Bulletin of Environment, Pharmacology and Life Sciences
Bull. Env. Pharmacol. Life Sci., Vol 3 [9] August 2014: 176-182

Online ISSN 2277-1808
Journal's URL:http://www.bepls.com
CODEN: BEPLAD
Global Impact Factor 0.533
Universal Impact Factor 0.9804

ORIGINAL ARTICLE

Concurrent treatment with Ascorbic acid and A1 Adenosine receptor on Hippocampus in gamma irradiation

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ABSTRACT
Unwanted irradiation can cause a lot of problems such as the types of irradiation syndromes and even death. Hippocampus is affected by degenerative disease and environmental stresses such as irradiation. Adenosine receptors agonist is belonging to a large family of adrenergic receptors. Ascorbic acid is known as a potent antioxidant and also is an anti-cancer substance. We have studied the protective role of separate and concurrent treatment of ascorbic acid and Adenosine receptor agonist on hippocampus neurons of mice following irradiation with gamma ray. For this study, 8 groups were designed. In each group, the mice exposed to 6 Gray radiations in one fracture. After a week, the drugs applied daily by intra-peritoneal injection for a week. After two week from irradiation, Y-maze and shuttle box memory tests were applied to scale short term memory. Finally nissl staining used for cell counting and Tunnel kit utilized to assess apoptotic cell death. Our finding offered that following irradiation short-term memory score was improved after treatment with ascorbic acid and agonist of A1 Adenosine receptor. Cell counting and tunnel test displayed a significant protective role of both factors. Concurrent usage of them can be considered as an effective pharmaceutical approach to reduce hippocampus neuron damages after irradiation.
Keyword: Hippocampus, irradiation, ascorbic acid, Adenosine receptor agonist, neuroprotective effect

Received 10.05.2014 **Revised 22.06.2014** **Accepted 19.07.2014**

INTRODUCTION

Hippocampus is Located in inferior horn of Lateral ventricles in the brain's temporal lobe. This structure plays a key role in maintaining of memory [1]. It also plays a role in organizing and storing information. Damage to the hippocampus causes amnesia and loose later memory [2]. Central nervous system is irradiated in some malady. The side effects of irradiation are depending upon the radiation dose and the size of the exposed zone [3].Gamma irradiation assertively conducts tissue damages through free radicals production. Studies have shown oxygen free radicals cause lipid peroxidation and oxidative stress. A goal of research in radiobiology is to recognize the radiation-sensitive targets in cells and describe the mechanism of damage and repair to modify treatment pathways [4, 5]. Exposure to different types of radiations produces different types of injuries [6]. It has been well known that radiation therapy is one of the major effective treatments for cancers. However, due to the high dose of radiation, patients frequently suffer from toxic effects of irradiation. There is an urgent need for novel approaches to improving response of cancer cells and healthy cells to radiation therapy [7]. Dietary antioxidants (vitamin E, ascorbic acid, selenium, β-carotene and …) as well as endogenous antioxidants (glutathione) neutralize or trap the reactive oxygen species [8, 9]. Treatment with radiation agents to eradicate the cancer cells is depending on their oxidative damage [10]. New study show that some dietary antioxidants may have potential as adjuvant in cancer therapy by their ability to induce programmed cell death [11, 12]. Studies in cell cultures showed that vitamin E, ascorbic acid, selenium, and some photochemical selectively induce apoptosis in cancer cells while sparing normal cells [13]. Study showed that Vitamin E and C significantly reduced the frequencies of micronuclei and chromosomal aberrations in bone marrow cells following gamma radiation [14]. The A1 adenosine receptor (A1AR) is a G-protein-coupled receptor that

mediates many of the physiological effects of adenosine in the brain. The binding of agonist to A1AR induces inhibition of adenylate cyclase and leading to a decrease in intracellular cyclic adenosine monophosphate levels or stimulation of phospholipase C [15]. This implies that adenosine acting via A1AR impairs glioblastoma growth [16]. Hippocampus is among the first areas of the brain affected by degenerative diseases such as Huntington's, Parkinson's disease and it injury from trauma and ischemia and other stress such as irradiation. This area express a lot of A1AR and have a sensitive pyramidal neurons that we used of this situation to design this study [17]. The goal of this study is find an approach to treatment unwanted irradiation effect in hippocampus with usage of ascorbic acid and A1 receptor agonist (CPA) separately and simultaneously.

