dispersion of neurons in hippocampus at 24 h after irradiation. Most of the cellular and nuclear damage in these brain parts had shown increasing trend up to day 5. In irradiated animals, maximum CTA acquisition {25.6 \pm 3.6% SPR [t(6)=3.499, p <0.05]} was at 24 h (day1) after irradiation, which did not change from day1 till day 5 ($F_{4, 4}$ =2.772, p =0.1736). This study suggested that whole body exposure to rats at clinically relevant dose of ^{60}Co - γ -ray caused lesions in cerebral cortex, hippocampus and amygdala, which did not prevent acquisition of CTA. Further, post-irradiation time dependent histological changes in these brain parts did not influence the retention of CTA.

Keywords: Brain, conditioned taste aversion, nuclear degeneration, neuronal dispersion, saccharin

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INTRODUCTION

Low linear energy transfer (LET) ionizing radiations are being used increasingly for diagnostic and therapeutic purposes. Tumor patients undergoing radiotherapy commonly display the ill effects of radiation such as neurotoxicity, nausea and vomiting [1]. Efforts have been in progress to develop the drugs which could counter the ill effects of radiation. However, despite decades of research, no safe and efficacious radioprotective drug has been approved for human use till date. Incomplete understanding of mechanisms which lead to ill effects of radiation (neurotoxicity, nausea, vomiting etc.), are some of the important reasons for limited progress in this field. Conditioned taste aversion (CTA) in rats is a considered parallel process to nausea and vomiting in humans, especially with regard to afferent pathways and central processing [2]. CTA is an aversive behavior and is a kind of fear learning, where the ingestion of a 'preferred food', the conditioned stimulus (CS), if paired with malaise/toxins, which provoke the unconditioned stimulus (US) such as nausea, an association learning between the ingested substance and internal consequences is quickly established and intake of 'preferred food' is avoided. Garcia and his co-workers in 1950s reported that small dose of ^{60}Co - γ -radiation induced CTA in rats. Study of CTA in experimental rats is often the method of choice to evaluate toxicity of radiation and/or anti-radiation drugs, because it is unethical to irradiate healthy human subjects [3, 4].

The low LET ionizing radiation damage the important biomolecules such as nucleic acids, proteins, lipids by direct deposition of energy as well as by generating a great flux of reactive oxygen species (ROS) and other free radicals. The damaging effects are dependent on the absorbed radiation dose. Brain is considered resistant to smaller doses of low LET radiation and has, therefore, received rather less attention regarding the injuries sustained and their role in CTA in irradiated experimental models. Most of the studies pertaining to radiation CTA are explained on the basis of visceral injuries/toxicity to gastrointestinal (GI) tract, because GI tract is known to be more radio-sensitive [3, 4]. There are studies reporting radiation damage in adult brain at higher doses of low LET ionizing radiation (6.75 Gy and 5 Gy) [5, 6,]. At lower doses of ionizing radiation, damage to brain cortex was reported mostly in prenatal rats [7]. Nonetheless, some experimental studies with rats demonstrated that lesions in the area postrema (AP) of rat brain, could attenuate the radiation (1 Gy) CTA [8]. Further, the literature is replete with a number of reports on changes in the taste perception in cancer patients treated with radiotherapy in head and neck area only, indicating that radiation induced changes in brain and/or neck region could contribute to taste and other behavioral issues [9, 10,]. The only anti-radiation drug WR-2721, approved for use in clinics in conjunction with radiotherapy only, was neurotoxic in humans [11] and had failed to ameliorate radiation CTA in rats [2]. While evaluating the effects of anti-radiation drugs in our laboratory, it was observed that one time exposure of rats to ionizing radiation dose (2 Gy), resulted in CTA, where the experimental rats avoided a 'novel taste' (saccharin), which was otherwise preferred by animals [4]. The radiation induced CTA, since, was believed to be interplay of signals between GI tract and brain, it was considered important to understand the changes caused by total body irradiation (2 Gy) in GI tract as well as in brain of rat showing radiation CTA. Earlier we reported that acquisition of CTA in rats after total body irradiation (2 Gy), was associated with increase in levels of serotonin (5-HT) in GI tract and plasma; increase in levels of corticosterone as well as decrease in levels of antioxidants in plasma [12]. The objective of present study was to investigate whether

one time total body exposure to small dose of ^{60}Co - γ -radiation (2 Gy) to rats caused histological changes in brain parts (cortex, hippocampus and amygdala) and also to study whether such changes affected the acquisition and/or retention of conditioned taste aversion (CTA) to saccharin. The representative brain parts (cortex, hippocampus and amygdala) were chosen because of their general association with multiple aversive signals as well as their role in processing of memory, attention, thought and consciousness [13, 14, 15].

