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Modulation of FOXO1/NF-κB Signaling via Inositol Alleviates the Radiation-Induced Brain Injury

Ghada M. Shafey[1], Marwa M. El-Sheikh[1], Tarek El-Hamoly[1,2*]
[1]Drug Radiation Research Department, National Center for Radiation Research and Technology, Egyptian Atomic Energy Authority, Cairo, Egypt.
[2]Cyclotron Facility, Nuclear Research Center, Egyptian Atomic Energy Authority, Cairo, Egypt.

THE HAZARDS emerging in the setting of ionizing radiation exposure emphasize a potential clinical predicament, and many protective trials have been challenged, compromising the mitigation of such complications. The present study elucidated the role of inositol (INS) in modifying the brain cytotoxicity induced by acute exposure to radiation.
In male Wister rats, brain cytotoxicity was triggered by an acute single dose (6 Gy) of IRR, while administration of INS (30 mg/kg) was conducted before IRR or both before and after exposure. On day eight, the animals were sacrificed, and brain tissues were collected for evaluation of histological changes, oxidative stress, and inflammatory responses by estimation of reduced glutathione (GSH), thiobarbituric acid reactive substances (TBARS), nitrite (NOx) contents, and myeloperoxidase (MPO) activity. Moreover, Forkhead box protein O1 (FOXO1) and nuclear factor kappa B (NF-κB) levels were evaluated by ELISA technique.

Inositol exerted protection against gamma radiation as indicated by reduced tissue contents of either TBARS or NOx, with a significant increase in GSH levels, accompanied by a decrease in MPO activity and an improvement in histological alternation. These findings were interestingly observed upon pre- and post-administration of INS compared to its pre-administration for only three days. The overall molecular impact of radiation on damaging the brain tissue could be explained by modifying the expressions of the upregulated FOXO1 and NF-κB proteins.

The present study revealed that the anticipated adverse reaction to brain tissue in course of irradiation could be effectively protected by daily pre- and post-administration of INS supplement.

Keywords: Irradiation; Inositol; Brain; FOXO 1; NF-κ B

Introduction

The routine use of radiotherapy or even vocational radiation exposure is commonly manifested with multiple organ cytotoxic risks. The brain is a dose-limiting organ extremely affected by type and dose of ionizing radiation in the regular course of cerebral radiotherapy. Pursuing novel therapeutic agents is an ultimate goal for these adverse effects during the course of ionizing radiation exposure.

Several endeavors have been enrolled to develop innovative radio-protectants that are mainly retrieved from natural sources as an attempt to prevent these deleterious effects of radiation-induced harmful free radicals (Arora et al., 2006; El-Hamoly et al., 2019; El-Sheikh et al., 2023). In such context, potential beneficial strategy has been approved by FDA on introducing a combined adjuvant therapy during the treatment regimen of solid tumors to overcome the anticipated toxicity (Arora et al., 2005). Molecular and cellular impairments during exposure to ionizing radiation may be aggravated

*Corresponding author: E-mail: tahamoly@hotmail.com,ORCID iD: https://orcid.org/0000-0001-5246-2168
Authors' E-mails: ghada.shafey@gmail.com, ORCID iD: https://orcid.org/0000-0003-2208-560X,
E-mail: meroelshiekh@yahoo.com, ORCID iD: https://orcid.org/0000-0002-1582-8818,
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through promotion of significant pathways such as PARylation, NF-κB-dependent inflammatory response, SIRT-1 activity, Akt/GSK-β/Notch-1, and autophagy (El-Hamoly et al., 2019; El-Sheikh et al., 2023; Abdel-Naby et al., 2024; Vikram et al., 2024).

By virtue of its carbocyclic sugar polyalcohol chemical structure, inositol mediates many molecular pathways in response to cellular exposure to various hormones, growth factors and transmitters. Through its metabolism, inositol can be converted into nine different stereoisomers and finally accumulated in organs including brain, liver, and kidney (Facchinetti et al., 2015). Inositol is a natural constituent that is basically found in many foods such as vegetables, milk, meat, fish, and eggs (Kiani et al., 2021).