MATERIALS AND METHODS

Animals:

56 adult bulb-c mice, weighting 35 g _40 g, were obtained from Iranian Razzi Institute and were maintained in one colony room at temperature of 21 ± 1C (50 ± 10% humidity) on a 12-h light/12-h dark cycle with access to water and food. The experimental protocol for animal care and handling was according to the guidelines of the National Institutes of Health for the use of live animals and those of the research council of Tehran University of Medical Sciences (Tehran, Iran). Principle of research ethics when working with laboratory animals was done according to the statute of Tehran University of Medical Ethics.

Experimental design:

The mice were assigned as follow:

groups	Sub groups	number	Dosage of drug
1- Intact groups	-	7	-
2- Irradiation control groups	-	7	-
3- irradiation control with vehicles	-	7	(2mg/kg)
4- Treatment groups	A- treatment group that receive ascorbic acid(AA) from 1 week after irradiation for 7 days	7	(100mg/kg)
	B- treatment group that receive A1 receptor agonist(CPA)	7	(1mg/kg)
	C- Combine treatment group with ascorbic acid and agonist A1 receptor	7	(AA: 100mg/kg) and (CPA: 1mg/kg)
	D- Post treatment group with antagonist of A1 receptor (DPCPX)	7	(2.25mg/kg)
	E- Combine treatment group with ascorbic acid and antagonist of a1 receptor	7	(AA: 100mg/kg) and (DPCPX: 2.25mg/kg)

Bulb/c Mice were subjected to whole body irradiation (6 Gray) with gamma ray (cobalt 60). in treatment groups, the drugs were injected one week after irradiation as intra peritoneal, and Continued for a week. The y-maze test and shuttle box test performed two weeks following irradiation and brains prepared for microscopic studies at the end of memory tests (Nissl staining and Tunnel test).

Irradiation: Mice were irradiated whole body with 6Gray of gamma-rays generated from a cobalt-60 source (Theratron II, 780 C, Canata, ON, Canada) at a dose rate of 76.66 cGray/min, with source sample distance =82 cm, field size: 35×35 cm and at room temperature 23 ± 2 ºC.

Nissl staining: This stain is commonly used for identifying the basic neuronal structure from necrotic neurons in brain and spinal cord. Deparaffinize sections in xylene and then Hydrate. Rinse in tap water and then in distilled water. Stain in 0.1% cresyl violet solution for 3-10 minutes. Rinse quickly in distilled

water. Then differentiate and dehydrate in alcohol then Clearing and finally mount with permanent mounting medium.

Tunnel test: The Tunnel Apoptosis Detection Kit is one of Gen scripts newly introduced products. The kit can detect fragmented DNA in the nucleus during apoptosis. Deparaffinize sections with heater 60^0 C and xylene and then Hydration. Incubate in proteinase k(30 minute) then blocking the endogenous peroxidase with use of H_2O_2 in methanol in dark room then wash in tris buffer and then incubate in Tunnel reaction mixture for 60 min in moisture condition then wash in tris buffer and then detection with incubate in Proper Orthogonal Decomposition.

Y-maze test: This working memory test is based on spontaneous exploration and alternations between arms – neither training nor food restriction are required. Three identical arms are mounted symmetrically on an equilateral triangular center. Mice walk between the arms and we recorded the arm name in 300 second. Finally ever three arms name that not similar is one correct number and another are wrong number. This finding analyze with this formula: Percent Alternation = $PA = x/(y-2) \times 100$ (x=number of correct and y = correct + wrong number). The working memory test was carried out two week after irradiation.

Shuttle box: The Passive Avoidance System has two compartment animal enclosures with black and white compartments, as the Shuttle Box System, with a sliding door partition. In four days this test in performed. In two days the mice get the habit and training with this system. Then in ternary day (Third day) they have an electrical shock (0.3mA in 1 sec) in dark room and ejected to cage. In forth day the entrance time from laminate compartment to dark compartment was counting. The Passive Avoidance test was carried out two week after irradiation.

Statistical analysis: Data were statistically analyzed by SPSS software (version16). All data were expressed as Standard error of the mean (SEM). For within group and intergroup comparisons, two-tailed paired and unpaired Student's t tests were used respectively. One way ANOVA, followed by Tukey post hoc test, was used for each group at different time points. In all analyses, the null hypothesis was rejected at the level of 0.05.

RESULTS

In this study we used the Nissl staining to count the necrotic cell and used the Tunnel kit to detect the apoptotic cell in the hippocampus region. The pictures in bellow show the result of these methods.

