



Research article

Gamma radiation improves AD pathogenesis in APP/PS1 mouse model by potentiating insulin sensitivity

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ARTICLE INFO

Keywords:

Alzheimer's disease
Brain insulin resistance
Gamma radiation
APP/PS1 mice
Cognition
Neuroscience
Biochemistry
Molecular biology
Neurology
Radiology

ABSTRACT

Alzheimer's disease (AD) is the largest unmet medical complication. The devastation caused by the disease can be assumed from the disease symptoms like speech impairment, loss of self-awareness, acute memory loss etc. The individuals suffering from AD completely depend on caregivers and have to bear the high cost of treatment which increases the socio-economic burden on the society. Recent studies have shown that radiation exposure can have therapeutic effects when given in suitable amount for a specific time period. Therefore, we investigated the role of gamma irradiation in AD pathogenesis. The effect of radiation on amelioration of disease progression was studied in AD transgenic mice model (APP/PS1). Our *in-vivo* studies using APP/PS1 mice demonstrated that a single dose of 4.0 Gy gamma irradiation improves AD associated behavioral impairment. Radiation exposure also increased the level of anti-oxidant enzymes and reduced the astrocyte activation in the brain of APP/PS1 mice. A significant reduction was observed in AD associated proteins (APP, pTau, BACE) and neurofibrillary tangle formations (NFTs). Exposure to a single dose of 4 Gy gamma radiation also increased glucose metabolic functionality in AD transgenic mouse model. The kinases involved in insulin signaling such as GSK, ERK and JNK were also found to be modulated. However, an increased level of GSK3 β (ser 9) was observed, which could be responsible for downregulating ERK and JNK phosphorylation. This resulted in a decrease in neurofibrillary tangle formations and amyloid deposition. The reduced hyperphosphorylation of Tau can be attributed to the increased level of GSK3 β (ser 9) downregulating ERK and JNK phosphorylation. Thus, a single dose of 4 Gy gamma irradiation was found to have therapeutic benefits in treating AD *via* potentiating insulin signaling in APP/PS1 transgenic mice.

1. Introduction

Alzheimer's disease (AD) is the most devastating neurodegenerative disorder characterized by A β ₄₂ accumulation in the brain. Other pathophysiological features are hyperphosphorylation of tau protein and neurofibrillary tangle formations (NFTs). According to the WHO, AD has become the 6th leading cause of death in the United States particularly at the age of 65 or above [1]. The current cost of treatment is \$36 million dollars, with 5.8 million sufferers and is predicted to increase three times by 2050 [2]. Amyloid deposition due to A β aggregation significantly affects cognition and memory [3]. Moreover, patients suffering from AD have also been diagnosed with neuronal/brain insulin resistance [4]. Therefore, improving insulin resistance by insulin sensitizing molecules could be a potential target for treating AD. Most of the

anti-amyloidogenic therapies fail due to the inability of drugs to cross the blood-brain barrier [5]. Currently, all the available treatments provide only symptomatic relief rather than curing the disease. Recently, ionizing radiation has been reported to potentiate insulin signaling and improve insulin sensitivity by targeting Akt/MDM2/P53-mediated anti-apoptotic and Akt/Nrf2-mediated anti-oxidant pathways simultaneously [6]. Furthermore, Cuttler *et al.* reported that low ionizing radiation from CT scans improved the memory deficits, mobility and communication in 81 years old female patient suffering from AD [7]. Low dose ionizing radiation has also been reported to cause adaptive protection by stimulating about 150 genes responsible for the growth and oxidative response in the brain which may be essential for the reversal of neurodegeneration [8, 9] in contrast to high dose damaging effects [10]. Recently, Marples *et al.* showed that fractionated doses (2 Gy X 5) of cranial irradiation are

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beneficial in reducing insoluble A β ₄₂ and improving cognition in AD transgenic mice [11]. Clinical studies have also shown that high dose irradiation of 30–60 Gy adversely affects cognition while 12–20 Gy irradiation in 2 Gy fractions are effective in improving cognition in AD patients [12].

Therefore, the aim of the present study was to investigate the effect of single and fractionated doses of gamma irradiation on APP/PS1 mouse model of AD, and its correlation with glucose metabolism/homeostasis and insulin sensitivity.

2. Material and methods

2.1. Mice

All animal experiment studies were performed after approval from the Institutional Animal Ethics Committee (IAEC) (345/14) of National Institute of Immunology and according to ARRIVE (Animal Research: Reporting of In-Vivo Experiments) guidelines. The transgenic mice were procured from Jackson's laboratory and maintained at the animal facility of National Institute of Immunology, New Delhi. For all the animal experiments APP/PS1 mouse model (stock no. 004462, B6C3-Tg (APP^{swe}, PSEN1^{dE9})85Dbo/Mmjax) was used. The AD transgenic mouse model expresses a chimeric mouse/human amyloid precursor protein (Mo/HuAPP695^{swe}) and a mutant human presenilin 1 (PS1-dE9) both directed to CNS neurons. The animals were kept in 12 h light/dark cycle with free access to food and water *ad-libitum*.

2.2. Genotyping

APP/PS1 mice were segregated into wild type and transgenic mice using genotyping. The DNA was isolated from the tail of these animals using DNA extraction kit. APP and PSEN genes were screened in these animals using specific primers (Table 1). Mice expressing both the genes were considered as transgenic and others as wild type. The protocol for DNA extraction was followed as per the manufacturer's instructions.

2.3. Glucose and insulin tolerance test

Glucose tolerance test (GTT) and insulin tolerance test (ITT) were performed when mice were 14 week old (Wt; n = 10, Tg; n = 50) and then again at 17 week (Wt; n = 10, Tg; n = 50) to identify the optimal time at which glucose metabolism was impaired in APP/PS1 mice. The mice were irradiated at 17 weeks of age as glucose metabolism was found to be debilitated at this stage. To study the effect of irradiation, GTT was again performed after 4 weeks, i.e. after the competition of radiation treatment, when mice were 21 weeks old (Wt; n = 10, Tg; n = 10 per group). For performing GTT, mice were fasted overnight (16 h) with full access to drinking water. Glucose was injected intraperitoneally at a dose of 2 mg/gm body weight. Glucose levels were then monitored using a glucometer (Roche) at various time intervals such as 0, 15, 30, 45, 60, 90 and 120 min.

For ITT, animals were fasted for 6 h, followed by intraperitoneally humanized insulin injection at the dose of 0.75 IU/kg body weight. The

glucose levels were monitored using a glucometer (Roche) at different time intervals such as 0, 15, 30, 45, 90 and 120 min.

2.4. Gamma irradiation

17 week old APP/PS1 mice (showing impaired glucose metabolism) were whole body irradiated to different doses of gamma radiation (0.5–4 Gy) at a dose rate of 1.5 Gy/min (Cobalt ⁶⁰ source). Mice were not anesthetized during radiation treatment. Each mouse was restrained in an irradiator made of glass with proper ventilation and exposed to radiation in a Co⁶⁰ gamma chamber at National Institute of Immunology, New Delhi, India.

2.5. Experimental design

The mice were divided into six groups of 10 mice each (Table 2). Group 1 and Group 2 were sham irradiated while as Group 3 to 6 were exposed to Co⁶⁰ gamma radiation. S represents single exposure to gamma radiation and M represents the exposure to gamma radiation more than once. The animals in Group 6 died during the course of irradiation and therefore could not be included further. After observing disturbed glucose homeostasis (GTT and ITT) in APP/PS1 mice, radiation treatment was started at 17 weeks of age. After one month i.e. at 21 weeks of age, APP/PS1 mice were sacrificed by decapitation and brain tissue was isolated. Each control and the treatment group consisting of 10 mice were sub-divided into two groups of 5 mice each. Brain from one group (5 mice per group) was used for protein extraction (left hemisphere) and RNA extraction (right hemisphere) and the other group (5 mice per group) for immunohistochemistry.

2.6. Body weight and food intake

The weight of APP/PS1 mice (Wt; n = 10, Tg; n = 10 per group) was measured at different time intervals. To measure the total food intake after irradiation mice were housed singly, i.e. one mouse per cage. Each mouse was given 20 g of feed for 24 h after which the remaining feed was removed. The leftover feed was weighed and compared with other groups.