It was accepted that the Myo-inositol stereoisomer is one of semi-essential vitamin B complex members. Its *de novo* synthesis is typically generated from glucose. The end products after Myo-inositol catabolism are phosphatidylinositol, inositol phosphate and phosphoinositide. These second messengers are included in many physiological processes and biological factors (Facchinetti et al., 2015).

D-chiro-inositol is another isomer that is subsequently synthesized from myo-inositol by a specific epimerase during metabolic stress in response to increased insulin release. Thus, it is important in diabetes mellitus, owing to its capability to restore insulin sensitivity and hence reduce hyperglycemia (Sun et al., 2002). Furthermore, it shows significant antioxidant, anti-inflammatory and anti-aging effects on many pathological conditions (Hada et al., 2013; Hu et al., 2015). D-chiro-inositol also mediates the activation of PI3K/Akt signaling. Such an effect could suppress the FOXO1 activity, which can modulate the rate-limiting step for gluconeogenesis, inflammation, and many modes of cellular death (Cheng et al., 2019).

In view of these considerations, the present study was constructed to assess the FOXO1/NF-κB pathway participation in the pathogenesis of ionizing radiation exposure and the possible protective role of the INS against brain damage induced by such radiation insult, pursuing a more efficient INS-dose regimen that could successfully protect against brain cytotoxicity.

Materials and Methods

Drugs and chemicals

Inositol(1,2,3,4,5,6Hexahydroxycyclohexane, Cat. No. 1340960) was obtained from Sigma Chemical Co. (St. Louis, MO, USA). The enzyme-linked immunosorbent assay (ELISA) kits of FOXO1 (Cat. No. E1493Ra) and NF-κB (Cat. No. E0287Ra) were obtained from Bioassay Technology Laboratory (Zhejiang, China). All chemicals and reagents used in the current work were of the purest analytical quality accessible.

Animals

Male Wister rats 5 weeks-old (120 ± 30 g) were obtained from the animal house of NCRRT. Rats were kept at 25 °C with changeable 12 h dark/light cycles. They were fed on a standard diet containing not less than 5% fiber, 20% protein, 3.5% fat, and 6.5% ash, as well as a vitamin mixture and water ad libitum. All the experimental procedures were conducted following ethical guidelines. Moreover, the Research Ethics Committee of the NCRRT-EAEA has authorized the experimental procedures under Permit No. P/37A/24.

Irradiation of animals

Whole-body gamma-irradiation was used as a model of radiation-induced brain damage based on El-Sheikh et al. (2018). Briefly, exposure to gamma rays was carried out using a Gamma Cell-40 biological irradiator equipped with a Cesium (^{137}CS) source at the NCRRT, Cairo, Egypt. The animals were kept in well-ventilated cages and positioned in a chamber fixed to the irradiation equipment. The animals were exposed to a single dose of 6 Gy gamma-rays for induction of brain damage, delivered at a dose rate of 0.47Gy/min in a field size of about 25×25 cm^2 and at 70 cm distance from the source.

Experimental design

Animals were acclimatized for a week before induction of the model. Rats were divided into 4 experimental groups each of 6 rats categorized as follows:

1. Control (Ctrl) group: Animals were given 1.0 mL of intraperitoneal (i.p.) saline/kg b.wt daily as an inositol vehicle for seven days.
2. IRR group: Animals were given 1.0 mL of i.p. saline/kg b.wt./day as an inositol vehicle for three days. On the 4th day, they were exposed to 6 Gy of acute whole-body gamma radiation, followed by three days of receiving the vehicle.

3. INS/3Days+IRR group: Animals were pre-treated with inositol (30 mg/kg b.wt., i.p.) for three consecutive days, according to the study of Nozadze et al. (2011). On the 4th day they were exposed to 6 Gy acute whole-body gamma radiation, followed by three days of receiving the vehicle.