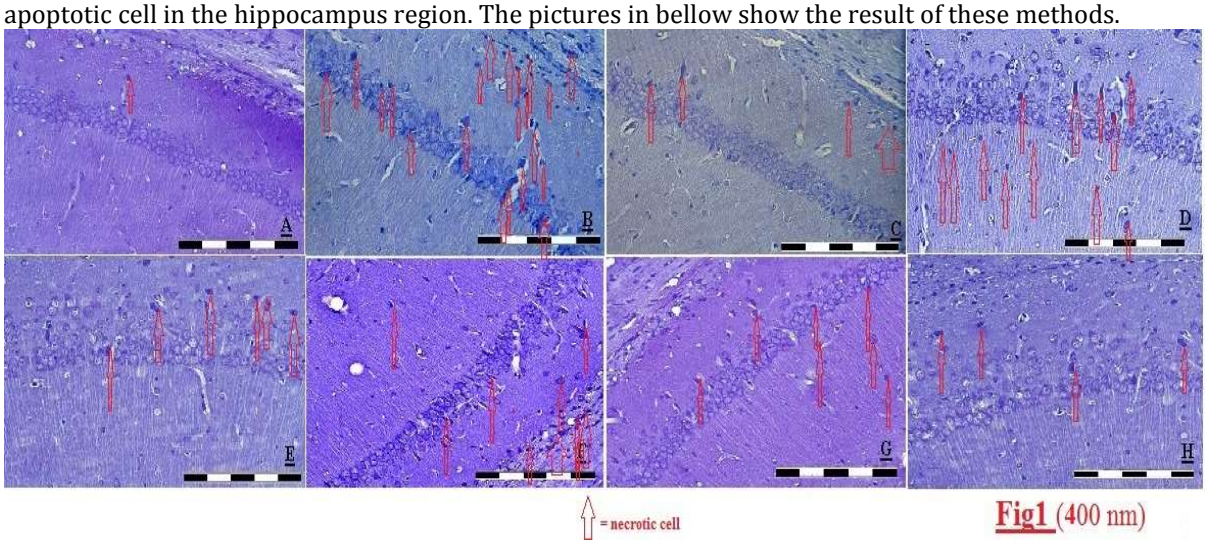

Figure1: Nissl staining (400x). In intact group, this nucleus of cells in image shows all the cells are intact (A). Dark core of necrotic cells is indicated after radiotherapy (B). In DMSO group, the number of necrotic cells shows effect of DMSO in irradiation complications (C). In AA group, Ascorbic acid is reduced the number of necrotic cells (dark) and it is maintained density of healthy (clear) cells (D). In CPA group, agonist adenosine receptor leads to reduced cell density and (dark) necrotic cells in the hippocampus lobe (E). In DPCPX group, antagonist adenosine receptor was caused severe cell death in the irradiation area (F). In DPCPX+AA group, Ascorbic acid has almost adjusted destructive effects of adenosine receptor antagonists (G). In CPA+AA group, Ascorbic acid and adenosine receptor agonist reconciliation strongly reduced of necrotic cells (H).

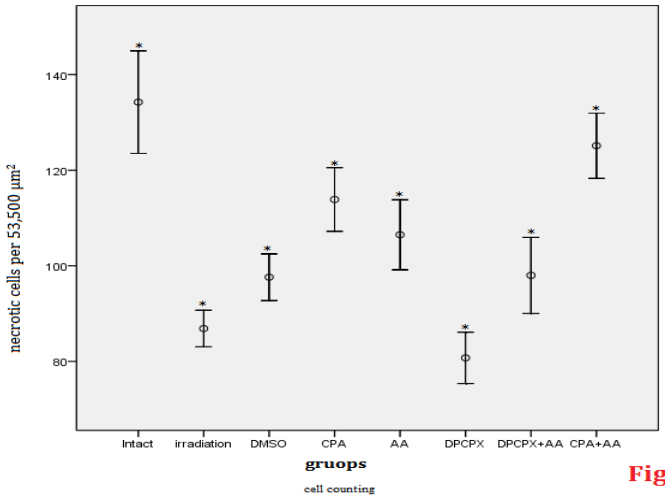

Figure2: In cell counting curve**:** The number of intact cells group and treatment groups with CPA, AA, CPA + AA shows significant difference compared with irradiation group. (P<0.05) *.

Tissue stained with cresyl violet (Nissl) Showed necrotic cells than healthy cells in a field of 53,500 micrometers square in tissue sections. Necrotic cells in the treatment groups significantly were lower (agonist with/and ascorbic acid) and cell density were more in the irradiation control group. Necrotic cells in the combination group were lower than other groups. The A1 receptor antagonist caused a greater complication, even was more necrotic cells than irradiation group and cell density was more reduced.