2.7. Behavioral studies

2.7.1. Morris Water maze

Spatial learning and memory were assessed in irradiated, 21 week old APP/PS1 mice (Wt; n = 10, Tg; n = 10 per group). It consisted of a circular tank of 100 cm in diameter. The tank was divided into four quadrants and labelled as A, B, C and D. The quadrant in which the platform is placed is known as target quadrant/zone. Before the actual trials mice were trained to find the hidden platform, two trials per day for 2 days. During the training period, the mice were allowed to swim freely for 60 s to find the hidden platform and to show them that, there is a place in a pool where they can come and rescue themselves. Upon the actual start of the testing, the platform was placed 1.5 cm below the water level. Animals were given two trials per day for 3 days consecutively. The escape latency was recorded, which is defined as the total time taken (sec) to

Table 1. List of primer sequences used for genotyping of APP/PS1 transgenic mice model.

Primer	Sequence 5' → 3'	Base pair
PSEN FP	CTAGGCCACAGAATTGAAAGATCT	608 bp
PSEN RP	GTAGGTGGAAATCTAGCATCATCC	
Internal Control FP	AATAGAGAACGGCAGGAGCA	324 bp
Internal Control RP	GCCATGAGGGCACTAATCAT	
APP FP	CCGAGATCTCTGAAGTGAAGATGGATG	377 bp
APP RP	GTGGATACCCCTCCCCAGCTAGACC	

Table 2. Experimental design of gamma irradiation regime followed in mice.

Group	Animal (Age at the start of exp.)	Gamma radiation	No. of mice (n)	Duration of exp.	Total radiation	Response	
1	Wild Type (Wt)	Wt (17 weeks)	-	10	4 weeks	0	Survived
2	Transgenic (Tg)	Tg (17 weeks)	-	10	4 weeks	0	Survived
3	Tg+ 0.5 Gy (S)	Tg (17 weeks)	0.5 Gy	10	4 weeks	0.5 Gy	Survived
4	Tg+ 0.5 Gy (M)	Tg (17 weeks)	0.5 Gy X 8	10	4 weeks	4 Gy	Survived
5	Tg+ 4 Gy (S)	Tg (17 weeks)	4 Gy	10	4 weeks	4 Gy	Survived
6	Tg+ 4 Gy (M)	Tg (17 weeks)	4 Gy X 8	10	4 weeks	32 Gy	Died

reach the platform in 5 consecutive days. On 6th day, probe test was performed in which platform was removed and the animal was allowed to swim freely for 60 s to calculate the total time spent in the target quadrant. Data were analyzed using ANY maze software (Stoeling Co., USA).

2.7.2. Y maze

Y maze test was used to assess the spontaneous short term memory in 21 week old APP/PS1 mice (Wt; n = 10, Tg; n = 10 per group). The maze contains three smooth arms separated at 120° and a depth of 2.5 cm. Each arm was labeled as A, B and C from outside. The apparatus arms do not contain any clues and each mouse entry was unbiased. The mouse was left in one of the arm for 8 min to assess the total arm entries and the sequence of the arm in which the mouse enters. The percent alternation was calculated by the proportion of arm choices that differ from the last two choices to total arm entries. Data were analyzed using ANY maze software (Stoeling Co., USA).

Percent Alternation = (Arm choices in triplets / Total arm entries)*100

To study the exploratory behavior of animals one of the three arms were closed and the mouse was allowed to move in the remaining two arms for 1 min. After 1 min mouse was taken out and arms were cleaned with 70% ethanol to remove any traces of smell of the previous animal. The animal was again placed in the arm for 7 min with all the arms open. The total arm entries and time spent in the novel arm/closed arm was calculated. Data were analyzed using ANY maze software (Stoeling Co., USA).

2.8. Total protein extraction and western blotting

For assessing the protein levels brain tissue from (left hemisphere) of 21 week old APP/PS1 mice (Wt; n = 5, Tg; n = 5 per group), was homogenized using RIPA lysis buffer with protease and phosphatase inhibitors. Tissue homogenates were centrifuged at 12,000 rpm at 4°C for

30 min. The supernatant was collected and the pellet was discarded. Protein was then estimated using Bradford assay and absorbance was measured at 595 nm.

For western blotting, total 20 µg protein was loaded for polyacrylamide gel electrophoresis (PAGE). The gel percentage was varied from 8 to 12, depending upon the molecular weight of the protein to be identified. For higher molecular weight proteins, 8% gel was used while for low molecular weight proteins, 12% of gels were employed. The protein was then transferred onto 0.22 micron nitrocellulose membrane and blocked with 5% BSA at room temperature for 1hr or overnight at 4°C. After blocking the membrane, primary antibody (Table 3) in 1% BSA was added overnight at 4°C. The membrane was then washed thrice with 1X TBST followed by secondary antibody incubation for 1 h at room temperature. After secondary incubation, the membrane was washed thrice with 1X TBST and bound antibody was visualized using a gel documentation instrument (LAS 4000). The densitometric analysis was performed using Multi Gauge software, version 3.0 (Fujifilm).

2.9. Real time PCR

Total RNA was isolated from right hemisphere of control and irradiated 21 week old APP/PS1 mice (Wt; n = 5, Tg; n = 5 per group) using a standard trizol method. cDNA was then prepared using 1 µg RNA using Verso cDNA synthesis kit. PCR reaction was set up with cDNA using templates with a specific primer sequence. Fold change was calculated using the formula $2^{-\Delta\Delta Ct}$ (Table 4).

2.10. Immunohistochemistry

Irradiated 21 week old APP/PS1 mice (Wt; n = 5, Tg; n = 5 per group) were sacrificed by decapitation. The brain tissue was dissected out and kept in 4% PFA overnight at 4°C. Fixed brain tissue was dehydrated with gradient ethanol followed by xylene and then embedded in paraffin blocks. 10 micron thick sectioned were prepared and rehydrated using

Table 3. List of antibodies.

ANTIBODY	COMPANY	CATALOG No.	MOLECULAR WEIGHT (kDa)
pAKT (Ser 473)	Cell signaling	4060	60
AKT	Cell signaling	4691	60
pGSK3β	Cell signaling	9336	46
GSK3 β	Cell signaling	12456	46
pJNK	Cell signaling	4668	46, 54
JNK	Cell signaling	9258	46, 54
pERK	Cell signaling	9101	42, 44
ERK	Cell signaling	9102	42, 44
pTau (Ser 199)	Sigma	T6819	45–68
Tau	Cell signaling	46687	50–80
BACE 1	Cell signaling	5606	70
GFAP	Cell signaling	3670	50
APP	Cell signaling	2450	100–140
Amyloid beta (DE2B4)	Santa Cruz	sc 58508	132
β -actin	Santa Cruz	sc 47778	45

Table 4. List of Primer sequence used in Quantitative Real Time PCR.

Gene	Accession number	Forward Primer (5'- 3')	Reverse Primer (5'- 3')
Catalase	NM_009804	AGCGACCAGATGAAGCAGTG	TCCGCTCTCTGTCAAAGTGTG
SOD	NM_01143/4	AACCAGTTGTGTGTGTCAGGAC	CCACCATGTTTCTTAGAGTGAGG
GPX	NM_008160.6	CCACCGTGTATGCCCTTCTCC	AGAGAGACGGCATTCTCAAT
GST	NM_013541.1	ATGCCACCATACACCATTGTC	GGGAGCTGCCATACAGAC
18S	NR_003278.3	CGAAAGCATTGCCAAGAAT	AGTCGGCATCGTTTATGTC

xylene and graded ethanol to water (xylene 100%, xylene 50%, ethanol 100%, 95%, 80%, 70%). After antigen retrieval in boiling sodium citrate buffer for 20 min, blocking was done with 5% BSA at room temperature for 1 h. The sections were incubated overnight at 4°C in primary antibody followed by washing of slides with 1X PBS thrice for 5 min each. The secondary antibody (Alexa fluor) was added for 1 h at room temperature followed by washing thrice with 1X PBS. Slides were mounted with coverslips and analyzed under a confocal microscope (Karl Zeiss, LSM 4000).