MATERIALS AND METHODS

Experimental animals

The inbred 6 weeks old male Sprague-Dawley rats, weighing 180 ± 20 g, were obtained from the breeding colony maintained at the Institute's Animal Experiment Facility, after the approval from Institutional Animal Ethical Committee. The experiments were conducted in accordance with the regulations specified by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India and approved protocol No. 8/Go/a/9a/CPCSEA.

Experimental Design

All the animals were housed individually in polyvinyl cages kept in experimental room, and were used after two week of acclimatization to the laboratory conditions ($25\pm 2^\circ\text{C}$ and 12 h light-dark cycle). The animals had free access to food and water during acclimatization period. The procedure described in detail earlier [12] and summarized in Fig. 1A, was employed for inducing radiation CTA. After acclimatization, all the rats were subjected to water deprivation schedule for 8 days, wherein each animal was offered tap water orally once a day for 30 min at a specified time (10:00 h - 10:30 h) of the day (i.e. one bottle regime). Water consumption by individual animal was recorded daily. On the 9th day (test day or D0), all the rats were given two bottles simultaneously for 30 min, where one bottle contained 0.1% saccharin solution (generally preferred taste) and another bottle contained tap water. The intake of saccharin solution as well as tap water was recorded. The animals showing preference for saccharin ($\geq 50\%$ saccharin intake of their total fluid intake), were selected for further experiment. This procedure was referred as two bottle regime.

The selected rats were divided into two groups where, Group I was treated as conditioned unirradiated controls (UIC, $n=6$) and were not given any further treatment; while Group II was treated as radiation group (R, $n=30$). The animals of group II were given total (whole) body exposure to ^{60}Co - γ -radiation (2 Gy) soon after grouping. For irradiation, each rat was placed in a wire gauze container and placed in the ^{60}Co -Gamma cell (Model GC-220, Atomic Energy Commission, Canada, dose rate of 0.23 rads/sec). On day 1 (D1) all the animals were again presented 0.1% saccharin solution and tap water simultaneously, for 30 min only. The intake of saccharin and water was recorded. Six animals at random were sacrificed by cervical dislocation, for histological study. The procedure of presenting saccharin and water simultaneously, recording the intake of saccharin vis a vis, total fluid intake was recorded after each day [day 2 (D2), day 3 (D3), day 4 (D4), day 5 (D5)]. For determining CTA, saccharin preference ratio (SPR) was calculated $\{\% \text{SPR} = [\text{saccharin solution}$

intake (SI)/ (water intake + SI)] X 100}. Decrease in SPR indicated CTA. In addition to UIC and R group, another group of 6 animals was kept as unconditioned untreated control group (UC). These animals were acclimatized to laboratory conditions for 2 weeks, but were not subjected to water deprivation schedule, saccharin treatment or irradiation. This group was used as control for histological studies only.

Histological Analysis

For histological studies, animals were first anesthetized and then sacrificed humanely by cervical dislocation. All animals of group UIC and UC were sacrificed on D1. The animals of group R were sacrificed on D1, D2, D3 and D5 for histological studies. The brains were extracted carefully and frozen immediately in NEG 50 medium at -20°C . Sections were coronally cut (6μ thick serial sections) from the frozen blocks using cryostat (Leica Systems, Germany) and mounted on the slides. All the coronal sections were stained with hematoxylin and eosin as per standard protocol. Observations were recorded under a light microscope (A1 Axio Scope Microscope, Germany) at magnification x50 and x200.

Statistical Analysis

Data for %SPR were analyzed with ordinary two-way Analysis of Variance (ANOVA), followed by post hoc Bonferroni's multiple comparison tests using Graph Pad prism 6.0. Data are presented as Mean \pm Standard Error (SE) of six samples. Value of $p < 0.05$ was considered as statistically significant.