Tunnel test results:

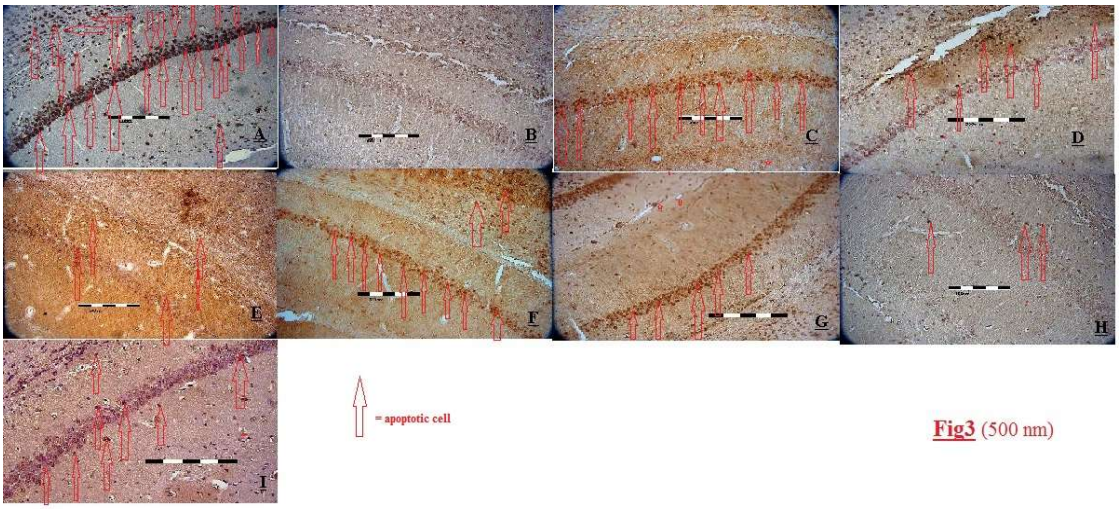

Figure3: Tunnel test (500 nm). In Positive control group, large numbers of Tunnel -positive cells are seen. (A). in Intact group Tunnel positive cells are not seen. (B). in irradiation control group Large numbers of Tunnel -positive cells are seen with brown nucleus. (C). in AA group Few Tunnel -positive cells are seen. (D). in CPA group Few Tunnel -positive cells are seen. (E). in vehicle (DMSO) group Large numbers of Tunnel -positive cells are seen (F). In DPCPX group large numbers of Tunnel -positive cells are seen. (G). In CPA + AA group more few Tunnel -positive cells are seen. (H). in DPCPX+AA group a lot of Tunnel -positive cells are seen. (I).

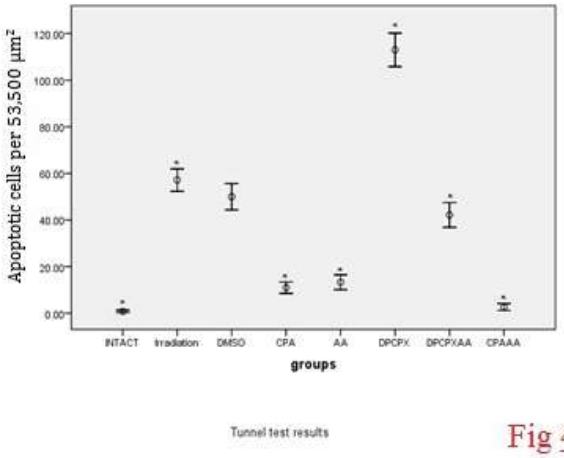

Figure 4: In Tunnel test curve: The numbers of cells in the intact and treatment groups are significantly different from irradiation group. (P<0.05) *.

Photographs related to the Tunnel test in the groups under investigation shows apoptotic cells. In this test, cells are seen as nuclei with small round brown color. Following the use of medications, the apoptotic and necrotic cells are decreased significantly which this effect is due to these two drugs in reducing the irradiation complications. This two-drug combination treatment is more effective than treatment with two drugs separately.