2.11. Statistical analysis

Two-tailed, unpaired student t-test was used to determine the statistical difference between the mean values of two different groups. One

way ANOVA followed by Duncan post-hoc test was employed in case of more than two groups. Differences were considered significant for $p < 0.05$ and results were expressed as mean \pm SEM.

3. Results

3.1. Gamma radiation improves cognitive and behavioral parameters

The behavioral deficits and cognitive functions were studied in 21 week old APP/PS1 mice following various radiation exposures regimen (0.5 Gy, 0.5 Gy X 8 and 4 Gy). In order to explore the spontaneous and working memory status in irradiated animals, Y maze and water maze tests were performed.

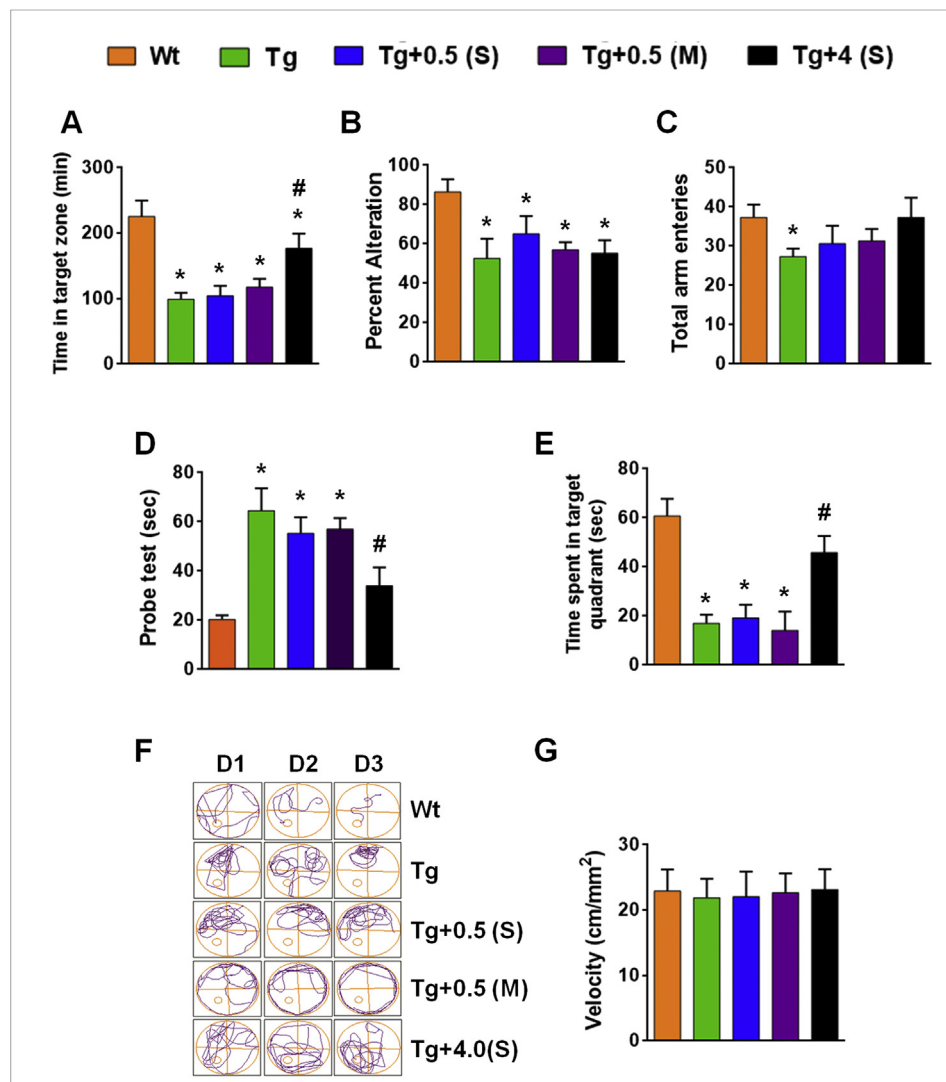


Figure 1. Effect of single and multiple exposure of gamma irradiation on behavioral and cognitive function in APP/PS1 mice. Behavioral testing was performed using Y and water maze to study the memory exploratory and retention functionality. Y maze: (A) Time in target zone (min) (Wt; n = 10, Tg; n = 10 per group), (B) Percent Alteration (Wt; n = 10, Tg; n = 10 per group) and (C) Total arm entries (Wt; n = 10, Tg; n = 10 per group). Water maze (Wt; n = 10, Tg; n = 10 per group): (D) Probe test (sec) (Wt; n = 10, Tg; n = 10 per group), (E) Time spent in target quadrant (sec) (Wt; n = 10, Tg; n = 10 per group), (F) Diagrammatic representation of animals finding the platform (Wt; n = 10, Tg; n = 10 per group) and (G) velocity (cm/mm²) (Wt; n = 10, Tg; n = 10 per group). Different groups are indicated as Wt (Wild type), Tg (Untreated Transgenic), Tg+0.5 (S) (Transgenic mice received single dose of 0.5 Gy radiation), Tg+0.5 (M) (Transgenic mice received 0.5 Gy radiation twice a week for one month), Tg+4 (S) (Transgenic mice received single dose of 4 Gy radiation). Values are expressed as mean \pm SEM. * $p < 0.05$ compared to wild type and # $p < 0.05$ compared to untreated APP/PS1 mice (Tg).

The Y maze test determines the parameters such as time spent in target arm, total arm entries and percent alternation. The APP/PS1 mice receiving a single dose of 4 Gy irradiation spent more time in the target arm (176.67 ± 22.55 min) as compared to the untreated transgenic control (99.02 ± 9.96 min) (Figure 1A). However, no significant change was observed in the case of percent alteration (Figure 1B) and total arm entries within the groups (Figure 1C) compared to untreated transgenic control.

In Morris water maze test, mice had to find the hidden platform in the given time period. The testing determines the animal's spatial memory and learning.

We observed that transgenic mice receiving a single dose of 4 Gy irradiation [4 Gy (S)] spent more time in the target quadrant (45.82 ± 6.79 s) as compared to untreated transgenic control (17.03 ± 3.44 s) While as other treatment groups could not memorize the target quadrant (Figure 1E). This is also clearly visible through track diagram (Figure 1F). However, the swimming speed of these animals remained unaltered (Figure 1G). Moreover, on the final day probe test was conducted in which platform was removed to identify the memory retention ability in these animals. Mice receiving 4 Gy irradiation were able to find the hidden platform on day 6 (34.01 ± 7.55 s) in lesser time as compared to untreated transgenic control (64.49 ± 9.14 s) (Figure 1D). No significant change in the body weight and/or food intake was observed in treated and untreated APP/PS1 mice (data not shown). Thus, these results indicate that APP/PS1 mice exposed to 4 Gy gamma irradiation were able to retain their memory and learning ability.

3.2. Gamma irradiation decreases A β plaque deposition

In AD, amyloid deposition/accumulation is the key event which occurs during disease progression resulting in neuronal toxicity and injury. Therefore, we examined the amyloid deposition/accumulation in the brain sections of 21 week old APP/PS1 mice in gamma treated and untreated groups. The A β_{42} plaques were studied using A β specific antibody using immunohistochemistry (IHC) and Thioflavin S (ThS) staining (data not shown). The mice exposed to a single dose of 0.5 Gy did not show a significant change ($95.67\% \pm 12.9$) as compared to untreated transgenic control. However exposure to 8 fractions of 0.5 Gy radiation significantly increased percentage of plaque formation/accumulation to 153.33%

(± 25.17). In contrast mice exposed to a single dose of 4 Gy radiation showed a significant reduction in plaque formation i.e. 48.33% (± 12.58) (Figure 2). Thus, these results clearly indicate that a single dose of 4 Gy irradiation was effective in breaking/clearing amyloid burden in APP/PS1 mice.