RESULTS

Whole body exposure to ^{60}Co - γ -irradiation (2 Gy) caused acquisition of significant ($p < 0.05$) CTA (decrease in %SPR) in rats, in comparison to UIC. The irradiated rats showed only $25.6 \pm 3.6\%$ [$t(6) = 3.499$, $p < 0.05$] SPR 24 h after irradiation on D1. The CTA in irradiated rats was retained till D5 and beyond, without any significant change in comparison to D1 (Figure 1B). Two-way ANOVA of %SPR showed significant difference between the animals of group UIC and R, on all the days (D1-D5) ($F_{1, 4} = 494.3$, $p < 0.0001$) but no significant difference was observed among different days of treatment in groups UIC and R ($F_{4, 4} = 2.772$, $p = 0.1736$). In other words, the effect of irradiation on CTA did not differ amongst the study days (D1-D5) ($F_{4, 4} = 2.772$, $p = 0.1736$).

The structural changes observed in the selected brain regions were tabulated in Table 1 by following a scoring system of 0-6 where 0 showed no lesions or structural change and 6 showed the maximum lesion/observed change (in this study), in comparison to the UIC. Because no difference was observed between the animals of UC and UIC group, the comparison of irradiated group (R) with UIC group only is shown in Figure 2 and Table 1. The arrows were used to depict the trends of increase (\uparrow), decrease (\downarrow) and no change (\leftrightarrow).