4. INS/6Days+IRR group: Animals were pre-treated with inositol (30 mg/kg b.wt., i.p.) for three consecutive days. On the 4th day, they were exposed to 6Gy acute whole-body gamma radiation. Twenty-four hours after IRR, rats received inositol (30 mg/kg b.wt., i.p.) for a further three consecutive days.

At the end of the experiments, after 24 hours, rats were sacrificed via cervical dislocation under deep urethane anesthesia. Following that, brain tissue was dissected, washed with ice-cold saline, weighed, and then homogenized in various media based on the parameter to be evaluated using a Glass-Col homogenizer (Terre Haute, Indiana, USA) to prepare tissue homogenate (20 % weight/volume). The homogenized tissues were centrifuged at 3000 rpm for 15 min using a cooling centrifuge (HettichVR Zentrifugen, Mikro 22/22 R, Germany) and the supernatants were stored at -80 °C. Furthermore, additional brain tissues were fixed in 10% formalin for hematoxylin and eosin (H&E) staining evaluation. The disposal of animals' carcasses was carried out by burying in quick lime.

Biochemical analysis

Total nitrate/nitrite (NOx) content was measured according to the previous method of Miranda et al. (2001). Briefly, the protein content was precipitated in prepared homogenates by ethanol for 48 h. The separated supernatants were incubated with vanadium trichloride and Griess reagent [0.1% N-(1-naphthyl) ethylenediamine dihydrochloride, 1% sulfanilamide] at 37 °C for 45 min. All generated nitrite concentrations were evaluated calorimetrically at 540 nm using a Unicam 8625 UV/Vis spectrophotometer (Cambridge, UK). According to the previous method of Beutler et al. (1963), the content of GSH in brain tissues was determined by adding (5,5`-dithiobis 2-nitrobenzoic acid) (DTNB) into the tissue homogenates that were prepared in meta-phosphoric acid. The final product was quantified at 412 nm. Lipid peroxidation was determined according to a previous method (Uchiyama and Mihara, 1978). Briefly, the brain tissue was homogenized in a buffer containing 1.5% potassium chloride to determine the level of TBARS. The final product was measured calorimetrically at 535 nm and expressed as mg/g tissue weight. Regarding MPO, a marker of neutrophil infiltration, it was measured in brain tissue homogenates prepared in a 50 mM potassium phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide, as stated by Bradley et al. (1982). The absorbance of MPO was measured calorimetrically at 450 nm, and the results were expressed as U/g wet tissue.

Evaluation of FOXO 1 and NF-κB activity using the ELISA assay

Following the manufacturer's instructions, a rat-specific ELISA kit assessed the contents of FOXO1 and NF-κB in brain homogenates. An ELISA plate reader was used to measure the optical density of each sample set at 450 nm.

Histopathological examination

Tissue samples from the cerebral cortex and hippocampus were collected and fixed in a 10% neutral buffered formalin solution for histopathology. Tissue specimens were processed as follows, dehydrated in an ascending concentration of ethanol, cleared in xylene, embedded in paraffin wax, and sectioned at a 5-micron thickness. Prepared slide sections were stained with haematoxylin and eosin for histopathological examination using an Olympus BX50 light microscope (Olympus Co. of America, Melville, NY, USA) according to the method of Bancroft et al. (2013). Parameters for whole samples were evaluated for microglial reaction, inflammatory reaction, vascular reaction, edema, and axonal damage. The damage severity in tissues and cells was scored from 0 to 3 (0 = normal; 1 = mild damage; 2 = moderate damage; 3 = severe damage) based on the study of Takahashi et al. (2002).

Statistical analysis

All data mentioned were compared via a one-way analysis of variance (ANOVA), followed by the Tukey-Kramer test for multiple comparisons. The results were expressed as means ± SEM and considered statistically significant at $p<0.05$ or less. Statistical analyses were conducted using the software of GraphPad version 6, La Jolla, CA.