3.3. Gamma irradiation promotes non-amyloidogenic processing of APP

In order to determine the neuropathological role of radiation, the key proteins involved in APP processing (APP and BACE) were studied in irradiated 21 week old APP/PS1 mice. These proteins play an important role in regulating amyloidogenic and non-amyloidogenic processing of APP. In diseased condition, the level of these proteins is higher and promotes amyloidogenic processing of APP resulting in amyloid deposition in the brain, thereby accelerating disease progression. Untreated transgenic mice exhibit increased levels of APP and BACE proteins by 6.33-fold (± 0.71) and 1.10-fold (± 0.20) as compared to wild type. However, a single dose of 4 Gy radiation significantly reduced APP and BACE expression to 3.23-fold (± 0.42) and 0.53-fold (± 0.15) respectively as compared to transgenic controls. However, the level of these proteins was significantly higher in other radiation treated groups of APP/PS1 mice (0.5 Gy, and 0.5 Gy x 8) (Figure 3).

3.4. Gamma radiation improves glucose tolerance in APP/PS1 mice

The APP/PS1 mice have been previously observed to have impaired glucose metabolism, an event which occurs prior to AD pathogenesis and cognitive impairment [13]. Therefore, we checked the effect of radiation on glucose metabolism and homeostasis by performing glucose tolerance test (GTT) and insulin tolerance test (ITT). To identify the actual age of mice showing disturbed glucose homeostasis so that radiation could be started from that particular age, GTT and ITT were performed at 14 and 17 weeks of age in APP/PS1 mice.

For GTT assay, animals were starved for 16 h to normalize the body glucose level and then injected with D-glucose intraperitoneally (i.p). The wild type (26702.13 ± 2433.60 A.U) and untreated transgenic APP/PS1 mice (26042.02 ± 1952.91 A.U) did not show disturbance in glucose metabolism at 14 weeks of age while as at 17 weeks of age glucose metabolism in wild type (23929.90 ± 1072.20 A.U) and untreated

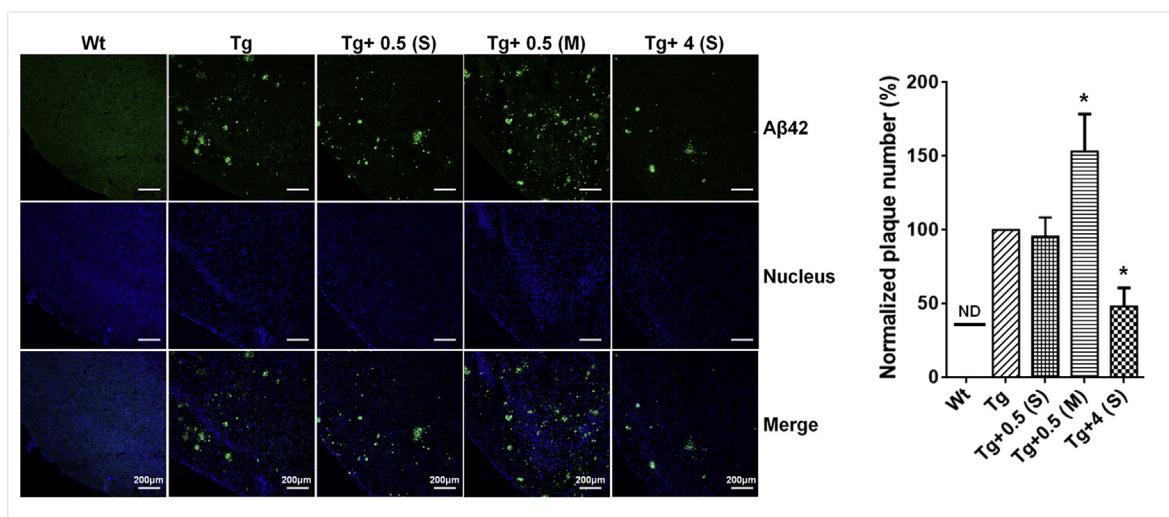


Figure 2. Effect of single and multiple exposure of gamma irradiation on amyloid burden. Brain was harvested at the end of experiment and embedded in paraffin. 10 μ m sections were obtained on poly-L-lysine coated slides for immunohistochemical analysis. A β_{42} plaques were probed with specific primary antibody and visualized on confocal microscope using secondary Alexa Fluor 488 (green) and nucleus with DAPI (blue). Representative images and densitometry of normalized plaque number (%) are shown. Different groups are indicated as Wt (Wild type), Tg (Untreated Transgenic), Tg+0.5 (S) (Transgenic mice received single dose of 0.5 Gy radiation), Tg+0.5 (M) (Transgenic mice received 0.5 Gy radiation twice a week for one month), Tg+4 (S) (Transgenic mice received single dose of 4 Gy radiation). Values are expressed as mean \pm SEM (Wt; n = 5, Tg; n = 5 per group). *p < 0.05 compared to untreated APP/PS1 mice (Tg).

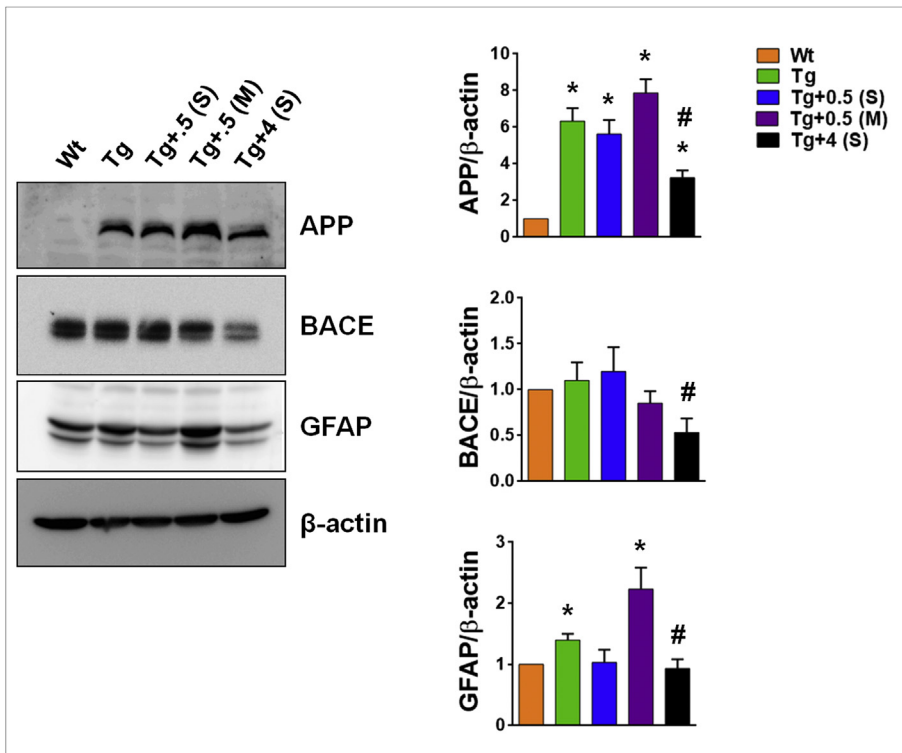


Figure 3. Effect of single and multiple exposure of gamma irradiation on APP processing and astrocyte marker, GFAP. Representative western blot images and densitometric analysis of APP, BACE and GFAP normalized by β -actin. Different groups are indicated as Wt (Wild type), Tg (Untreated Transgenic), Tg+0.5 (S) (Transgenic mice received single dose of 0.5 Gy radiation), Tg+0.5 (M) (Transgenic mice received 0.5 Gy radiation twice a week for one month), Tg+4 (S) (Transgenic mice received single dose of 4 Gy radiation). Values are expressed as mean \pm SEM (Wt; n = 5, Tg; n = 5 per group). *p < 0.05 compared to wild type and #p < 0.05 compared to untreated APP/PS1 mice (Tg).

transgenic APP/PS1 mice (28684.02 ± 2082.23 A.U) was altered (Figure 4A–C). This led us to select 17 weeks as the optimal age for irradiation. GTT assay was performed again at 21 weeks in mice receiving various doses of radiation (0.5Gy, 0.5 Gy X 8, 4 Gy). The results obtained indicate that APP/PS1 mice irradiated with 4 Gy (27792.10 ± 4123 A.U) were efficient in improving glucose metabolism and its utilization as compared to untreated transgenic control (39792.02 ± 5421.23 A.U). However, other treatment groups [0.5 Gy (37229.91 ± 2451.11 A.U), 0.5 Gy X 8 (37985.03 ± 1562.32 A.U)] did not show any significant improvement (Figure 4D and E).