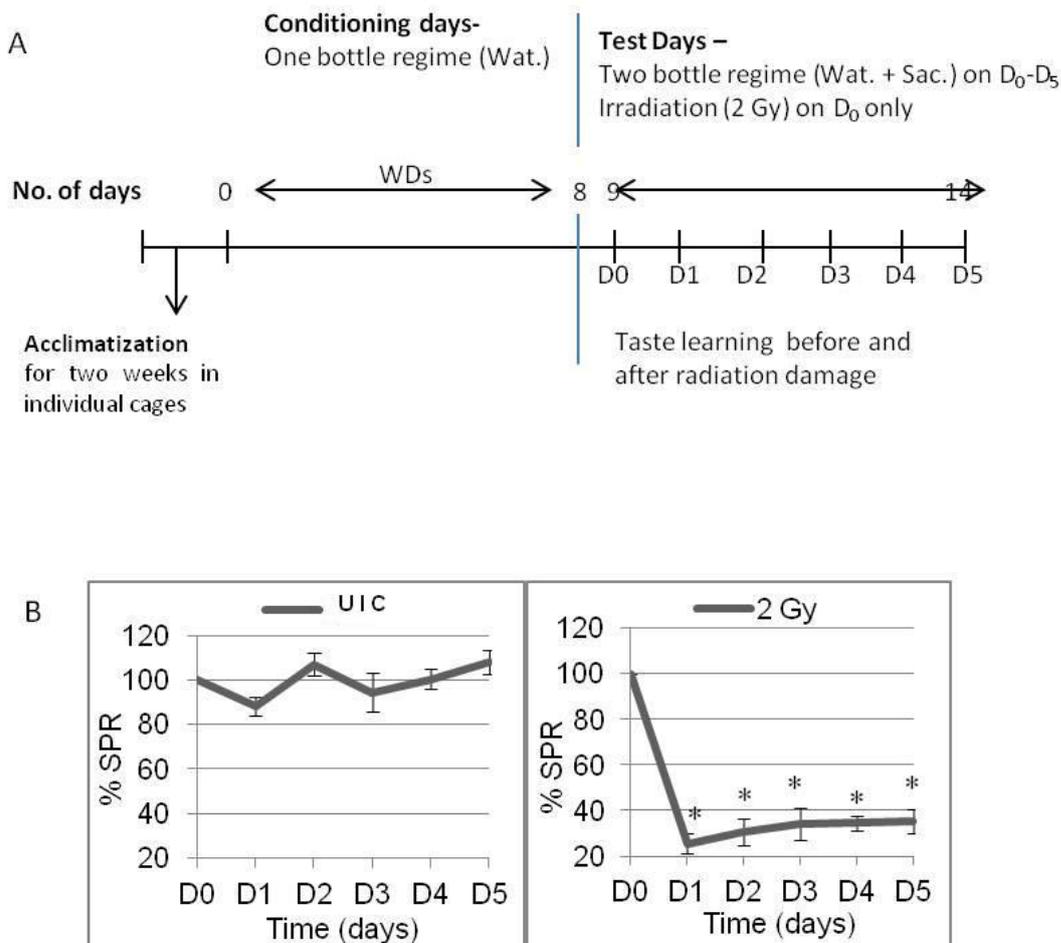


Figure 1. Conditioned taste aversion (CTA) experiments and results. A) Shows the experimental scheme. Rats were first acclimatized for two weeks in individual cages; then for next 8 days trained or conditioned to drink water once a day for 30 min only (water days or WDs). On test day (D₀), rats were given both water and saccharin (wat. and sac.) simultaneously once for 30 min. The animals showing preference for saccharin ($\geq 50\%$ intake of saccharin of the total fluid intake) were selected and divided in two groups. One group was kept as conditioned un-irradiated control (UIC) while all rats of the other group were total body ⁶⁰Co- γ -irradiated (2 Gy). On next five consecutive days (D₁-D₅), all rats were given both water and saccharin (wat. and sac.) simultaneously once a day for 30 min; intake of saccharin and water was recorded and Saccharin preference ratio (%SPR) was calculated; %SPR = [Sac. intake (SI)/ (wat. intake + SI)] X 100. B) Shows the day wise changes in %SPR in conditioned untreated control (UIC) and ⁶⁰Co- γ -irradiated (2 Gy) rats. Decrease in %SPR on D₁ indicated acquisition of CTA. UIC showed no change in %SPR from D₀-D₅. Irradiated animals (2 Gy) exhibited decrease in %SPR on D₁ in comparison to D₀; and no change in %SPR from D₁ to D₅. Data presented is the Mean \pm SE of 6 rats. (*) Significant in comparison to D₀ at $p < 0.05$.