Results

Inositol in different dosing regimens ameliorates oxidative stress and inflammatory response on radiation-induced brain damage

Overproduction of reactive oxygen species (ROS) and reactive nitrogen species (RNS) is deleterious for brain tissue. The exposure to ionizing radiation revealed a marked brain injury

revealed by significant elevation of TBARS and NOx contents, recorded 3- and 2.27- fold increase respectively. In addition, irradiation could decrease the GSH contents compared to normal control amounting to about 2.5-fold decrease. On the contrary, pre-treatment with INS before irradiation significantly decreased the elevated levels of TBARS and NOx, amounting to 50% and 30% decrease, as well as up-regulated the GSH content reached a 2-fold increase comparable to the IRR group. Furthermore, administration of pre- and post-administration of INS recorded about 50% decreases in levels of either TBARS or NOx with a 2.4-fold increase in GSH content (Fig 1 A, B, and C).

MPO is a product of activated inflammatory cells including tissue macrophages and neutrophils. Gamma irradiation triggered the activity of the MPO in brain tissue compared to non-irradiated normal animals, reached about 2.5-fold increase. Pre-administration of INS (INS/3Days+IRR) showed a marked decrease in MPO activity ($p < 0.05$) in comparison with IRR group. However, pre- and post-administration of INS (INS/6Days+IRR) markedly decrease the activity of MPO ($p < 0.001$) compared to IRR animals, amounting to about 50% decrease (Fig 1D).

FOXO1 and NF-κB activities are modulated by inositol different dosing regimens on radiation-induced brain damage

Based on the results of ELISA assay, expression of brain FOXO1 and NF-κB was significantly raised following gamma radiation exposure, amounting to about two-fold increase compared to the control non-irradiated group, respectively. Meanwhile, pre-administration of INS could down-regulate the expression of FOXO1 compared to the IRR group. In contrast, animals being pre- and pos-treated with INS (INS/3Days+IRR) depicted relatively lower expression of FOXO1 protein compared to IRR group. However, only INS/6Days+IRR group showed a significant decrease in NF-κB protein expression, in comparison, a restricted pre-administration of INS for only three days could not exert a significant decrease in such expression (Fig. 2 A and B).