For ITT, APP/PS1 mice were fasted for 6–8 h prior to insulin injection (0.75 IU/kg b.wt) followed by glucose level measurement at various time points such as 0, 15, 30, 45, 60, 90, 120 min. No significant difference in

glucose metabolism was observed in 14-week old wild type (19402.50 ± 1258 A.U) and transgenic APP/PS1 mice (19857.01 ± 1952 A.U). However, ITT at 17 weeks mice showed impaired glucose metabolism in transgenic mice (26894.08 ± 1121.82 A.U) compared to wild type mice (19403.41 ± 3321.02 A.U) (Figure 5A–C). Therefore 17-week old mice were selected further to study the effect of radiation treatment, and ITT was performed at 21 weeks of age. Results obtained from ITT assay showed that mice receiving 0.5 Gy X 8 (8269.10 ± 1124.10 A.U) and 4 Gy radiation (10010.81 ± 2154.91 A.U) were able to metabolize the glucose faster than mice receiving 0.5 Gy radiation (18690.20 ± 3251.33 A.U) as compared to untreated transgenic control (18174.62 ± 1232.43 A.U) (Figure 5D and E).

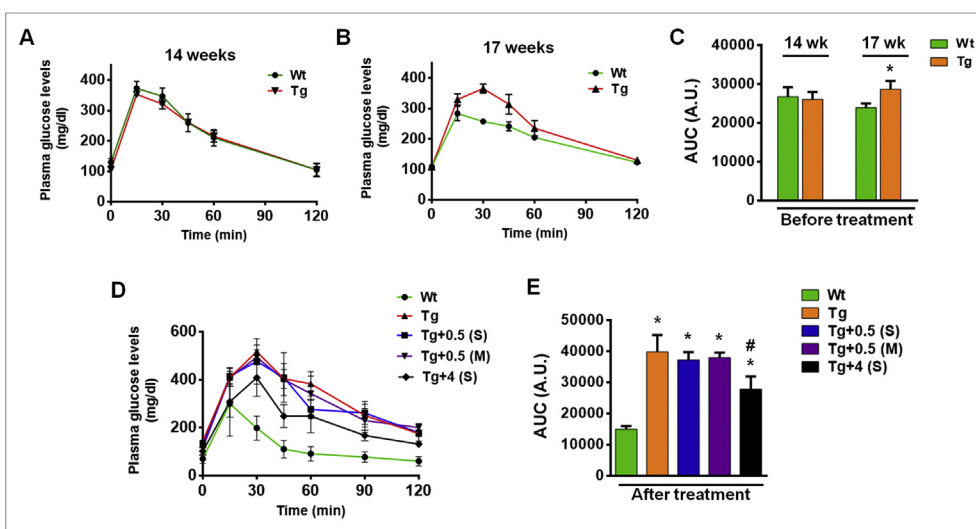


Figure 4. Effect of single and multiple exposure of gamma irradiation on glucose metabolism by GTT in APP/PS1 mice. Body glucose of normal and irradiated mice was measured after 16 h of fasting with free availability of water *ad libitum*. Glucose was intra-peritoneally injected at the dose of 2 mg/g body weight. GTT (before radiation exposure) (Wt; n = 10, Tg; n = 50). (A) 14 weeks, (B) 17 weeks, (C) Area under curve (AUC), at 14 and 17 weeks. (D&E) GTT and Area under curve (AUC) after radiation exposure at 21 weeks (Wt; n = 10, Tg; n = 10 per group). Different groups are indicated as Wt (Wild type), Tg (Untreated Transgenic), Tg+0.5 (S) (Transgenic mice received single dose of 0.5 Gy radiation), Tg+0.5 (M) (Transgenic mice received 0.5 Gy radiation twice a week for one month), Tg+4 (S) (Transgenic mice received single dose of 4 Gy radiation). Values are expressed as mean \pm SEM. *p < 0.05 compared to wild type and #p < 0.05 compared to untreated APP/PS1 mice (Tg).

3.5. Gamma radiation modulates phosphorylation/activation of GSK3 β , a key component of insulin signaling

Recently, studies have shown a strong correlation between insulin signaling and amyloid deposition in AD progression. Therefore, we further checked the phosphorylation of key intermediate molecules of insulin signaling pathway i.e. AKT and GSK3 β in the brain homogenates of 21 week old APP/PS1 mice post radiation exposure. Transgenic mice treated with various doses of gamma radiation (0.5 Gy, 0.5 Gy X 8 and 4 Gy) did not show a significant change in AKT phosphorylation (Figure 6A). Interestingly, the inhibitory phosphorylation of GSK3 β (Ser 9) increased by 1.87-fold (± 0.31) in mice exposed to a single dose of 4 Gy radiation while as it was reduced (0.93-fold ± 0.15) in untreated transgenic control mice as compared to wild type (Figure 6B). Thus, increased activation of GSK3 β (Ser 9) might have activated downstream signaling thereby increasing insulin sensitivity in an AKT-independent manner. No significant change was observed in pGSK3 β (Ser 9) in other treated groups such as 0.5 Gy and 0.5 Gy X 8 (Figure 6A and B).

3.6. Gamma radiation affects activation/phosphorylation of JNK and ERK and reduces Tau hyperphosphorylation

NFTs are the major neuropathological feature of AD composed of microtubule associated protein-Tau. The hyperphosphorylation of tau destabilizes the microtubule resulting in neurotoxic tangle formation, which leads to neurodegeneration. The activation of two stress related proteins ERK and JNK plays an important role in regulating tau hyperphosphorylation. We observed noticeable reduction in phosphorylation of stress activated molecules after irradiation with a single dose of 4 Gy in 21 week old APP/PS1 mice. Untreated transgenic control showed increased expression of JNK (1.97-fold ± 0.25) and ERK (1.81-fold ± 0.30) in comparison to wild type. This increased expression was restored to baseline values i.e. 1.17-fold (± 0.25) and 1.35-fold (± 0.12) for JNK and ERK respectively in transgenic mice irradiated with a single dose of 4 Gy (Figure 7A and B). Tau phosphorylation which is significantly high [1.87-fold (± 0.22)] in untreated transgenic mice (in comparison to wild type) was found to be reduced [1.10-fold (± 0.22)] significantly in transgenic mice exposed to a single dose of 4 Gy radiation (Figure 7C). The decreased level of stress related genes might have resulted in reduced tau hyperphosphorylation in APP/PS1 transgenic mice receiving 4 Gy exposure. These results indicate that a single dose of 4 Gy in APP/

PS1 mice could stabilize the microtubule by regulating Tau hyperphosphorylation, which could be ERK and JNK mediated.

3.7. 4 Gy gamma radiation increases anti-oxidant enzymes

Oxidative stress participates in AD pathogenesis by promoting A β deposition and tau hyperphosphorylation, which leads to impaired synaptic and neuronal functionality. Previous studies have shown that radiation causes oxidative stress leading to pathophysiological changes in the brain which are neurotoxic. In our study, we checked the effect of different doses of radiation [0.5 Gy (S), 0.5 Gy (M), 4 Gy (S)] on the anti-oxidant enzymes in APP/PS1 mice. The mRNA expression of major antioxidant enzymes such as SOD, Catalase, GPX and GST were assessed using real-time PCR. The diminished expression of catalase and SOD [0.61-fold (± 0.23) and 0.53-fold (± 0.16)] was observed in untreated transgenic mice (Figure 8A and B) while no significant change was observed in case of GST and GPX [0.93-fold (± 0.18) and 0.94-fold (± 0.28)] as compared to wild type (Figure 8C and D). APP/PS1 mice exposed to low dose radiation (0.5 Gy and 0.5 Gy X 8) did not show any significant change in the level of anti-oxidant enzymes with respect to untreated transgenic control however the levels were lower than the wild type control. Interestingly, a single dose of 4 Gy increased the level of antioxidant enzymes such as catalase, SOD and GPX [1.33-fold (± 0.19), 1.47-fold (± 0.15) and 1.27-fold (± 0.06) except for GST, 0.97-fold (± 0.21)] as compared to [0.61-fold (± 0.26), 0.53-fold (± 0.15) and 0.93-fold (± 0.17)] untreated transgenic control respectively (Figure 8). Thus, these results indicate that a single dose of 4 Gy exposure increases the level of anti-oxidant enzymes in APP/PS1 mice.