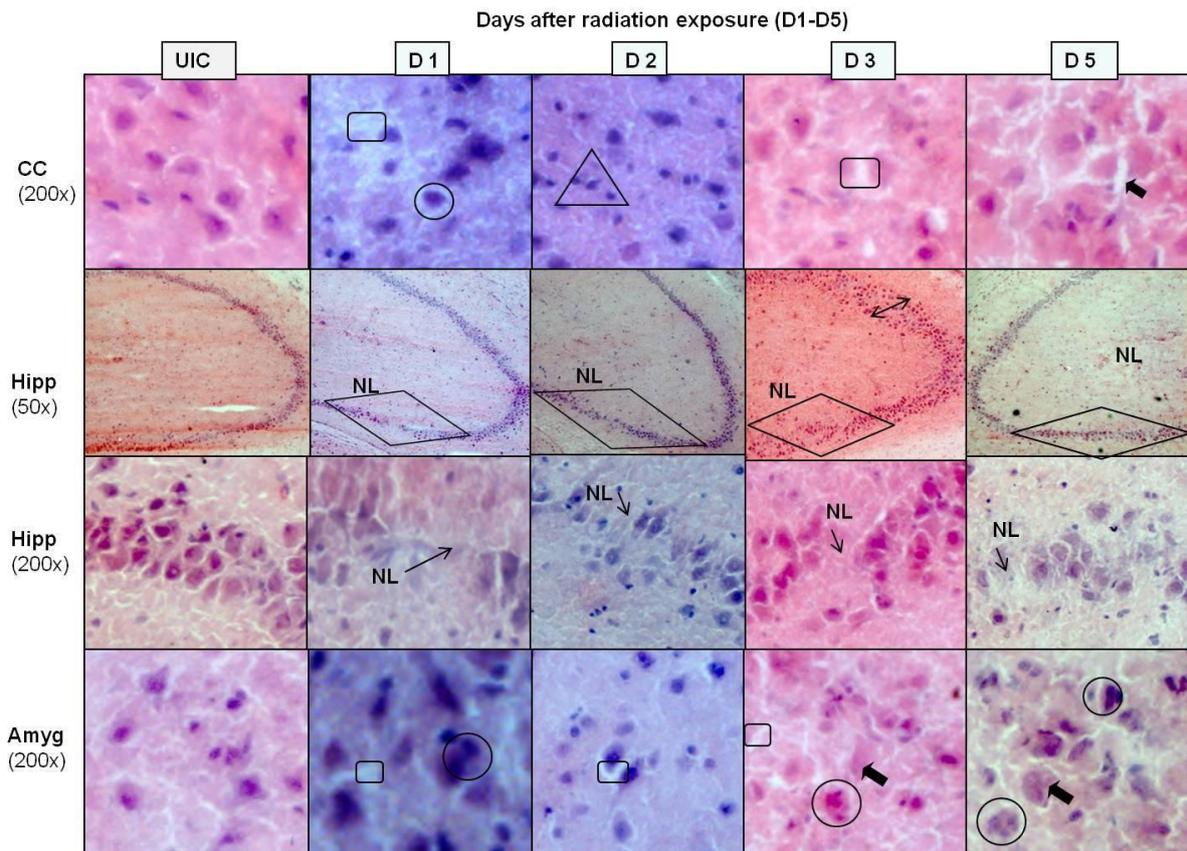


Figure 2. Histological changes after ^{60}Co - γ -irradiation (2 Gy) in cerebral cortex (CC), hippocampus (Hipp) and amygdala (Amyg) of rat brain. 6- μ thick serial coronal sections were cut from the frozen blocks stained with H&E and presented at 50x / 200x magnification. Conditioned un-irradiated control (UIC) shows the normal architectures. D1- D5 present changes from day1 to day 5 after irradiation. The area enclosing \bigcirc shows nuclear degeneration; \square shows increase intercellular space; \triangle shows glial cells. Enucleated cells are pointed as \uparrow and the neuronal loss (NL, is enclosed in \diamond , the \leftrightarrow shows dispersion in neuronal arrangement.

It was observed that in cortex and amygdala of UIC rats the neuronal cells were compactly placed, nuclear material was clearly demarcated from the cytoplasm and no nuclear degenerative changes could be observed; in hippocampus the neuronal cells were normal and neuronal arrangement was intact. ^{60}Co - γ -irradiation caused observable nuclear degeneration in cerebral cortex. Degenerative nuclei could be identified by intense and darkly stained nuclear material. The clear demarcation of nuclei from the cytoplasm was lost. The fragmentation of nuclear material was seen on D1. By D5 a large number of cells were found to be enucleated and most of the cells had lost their normal cell outline indicating increased manifestation of radiation damage. In addition, time dependent increase in intercellular spaces was observed at D1, D2, D3 and D5 (Table 1 and Figure 2).

Table 1. Histological changes in rat brain after total body ⁶⁰Co-γ-irradiation (2 Gy).

Brain regions	Parameters	Change*	D1	D2	D3	D5
Cerebral cortex	Nuclear degeneration	Trend	↑	↑	↑	↑
		Grade	5	4	4	4
	Increase in intercellular spaces	Trend	↑	↑	↑	↑
		Grade	2	1	4	6
	Enucleated cells	Trend	↑	↑	↑	↑
		Grade	1	1	4	5
	Increase in glial cells	Trend	↔	↑	↔	↔
		Grade	0	5	0	0
Hippocampus	Neuronal Loss	Trend	↑	↑	↑	↑
		Grade	5	4	4	5
	Dispersion of neuronal cells	Trend	↑	↑	↑	↑
		Grade	0	0	4	2
	Neuronal density	Trend	↔	↔	↓	↔
		Grade	0	0	2	0
Amygdala	Nuclear degeneration	Trend	↑	↑	↑	↑
		Grade	4	4	3	3
	Increase in intercellular spaces	Trend	↑	↑	↑	↑
		Grade	3	3	2	2
	Enucleated cells	Trend	↔	↔	↑	↑
		Grade	0	0	4	5
	Increase in glial cells	Trend	↔	↔	↔	↔
		Grade	0	0	0	0

*Presented in comparison to conditioned un-irradiated control on number scale with 0 as minimum and 6 as maximum. ↔: no change; ↑ increase; ↓ decrease; D: days after irradiation

On D2, the glial cell number increased observably. The glial cells could be clearly distinguished from the neuronal cells and endothelial cells because of presence of very small nuclei, inconspicuous nucleolus and less endoplasmic reticulum [16]. The increased number of glial cells was reported in the rats exposed to higher doses of radiation (10-20 Gy) [17]. In comparison to UIC group, the amygdala region of irradiated rats showed prominent nuclear degeneration and increased intercellular spaces on all the observed days (Figure 2 and Table 1). Most of the cells were found to be enucleated on D1 and D2. In the hippocampus region, the irradiated rats showed prominent neuronal loss on all the observed days (Figure 2), in comparison to unirradiated controls. Further, observable dispersion of neurons and decreased neuronal density was seen on D3.