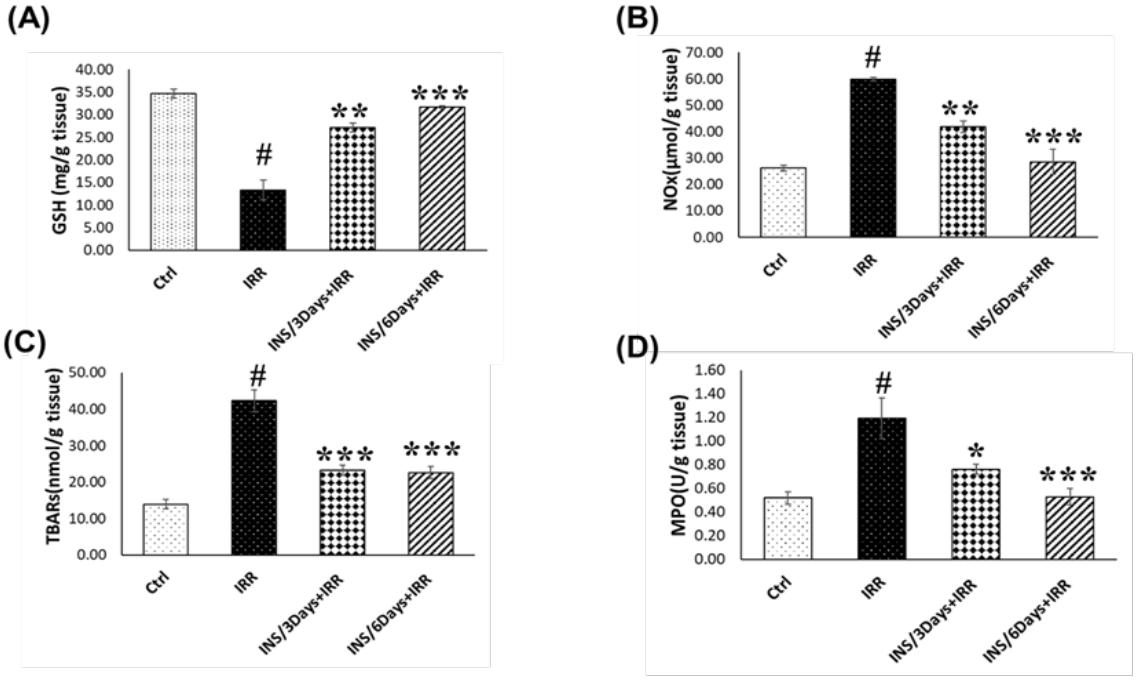

Fig. 1. Inositol in different dosing regimensameliorate oxidative stress and inflammatory response onradiation-induced brain damage.Biochemical evaluations of (A)GSH and(B)NOx were evaluated in brain tissues from all experimental groups. Values are expressed as mean ± SEM (n = 6). #Hashmark denotes statistical significance vs. Ctrl group ($p < 0.05$). *Asterisk indicates statistical significance vs. IRRgroup (* $p < 0.05$, ** < 0.01, *** $p < 0.001$).

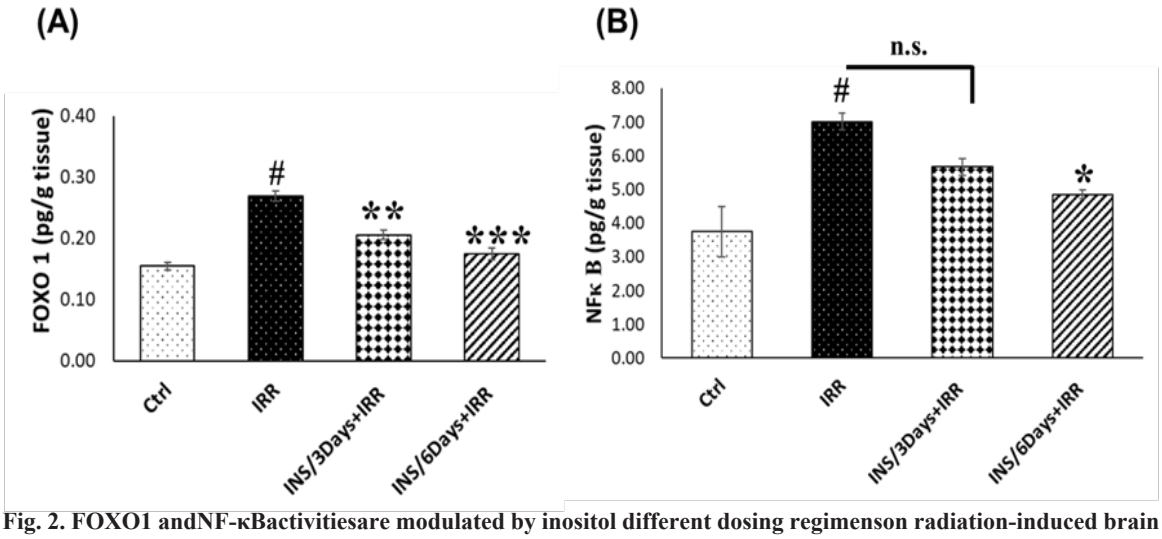

Fig. 2. FOXO1 andNF-κBactivitiesare modulated by inositol different dosing regimenson radiation-induced brain damage.ELISA assays of (A) FOXO1 and (B) NF-κB expressions were evaluated in brain tissues from all experimental groups. Values are expressed as mean ± SEM (n = 6). # Hashmark denotes statistical significance at p <0.05 vs. Ctrl. * Asterisk indicates statistical significance vs. IRR group (* p < 0.05, ** < 0.01, *** p < 0.001).