3.8. Gamma irradiation reduces neurotoxicity induced by activation of astrocytes

Persistent astrocyte activation causes neurotoxicity, a major pathophysiological event in AD. Neuroinflammation is characterized by the accumulation of activated astrocytes around A β ₄₂ deposits. The A β ₄₂ accumulation leads to neuronal injury and is assessed by the astrocyte activation in the brain. Immunohistochemistry of GFAP (a marker for astrocyte) in 21 week old APP/PS1 mice showed increased percentage fluorescent intensity 142.70% (± 15.10) in 0.5 Gy X 8 treatment group as compared to untreated transgenic control. Percentage fluorescent intensity was reduced in 0.5Gy [92.63% (± 17.80)] and this reduction was quite significant in 4 Gy [53.51% (± 8.91)] radiation exposed APP/PS1

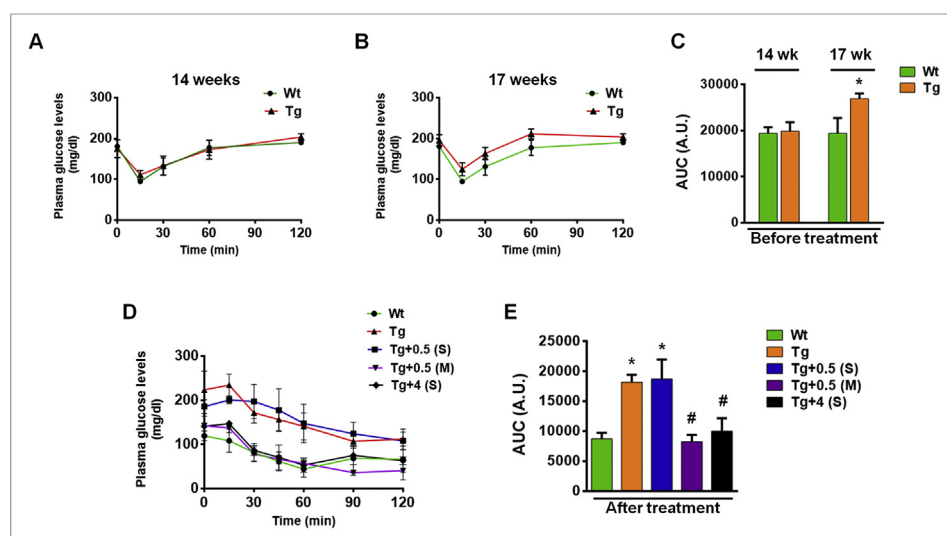


Figure 5. Effect of single and multiple exposure of gamma irradiation on glucose metabolism by ITT in APP/PS1 mice. Body glucose of normal and irradiated mice was measured after 6–8 h of fasting with free availability of water *ad libitum*. Insulin was intra-peritoneally injected at the dose of 0.75 IU/kg body weight. ITT (before radiation exposure), (Wt; n = 10, Tg; n = 50) (A) 14 weeks, (B) 17 weeks, (C) Area under curve (AUC). (D & E) ITT and Area under curve (AUC) at 21 weeks after irradiation (Wt; n = 10, Tg; n = 10 per group). Different groups are indicated as Wt (Wild type), Tg (Untreated Transgenic), Tg+0.5 (S) (Transgenic mice received single dose of 0.5 Gy radiation), Tg+0.5 (M) (Transgenic mice received 0.5 Gy radiation twice a week for one month), Tg+4 (S) (Transgenic mice received single dose of 4 Gy radiation). Values are expressed as mean \pm SEM. *p < 0.05 compared to wild type and #p < 0.05 compared to untreated APP/PS1 mice (Tg).

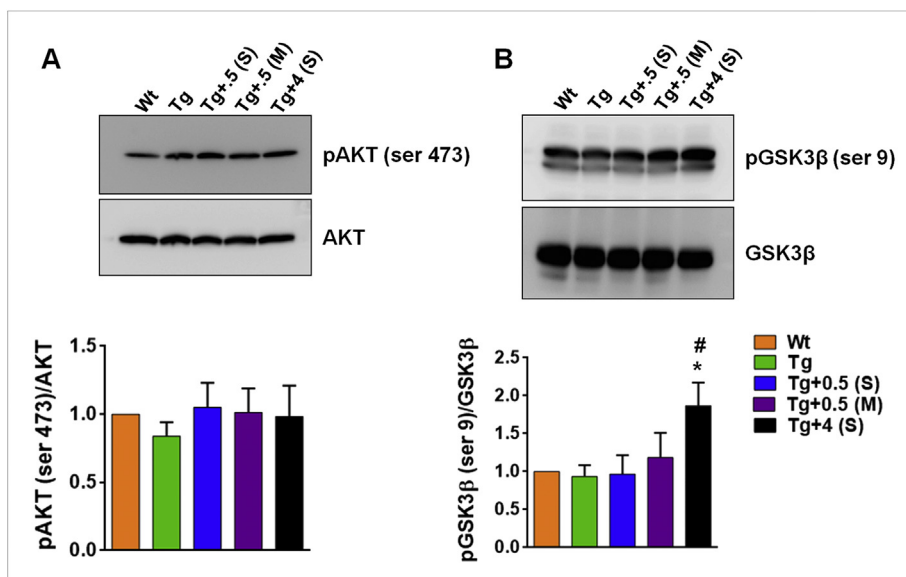


Figure 6. Effect of single and multiple exposure of gamma irradiation on insulin signaling molecules AKT and GSK3β. Brain homogenates from various experimental mice were studied to identify the phosphorylation status of key insulin signaling molecules. Representative western blots and densitometric analysis of (A) pAKT (ser 473) normalized by total AKT (Wt; n = 5, Tg; n = 5 per group) and (B) pGSK3β (ser 9) normalized by total GSK3β (Wt; n = 5, Tg; n = 5 per group). Different groups are indicated as Wt (Wild type), Tg (Untreated Transgenic), Tg+0.5 (S) (Transgenic mice received single dose of 0.5 Gy radiation), Tg+0.5 (M) (Transgenic mice received 0.5 Gy radiation twice a week for one month), Tg+4 (S) (Transgenic mice received single dose of 4 Gy radiation). Values are expressed as mean ± SEM. *p < 0.05 compared to wild type and #p < 0.05 compared to untreated APP/PS1 mice (Tg).

mice (Figure 9). The result is similar to the expression of GFAP protein in brain homogenates of the treated animals as shown in Figure 3. Thus our results indicate that a single exposure of gamma radiation at 0.5 Gy and 4.0 Gy have decreased GFAP expression. In contrast, multiple doses of gamma radiation at 0.5 Gy X 8 increased the expression of GFAP. It may be concluded that a single exposure to 4 Gy gamma radiation is non-lethal and reduces astrocyte activation in APP/PS1 mice.

4. Discussion

The drugs available for treating AD only provide symptomatic relief while other newly developed drugs are expensive [14]. Therefore, different therapeutic strategies are being evaluated [15]. Recently, ionizing radiations have been reported to be effective in treating AD. Ionizing radiations have been found to significantly reduce the amyloid accumulation/load in extra-cranial sites of the brain [16, 17, 18]. AD pathogenesis is the result of an imbalance in amyloid deposition and its clearance. Recent studies further showed that radiation provides adaptive protection by activating the pathways and genes responsible for reducing disease progression [19]. Furthermore, ionizing radiations have also been shown to activate some of the protective mechanisms (e.g. AKT/MDM2/P53-mediated anti-apoptotic and AKT/Nrf2-mediated

anti-oxidant pathways) which help in the reversal of neurodegenerative diseases without causing adverse side effects [20].