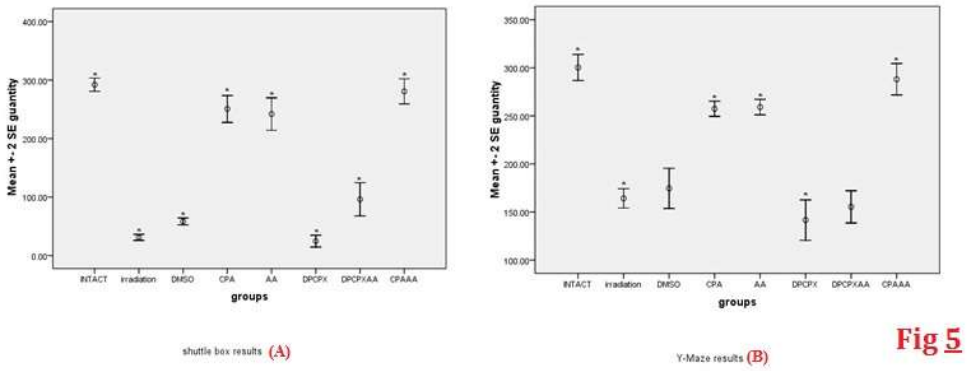

Figure5: in short term memory curve (Shuttle box, Fig 5A): Irradiation group compared with the intact group represents significant difference. (P<0.05) * . Irradiation group compared with treatment groups, except DMSO and DPCPX treated groups, represents significant difference. (P<0.05) *. In y-maze curve (Fig 5B): Irradiation group compared with the intact group represents significant difference. (P<0.05) *.

Y-maze and shuttle box behavioral tests showed that irradiation leads to severe damage to short-term memory. Best results achieved by treatment with this two-drug combination treatment. In Intact group this disorder failed to register.

DISCUSSION

Radiation-induced damage is considered as major contributory factor in treatment-induced neurotoxicity prevalent cancer and it impair long-term memory [18]. In apoptosis, there are many opportunities for cells to prevent from it and are compared their reasons to enzymatic cascade and reversible. [19]. in this study, we investigated the role of antioxidant against of irradiation and necrosis by injection ascorbic acid after induction of irradiation. With this substance, treatment group has a high cell density and were necrotic cells lower and had significant difference from the control irradiation group (Figure 1). These findings were consistent with findings by other researchers [20]. It was found, during radiotherapy vitamins protected hippocampus neurons in diabetic rats. [21]. In treatment group with CPA the number of apoptotic cells was lower than treatment group with ascorbic acid and it seems more effective than ascorbic acid to inhibit apoptosis. In one study, the role of CPA was demonstrating in protection of brain neurons in newborn rats. CPA prevents from activation of the caspase 3, also prevents from the release of cytochrome C from mitochondria and increases the opportunities of cellular repair. recently proven the effect of CPA to prevent cell death in hypoxic conditions in the cerebral cortex [22]. Also has been stated which the CPA have a minor antioxidant role [23]. We expect a significant reduction in radiation lesions in concurrent treatment with ascorbic acid and CPA which its

result was consistent with these tests (figure 2 and 4). According to studies on mechanisms of these materials and by findings from this study, it seem that total antioxidant and anti-apoptotic can prevent from injury to many neurons and decreases death of large numbers of neurons which are damaged following incident radiation. Both of these mechanisms prevent the death of these neurons. Finally, keep functioning of this area of the brain. Subsequent studies have shown which spatial memory and short-term memory are impaired by irradiation in the hippocampus and this disorder is also wider depending on the extent caused by neuronal injury (figure 5A and 5B) [24]. Hippocampus pyramidal neurons are as well as important of the neurons in this area. Major activities include short-term memory and spatial memory in hippocampus neurons are evaluated as its function [25]. Since the materials used in this study have different Neuroprotective effects on these neurons and maintain its density and prevent severe damage and death them. With used the drug, function of hippocampus is maintained after irradiation. Behavioral tests showed that the use of ascorbic acid after the occurrence of radiation can maintain the performance of the hippocampus in spatial memory and short-term to appropriate level (figure 5A and 5B). CPA also with maintain of neuronal density and prevent neuronal death, was caused the hippocampus maintain of memory performance and this information is consistent with results from other researchers [26]. The simultaneous use of concurrent these two drags is more effective rather than usage separate of them.

CONCLUSION

According to the anti oxidative and free radical scavenging properties of ascorbic acid and A1 receptor agonist, we suggest that usage of concurrent these two drags can able to reduce irradiation–induced toxicities in hippocampus neurons and may be in brain.

ACKNOWLEDGEMENTS

We want to thanks research council of Tehran University of Medical Sciences for financial support of the present work

AUTHORS' CONTRIBUTIONS

All authors had equal role in design, work, statistical analysis and manuscript writing.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

FUNDING/SUPPORT

Tehran University of Medical Sciences. Tehran - Iran.

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CITATION OF THIS ARTICLE

Hassanshahi J ,Arsang Sh, Zamani M , Gasemzadeh S. Concurrent treatment with Ascorbic acid and A1 Adenosine receptor on Hippocampus in gamma irradiation.Bull. Env. Pharmacol. Life Sci., Vol 3 [9] August 2014: 176-182