Inositol in different dosing regimens mitigates the histological changes of brain injury incurred by exposure to radiation

Light microscopic examination of hematoxylin and eosin-stained sections of cerebral cortex of control group revealed normal histological structure. Several layers of neuronal cells arranged with no sharp boundaries in association with small blood vessels were viewed. The neuronal cells contained oval or rounded open face nuclei with prominent nucleoli surrounded by basophilic cytoplasm score (0) (Fig. 3A). The Hippocampal section showed normal layers of compact granular cells with dark nuclei in dentate gyrus. The molecular layer showed glial cells as well as pyramidal cells score (0) (Fig. 3B).

Animals group exposed to radiation showed a swelling of neuronal cells with peripheral condensation of nuclear chromatin. Focal gliosis, perivascular oedema and apoptosis of neuronal cells appeared as densely basophilic bodies surrounded by halo zone were seen score (2) (Fig. 3C). The histological section of hippocampal dentate gyrus region revealed cellular disorganization and marked shrinkage in size of large pyramidal cells, with nuclear pyknosis were viewed. Vacuolar degeneration of molecular cells was also noticed score (2) (Fig.3D).

Administration of INS before irradiation displayed mild improvement in comparison with previous group. Cerebral cortex showed swelling of neuronal cells with focal gliosis and perivascular oedema score (1) (Fig.3E). The histological section of hippocampal dentate gyrus region demonstrated cellular disorganization and marked shrinkage in size of large pyramidal cells, with darkened basophilic nuclei were seen. Molecular cell layers also showed vacuolar degeneration score (2) (Fig.3F). On the other hand, animals administrated INS before and after irradiation showed an improvement in comparison with previous group appeared as mild vacuolar degeneration of neuronal cells. Some neuronal cells appeared darkly stained shrunken score (I) (Fig. 3G). The histological section of hippocampal dentate gyrus region revealed cellular organization of neuronal cells with shrinkage in size of large pyramidal cells which appeared as dark basophilic nuclei. Granular cell layers also showed normal arrangement grade (I) (Fig. 3H).

Discussion

Ionizing radiation is widely applied in medical applications as a diagnostic device or even as a treatment approach for cancer (e.g., radiotherapy with linear accelerators). Despite the outstanding protocols for controlling radiation doses associated with these internal or external radiotherapeutic techniques, the recurrent use of these radiation-dependent applications unveils much radiation-related pathology. The post irradiation amendments that could be observed are broadly extended to various levels of organs and biological systems (Moding et al., 2013). Pursuing a new prophylactic strategy, the neuroprotective effect of inositol on the harmful premises of gamma radiation was examined in the current study.