Behavioral impairment and cognitive dysfunction are the major events in AD sufferers. Marples *et al.* showed that a fractionated dose of 2 Gy, 5 times for 8 weeks reversed the cognitive functions in a murine model of AD with reduced Aβ plaque deposition. The probable rationale mentioned was the recruitment of pro-inflammatory phenotype (MIP2) for the reduction in Aβ plaques burden [11]. However, in our study we observed that a single dose of 4 Gy reduced amyloid plaque burden and improved memory functionality based on Y maze and water maze tests. Memory exploration and retention parameters like time spent in the novel arm, probe capability and time spent in target quadrant showed significant improvement. The observed differences between our findings and previous study by Marples *et al.* could be due to the multiple mechanisms involved in the reduction of amyloid burden and cognitive improvement. As observed in the present study, potentiation in insulin sensitivity and glucose homeostasis in the brain of AD mice after a single dose of 4 Gy irradiation could also be responsible for reduced amyloid plaque burden whereas Marples *et al.* showed improvement due to increase in pro-inflammatory cytokine (MIP2).

Many studies indicate that insulin signaling plays a significant role in AD [21, 22]. The diminished utilization of glucose by the brain during AD

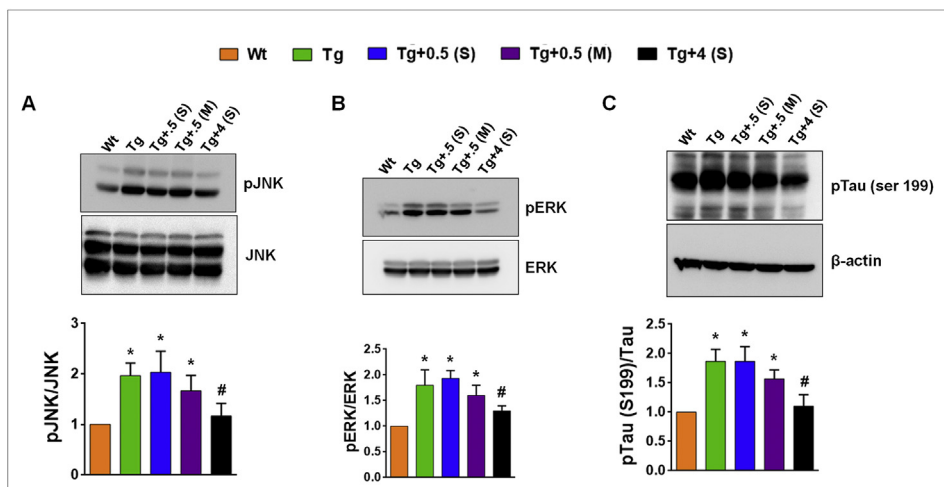


Figure 7. Effect of single and multiple exposure of gamma irradiation on phosphorylation of JNK, ERK and Tau proteins. Brain homogenates from various experimental mice were studied to identify the phosphorylation of stress related proteins such as JNK, ERK and Tau. Representative western blot and densitometric analysis of (A) pJNK normalized using total JNK (Wt; n = 5, Tg; n = 5 per group) (B) pERK normalized using total ERK (Wt; n = 5, Tg; n = 5 per group) and (C) pTau (ser 199) normalized using β-actin (Wt; n = 5, Tg; n = 5 per group). Different groups are indicated as Wt (Wild type), Tg (Untreated Transgenic), Tg+0.5 (S) (Transgenic mice received single dose of 0.5 Gy radiation), Tg+0.5 (M) (Transgenic mice received 0.5 Gy radiation twice a week for one month), Tg+4 (S) (Transgenic mice received single dose of 4 Gy radiation). Values are expressed as mean ± SEM. *p < 0.05 compared to wild type and #p < 0.05 compared to untreated APP/PS1 mice (Tg).

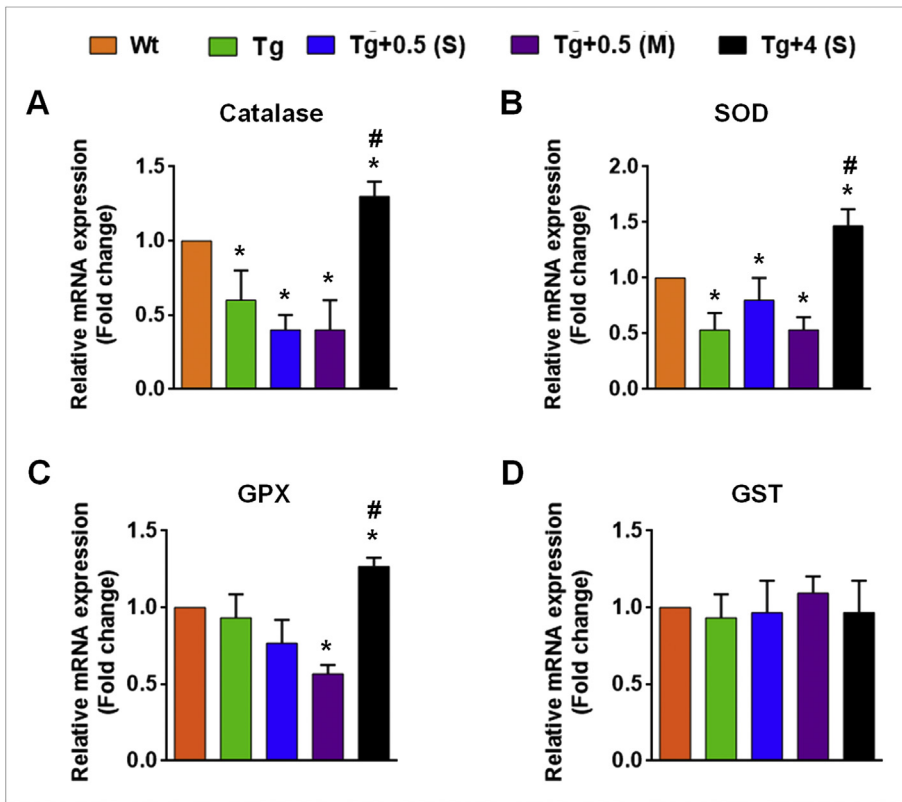


Figure 8. Effect of single and multiple exposure of gamma irradiation on the levels of anti-oxidative enzymes. Total mRNA was isolated from brain of experimental mice and gene expression of anti-oxidative enzymes studied. Relative mRNA expression (fold change) of (A) Catalase, (Wt; n = 5, Tg; n = 5 per group), (B) Superoxide Dismutase (SOD) (Wt; n = 5, Tg; n = 5 per group), (C) Glutathione peroxidase (GPX) (Wt; n = 5, Tg; n = 5 per group) and (D) Glutathione S transferase (GST) (Wt; n = 5, Tg; n = 5 per group). Different groups are indicated as Wt (Wild type), Tg (Untreated Transgenic), Tg+0.5 (S) (Transgenic mice received single dose of 0.5 Gy radiation), Tg+0.5 (M) (Transgenic mice received 0.5 Gy radiation twice a week for one month), Tg+4 (S) (Transgenic mice received single dose of 4 Gy radiation). Values are expressed as mean ± SEM. *p < 0.05 compared to wild type and #p < 0.05 compared to untreated APP/PS1 mice (Tg).