DISCUSSION

CTA, developed by single-trial training, is a long-lasting memory that provides a useful model for studying the different phases of memory, such as acquisition, consolidation and retrieval. Amygdala receives a variety of sensory inputs, including gustatory and visceral information. Cerebral cortex is the largest (> 70%) and highly developed part of the brain and is responsible for sensing and interpreting inputs from various sources and maintaining cognitive functions. Hippocampus is associated with learning and memory and plays an essential role in the formation of spatial, contextual and trace conditioning memories.

Although, a number of clinical studies exist wherein the patients undergoing radiotherapy in neck and head region only or with neurological disorders describe alterations in taste [9, 10], so far, there are not many studies to understand the role of various brain regions in the CTA induced by irradiation. Low LET ionizing radiation, since, is being utilized increasingly for therapy and diagnosis; it was considered important to understand the injuries caused, if any, to the brain parts such as cortex, hippocampus and amygdala, which are generally associated with multiple aversive signals, processing of memory, attention, learning, thought and consciousness, and their role in radiation CTA.

In this study it was observed that low LET ^{60}Co -gamma-irradiation (2 Gy) caused nuclear degeneration, formation of enucleated cells and intercellular spaces in the amygdala and cortex; neuronal loss and neuronal dispersion in hippocampus (Figure 2, Table 2). There were observable tissue lesions in the cerebral cortex, amygdala and hippocampus at 24 h (D1) after irradiation, which did not recover till D5 (Table 1 and Figure 2). These observations were rather unexpected, because the maximum CTA (% SPR) was also observed at D1 (Figure 1B), which did not recover till D5 and beyond. This study clearly demonstrated that the acquisition of CTA in the total body irradiated rats was not compromised by lesions in cerebral cortex, amygdala and hippocampus. Whole body exposure to ionizing radiation damages multiple body tissues and the effects are dose dependent. The manifestation of tissue damage is known to vary from one tissue to another depending upon the radiation sensitivity of tissue under investigation. Brain is considered as a radio-resistant organ and structural damage in adult brain is so far, reported only at higher doses of ionizing radiation (6.75 Gy and 5 Gy) [5, 6] or at lower doses in prenatal rats only [7]. Based on these studies it was hypothesized that radiation dose (2 Gy) was not sufficient to cause structural damage in brain; which could be the cause of acquisition of CTA in total body irradiated animals. However, our study demonstrated that structural change or lesions were observed in these brain regions and that these lesions had no direct bearing on acquisition and/or retention of CTA. This is the first study to report that cellular lesions in hippocampus, amygdala and cerebral cortex, caused by total body irradiation (2 Gy) did not directly affect the acquisition as well as retention of CTA.

Oxidative damage is an important etiological factor in radiation injury. Brain tissue, being highly enriched with polyunsaturated fatty acids, is likely to be susceptible to the oxidative damage. Radiation induced multiple DNA damage is commonly seen in all radiosensitive tissues leading to apoptosis or necrosis. The structural changes observed in amygdala, cerebral cortex and hippocampus of whole (total) body irradiated rats could be attributed to the intra-cellular reactive oxygen species and other free radicals generated by ionizing radiation. It is further logical to propose that the increased nuclear degeneration could have caused enucleation of cells, cytoplasmic shrinkage and cellular losses leading to increase in intercellular spaces. Increased glial cells in the cerebral cortex (Figure 2) could be due to the increased population of microglial cells, which are known to participate in the engulfment of cellular debris [18].

CONCLUSION

This study necessitates the need to examine the effects of small and clinically relevant doses of ionizing radiation on the structural, biochemical as well as functional

changes in various brain parts. Radiation dose at 2-3 Gy per fraction is most commonly used for treatment of brain tumors. The development of anti-radiation drugs is the need of hour and is gaining worldwide momentum [19] and therefore, this study has wider implications.

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