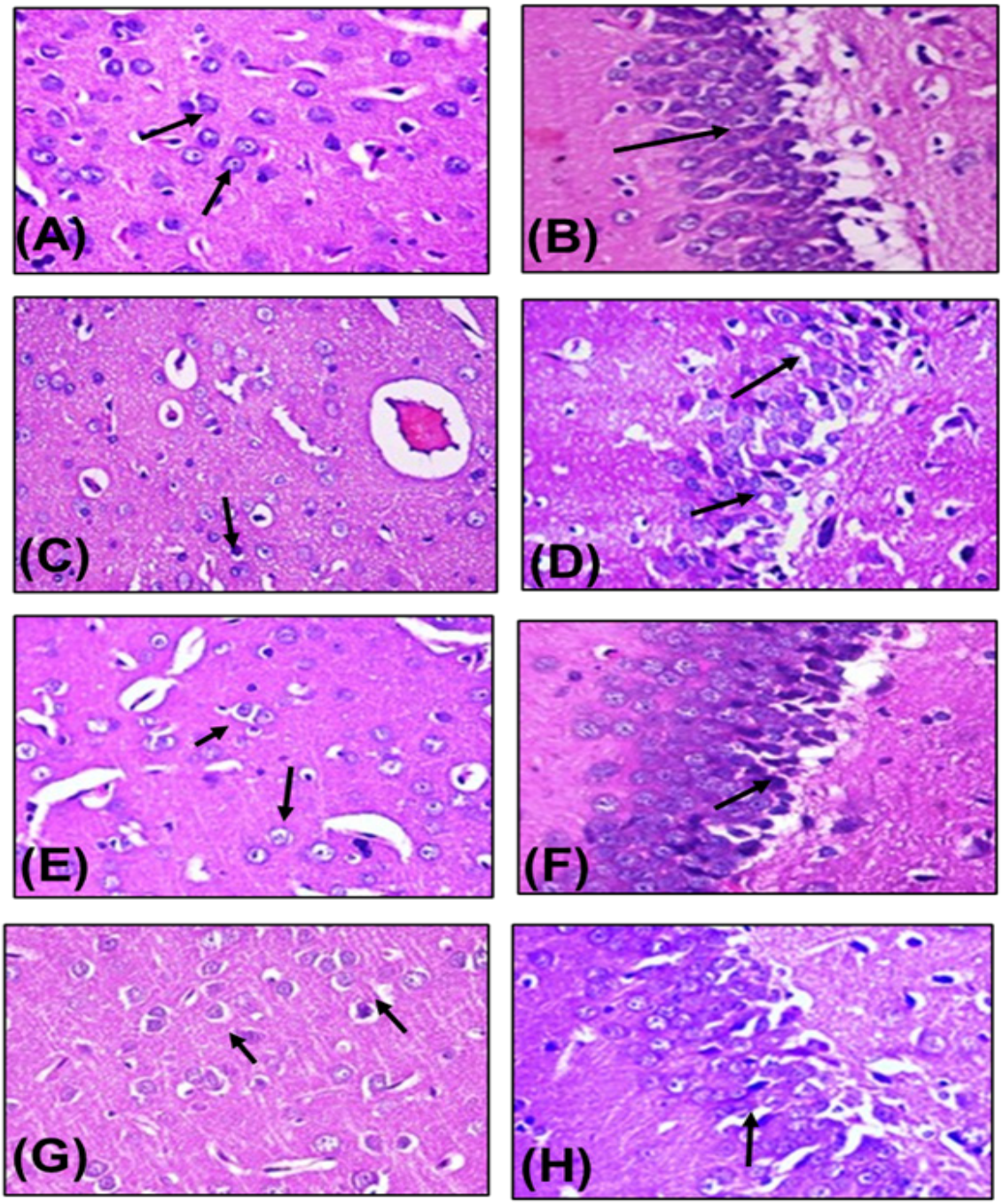

Fig. 3. Inositol in different dosing regimensmitigates the histological changesof braininjury incurred by exposure to radiation. (A)A Photomicrograph of the cerebral cortex showsthe normal histological structure of neuronal cells with a rounded open face nuclei arrow. (B) Aphotomicrograph ofthe hippocampus showsa normal cellular organization arrow. (C) A photomicrograph of the cerebral cortex showing apoptosis of neuronal cells arrow andperivascular oedema arrowhead. (D) A photomicrograph ofthe hippocampusshows acellular disorganization and vacuolar degeneration of granular cells"arrow". (E) A photomicrograph of the cerebral cortex shows a swelling of neuronal cells with focal gliosis "arrow". (F)A photomicrograph ofthe hippocampus shows acellular disorganization and a marked shrinkage in size of large pyramidal cells, with a darkened basophilic nuclei arrow. (G)A Photomicrograph of the cerebral cortex shows mild vacuolar degeneration of the neuronal cells"arrow". (H)A photomicrograph ofthe hippocampus shows a cellular organization of the neuronal cells with shrinkage in the size of large pyramidal cell "arrow"(H&Ex400).