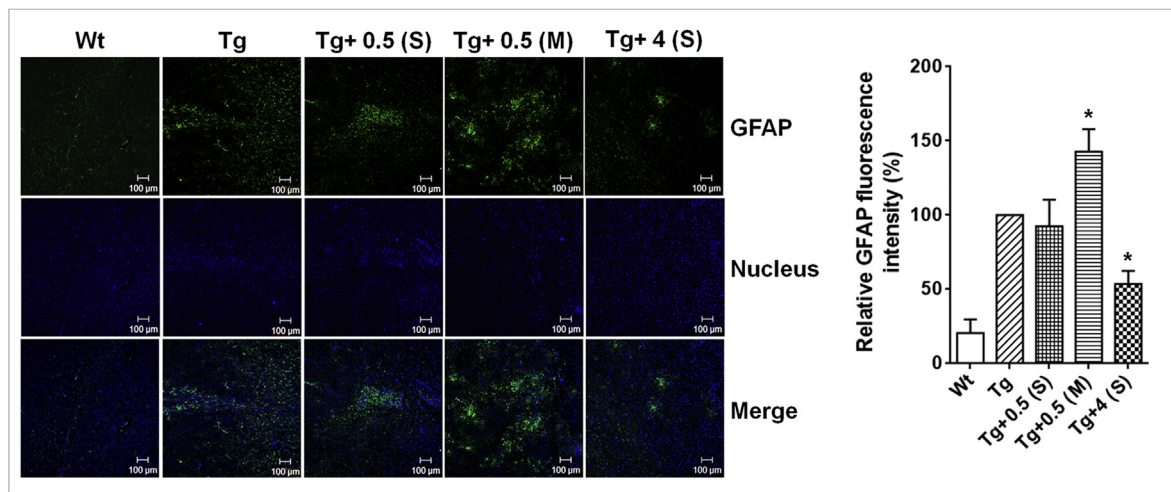


Figure 9. Effect of single and multiple exposure of gamma irradiation on astrocyte activation. Brain was harvested at the end of experiment and embedded in paraffin. 10 µm sections were obtained on poly-L-lysine coated slides for immunohistochemical analysis. GFAP was probed with specific primary antibody and visualized on confocal microscope using secondary Alexa Fluor 488 and nucleus with DAPI (blue). Representative GFAP images and densitometry of relative fluorescence intensity are shown (Wt; n = 5, Tg; n = 5 per group). Different groups are indicated as Wt (Wild type), Tg (Untreated Transgenic), Tg+0.5 (S) (Transgenic mice received single dose of 0.5 Gy radiation), Tg+0.5 (M) (Transgenic mice received 0.5 Gy radiation twice a week for one month), Tg+4 (S) (Transgenic mice received single dose of 4 Gy radiation). Values are expressed as mean ± SEM. *p < 0.05 compared to untreated APP/PS1 mice (Tg).

leads to reduced neuronal signal transmission and impaired cognitive abilities [23]. APP/PS1 mice are also reported to have disturbance in glucose tolerance prior to the mature amyloid deposition [13]. In our study, it was observed that glucose metabolism increased significantly after exposure to a single dose of 4 Gy gamma radiation. The major pathological features occurring during AD pathogenesis are tau hyperphosphorylation which lead to neurofibrillary tangle formation (NFTs) and amyloid deposition (Aβ₄₂) [24]. The major kinases GSK, ERK and JNK act as a substrate for tau phosphorylation [25]. These kinases are the

important players involved in insulin signaling. Previously, it has been shown that GSK3β is a regulator of ERK and their association primes the activation of these molecules [26, 27, 28]. In AD, due to the activation of stress responsive kinases such as ERK and JNK via phosphorylation of GSK, Tau gets hyperphosphorylated [29]. Le Corre et al. showed that inhibition of GSK3, ERK2, Cdk1, PKA and protein kinase C in a JNPL3 tau transgenic mice model decreased hyperphosphorylated tau and improved motor impairments [30]. In our studies, we observed that a single dose of 4 Gy gamma radiation reduced the levels of ERK, JNK,

pTau and increased inhibitory phosphorylation of GSK3 β (ser 9). However, AKT phosphorylation was not affected. Although canonical PI3K-AKT pathway involves the binding of insulin to insulin receptor and activation of AKT, a key molecule of insulin signaling in peripheral tissues, yet AKT independent activation of insulin signaling has also been reported [31, 32, 33, 34]. There could be several other probable reasons of AKT-independent downstream signaling like (a) Activation of the alternative pathway such as IGF-1, PTEN, PKC etc. upon irradiation which might be responsible for phosphorylating GSK3 β at ser 9, an inhibitory phosphorylation, without affecting AKT phosphorylation [35, 36, 37] (b) Sometimes undetected non-significant increase in phosphorylation and activation of AKT may be sufficient for phosphorylating GSK3 β (ser 9) (inhibitory phosphorylation) significantly [38] (c) Cross-talk between two pathways might result in activating GSK3 β [39]. Moreover, direct activation of GSK3 β without affecting AKT phosphorylation has also been reported and supports our finding [40].

The decreased phosphorylation of Tau results in a low level of neurofibrillary tangle formation and neurotoxic amyloid deposition [41]. Recently, Lisa Kirouac *et al.* (2017) showed that activation of GSK3 β and Ras/ERK signaling cascade promotes hyperphosphorylation of Tau protein, a major pathological event in AD [42]. Thus, it can be hypothesized that increased inhibitory phosphorylation of GSK3 β (ser 9) reduced the phosphorylation of ERK and JNK, resulting in downregulation of tau phosphorylation and NFT formation hence reducing AD progression. Studies also indicate that deficiency in insulin receptor substrate-2 which is regulated by insulin like growth factor (IGF-1) signaling cascade impairs brain functionality and deregulates Tau expression/phosphorylation resulting in impaired glucose homeostasis and neurodegeneration [43, 44]. Differential interaction of Tau and its phosphorylated forms with PI3K, an intermediate of the insulin signaling pathway, has been previously reported [45]. Moreover, PTEN, a negative regulator of insulin signaling, reduces Tau phosphorylation in AKT and GSK3 β independent manner [46]. In kidney, skeletal muscle and adipose tissue irradiation has been shown to modulate insulin signaling [47, 48] however, its role in the neuronal system is still less studied.

During early stages of AD, the glial cells induce repair by promoting amyloid clearance mechanism. However, as the disease progression occurs, the pro-inflammatory cytokines downregulate the genes involved in amyloid clearance due to deposition of amyloids in the brain [49]. Ionizing radiation has also been shown to increase amyloid clearance by biophysical effects like breaking of H-bonds and depolarizing the glucosamine glycans present in the β sheet structure of amyloid resulting in reduced AD pathogenesis [50]. We also observed a decrease in the APP and BACE proteins, which are involved in APP processing in, APP/PS1 mice exposed to a single dose of 4 Gy gamma radiation. The reduced level of these proteins indicates non-amyloidogenic processing of APP [51]. In the present study also non-amyloidogenic processing of APP resulted in the decreased level of A β ₄₂ (toxic).

Radiation has been reported to protect renal damage by reducing inflammation and oxidative stress in rats with Type 1 diabetes [47]. In diseased conditions like diabetes, neurodegenerative disorders, metabolic disorders etc. the level of ROS increases, thereby causing oxidative stress. The enzymes, such as SOD, catalase and GPX, act as primary antioxidant defense for inhibiting disease progression. Our findings indicate that exposure to a single dose of 4 Gy gamma radiation resulted in reduced astrocyte activation and increased levels of anti-oxidant enzymes in APP/PS1 mice. In contrast, mice receiving single (0.5 Gy) or multiple doses (0.5 Gy X 8) of gamma radiation showed increased astrocytosis (GFAP, a marker for astrocyte) resulting in neurotoxicity. Maximum activation and proliferation of microglial have been reported by a single dose of 5 Gy ionizing irradiation in comparison to fractionated dosage [52].

Besides increasing the levels of anti-oxidant enzymes and decreasing astrogliosis, it can be speculated that a single dose of 4 Gy irradiation can attenuate AD *via* potentiating insulin sensitivity. To the best of our

knowledge, this is the first report of modulation of AD pathogenesis *via* insulin signaling cascade in response to gamma irradiation.

5. Conclusion and future directions

We observed that APP/PS1 mice receiving a single dose of 4 Gy gamma radiation showed improved AD pathophysiological features such as reversal of cognitive dysfunction, improved glucose metabolism, decreased amyloid burden, increased anti-oxidative enzymes and decrease in astrocyte activation. These studies need to be further validated in future in insulin resistant neuronal cell model in *in-vitro* conditions where the effect of radiation on different signaling molecules and their interplay could be directly investigated using specific inhibitors and/or activators of specific intermediate molecules. Furthermore, transcriptome analysis of irradiated AD brain will provide better understanding of the complex signaling pathways modulated after radiation exposure. Radiation being non-invasive and cost effective, these studies need to be carried further in future as a potential treatment for patients with mild and severe forms of AD.

Declarations

Author contribution statement

Mayuri Khandelwal: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Kapil Manglani: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data.

Sarika Gupta: Contributed reagents, materials, analysis tools or data; Wrote the paper.

Ashu Bhan Tikku: Conceived and designed the experiments; Wrote the paper.

Funding statement

This work was supported by National Institute of Immunology (core grant) and Jawaharlal Nehru University (core grant), New Delhi, India. Mayuri Khandelwal was supported by Indian Council of Medical Research (ICMR) (Senior Research Fellowship).

Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

Acknowledgements

The authors thank all the technical staff of NII and JNU for their help.

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