Ionizing radiation may cause direct or indirect DNA damage in the CNS as well as have a negative impact on neurogenesis (Andres-Mach et al., 2008; Smart, 2017). The imbalance between oxidative and antioxidant systems results in excessive production of ROS in mitochondria, which in turn breaks the mitochondrial DNA and ultimately leads to tissue damage and cellular death (Islam, 2017; Chio et al., 2022). This fact was reflected in the current study, while exposure to radiation decreased the non-enzymatic antioxidant GSH in its reduced form. Because of excessive ROS generation, GSH was markedly consumed to compensate for such a hazardous oxidative pattern. Furthermore, overproduction of ROS increased cellular membrane lipid peroxidation, indicated by the high content of TBARS. Oxidative stress is a significant prognostic for most early-developed neurodegenerative disorders; it explicitly triggers various signaling pathways that eventually lead to progressive damage of the neuronal structure and eventually the loss of cognitive function (Niedzielska et al., 2016; Panahi et al., 2019). On the other hand, the authors presented that exposure to radiation exhibited an increase in total nitrite/nitrate contents. Radiation can stimulate the inflammatory response overwhelmed by up-regulation of inducible iNOS, which in turn could generate active nitrite/nitrate and finally unveil deleterious RNS in living cells. Moreover, irradiation may increase the activity of MPO which is commonly released by neutrophils and tissue macrophages as an immune and inflammatory response. Particularly, MPO catalyzes the formation of hypochlorite from hydrogen peroxide and halide ions (El-Sheikh et al., 2018). In a dose dependent effect, administration of inositol revealed a reserve in GSH contents with decreased levels of TBARS, NOx, and neutrophilic MPO activity. It was found that inositol, by virtue of its active metabolite, D-chiro-inositol, demonstrated a powerful antioxidant capacity by decreasing MDA content and increasing SOD activity in liver tissue after bile duct ligation. In addition, it modified the expression of a collection of genes related to oxidative stress (Zhao et al., 2018). In line with the present findings, INS administration could also improve histopathological alterations after irradiation of rats. Thus, it can be deduced that INS protects the brain partly by attenuating radiation-induced oxidative stress.

The activation of NF-κB is commonly aggravated by excessive ROS production following a stressful environment. After cerebral irradiation, brain tissue potentiates NF-κB activity, which in turn facilitates the expression of several pro-inflammatory cytokines and chemokines (Liu et al., 2020). Such observations are manifested through our findings, which revealed elevated NF-κB protein level in brain tissue based on ELISA analysis results. In the present study, treatment of gamma-irradiated rats with INS has noticeably decreased the transcription factor NF-κB, indicating that INS suppressed the inflammatory response. This was in parallel with previous studies on rat models of diabetes mellitus and hepatotoxicity, while INS administration could protect against the inflammatory response in target organs (Gao Et Al., 2016; Zhao et al., 2018). Additionally, in the current study, exposure to radiation exhibited an up-regulation of FOXO1 expression in brain tissue. In general, FOXO1 proteins play a pivotal role in the transduction of several signaling pathways, including PI3K/AKT, MAPK, and NF-κB, with a significant crosstalk among these pathways (Yang et al., 2008; Lin et al., 2014). FOXO1 is one of the isoforms in the FOXO family that stimulates the activation of NF-κB, the upstream regulator for production of cytokines such as IL-6 and TNF-α pro-inflammatory mediators (Sirwi et al., 2021). This can explain the increased expression of NF-κB in association with the up-regulation of the FOXO1 protein. Treatment with INS could decrease FOXO1 expression, suggesting its downstream regulation of the inflammatory response via NF-κB inhibition.

Conclusion

The present research is the first to elucidate the putative neuroprotective influence of INS against radiation-induced brain injury. This effect was explicitly demonstrated after the treatment with INS for a further three consecutive days post-irradiation compared with only pre-administration. The neuroprotective effects of INS treatment may be related to regulatory activity on the FOXO1/NF-κB signaling axis. Such a pathway could induce significant beneficial antioxidant and anti-inflammatory effects on brain tissue. Of note, further pre-clinical investigations may possibly be warranted, suggesting the capability of INS administration to modulate the potentially adverse effects of radiotherapy.

Declaration of competing interest

The authors declare that they have no conflicts of interest.

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