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Neuroprotective role of remote ischemic post-conditioning in radiotherapy-induced cognitive decline: Modulating hippocampal phospho-tau and autophagy via Nrf2-ARE pathway

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► ***Original article***

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ABSTRACT

Background: Cranial radiotherapy (RT), while critical for treating brain tumors, often leads to delayed cognitive dysfunction due to neuronal damage and Tau protein accumulation. Remote ischemic post-conditioning (RIPC) has emerged as a potential strategy to mitigate RT-induced neurotoxicity. This study explores the neuroprotective role of RIPC in modulating hippocampal autophagy and phospho-Tau (P-Tau) expression through the Nrf2-antioxidant response element (ARE) signaling pathway following cranial irradiation. ***Materials and Methods:*** Male Sprague-Dawley rats were subjected to focal cranial RT to induce radiation-associated hippocampal injury. A subgroup received RIPC through intermittent limb ischemia. Behavioral evaluations, including Morris water maze, elevated plus maze, and novel object recognition tests, were conducted at weeks 4 and 8 post-irradiation. Hippocampal expression levels of Nrf2, HO-1, SOD2, Tau, and P-Tau were assessed via qPCR and ELISA. Immunohistochemistry and immunofluorescence were employed to localize and quantify neuronal changes and autophagy markers. ***Results:*** Rats receiving RIPC demonstrated significant improvements in memory and spatial learning compared to non-conditioned controls. RIPC upregulated hippocampal Nrf2, HO-1, and SOD2, while decreasing Tau and P-Tau accumulation. Additionally, increased neuronal autophagy and reduced oxidative stress were observed, correlating with better cognitive outcomes. ***Conclusion:*** RIPC exerts neuroprotective effects in a RT-induced brain injury model by activating the Nrf2-ARE pathway, promoting autophagy, and reducing Tau pathology. These findings support the therapeutic potential of RIPC as an adjunct to RT for preserving cognitive function in cancer patients.

INTRODUCTION

Radiotherapy (RT) is a cornerstone modality in the treatment of both primary and metastatic brain tumors, offering significant survival benefits and local tumor control [1, 2]. However, despite these therapeutic advantages, the adverse effects of cranial irradiation on healthy brain tissue-especially within the hippocampal region-pose major limitations to its long-term application. Accumulating evidence suggests that radiotherapy-induced cognitive dysfunction (RICD) is a prevalent and debilitating side effect, manifesting as impairments in memory, attention, executive function, and spatial learning [3, 4]. Recent clinical efforts have aimed to minimize such damage through hippocampal-sparing radiotherapy protocols using advanced collimator systems like Elekta Agility [5].These deficits are often irreversible and can emerge months to years' post-treatment, severely impacting the quality of life in cancer survivors, particularly pediatric and elderly populations [6, 7].

The pathophysiological mechanisms underlying RICD are multifaceted, involving a complex interplay of oxidative stress, neuroinflammation, white matter degradation, reduced neurogenesis, and neuronal apoptosis [8, 9]. Among these, oxidative damage and proteinopathy-particularly involving the microtubule -associated Tau protein-are gaining increasing attention. Ionizing radiation induces a surge in reactive oxygen species (ROS), leading to mitochondrial dysfunction, lipid peroxidation, DNA strand breaks, and ultimately the activation of cell death pathways [10]. In parallel, abnormal post-translational modifications of Tau, especially hyperphosphorylation, result in its dissociation from

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microtubules and formation of insoluble aggregates known as neurofibrillary tangles (NFTs), which are a hallmark of neurodegeneration [11].

One of the central endogenous mechanisms for combating oxidative stress and promoting cellular resilience is the nuclear factor erythroid 2-related factor 2 (Nrf2) pathway [12]. Under basal conditions, Nrf2 is sequestered in the cytoplasm by Kelch-like ECH-associated protein 1 (Keap1). Upon oxidative insult, Nrf2 dissociates from Keap1, translocates into the nucleus, and binds to antioxidant response elements (ARE) within DNA, thereby upregulating the transcription of a battery of cytoprotective genes, including heme oxygenase-1 (HO-1), NAD(P)H:quinone oxidoreductase 1 (NQO1), and mitochondrial superoxide dismutase (SOD-2) [13]. These downstream targets work collectively to detoxify ROS, maintain mitochondrial function, and restore redox balance. Moreover, recent studies have shown that Nrf2 activation is closely linked with the induction of autophagy, an essential process for degrading damaged organelles and protein aggregates, including hyperphosphorylated Tau.

Remote ischemic post-conditioning (RIPC) is a novel, non-invasive therapeutic strategy that involves the application of brief, repetitive cycles of limb ischemia and reperfusion to confer systemic protection against ischemic and oxidative damage [14]. First explored in cardiology, RIPC has since been shown to trigger a cascade of protective responses, including modulation of inflammatory cytokines, enhancement of antioxidant defenses, and activation of cellular repair mechanisms. The mechanistic overlap between RIPC and the Nrf2-ARE signaling axis makes RIPC a compelling candidate for neuroprotection in contexts beyond stroke, including radiation-induced brain injury. However, few studies have investigated the neurobiological effects of RIPC in the context of cranial irradiation [15, 16].

In this study, we sought to investigate whether RIPC could attenuate cognitive decline and hippocampal damage induced by cranial radiotherapy. We hypothesized that RIPC would activate the Nrf2-ARE pathway, enhance autophagy, reduce the burden of Tau and phospho-Tau(P-Tau) proteins, and preserve neuronal structure and function in the hippocampus. To test this hypothesis, we developed a rat model of localized cranial irradiation and performed comprehensive behavioral, molecular, and histological assessments to evaluate cognitive function, oxidative stress response, autophagic flux, and Tau pathology. This study provides novel evidence demonstrating the neuroprotective effect of RIPC in radiotherapy-induced brain injury through coordinated modulation of the Nrf2-ARE signaling pathway, autophagy, and Tau protein clearance-an interaction not previously characterized in this context.

MATERIALS AND METHODS

Experimental animals and ethical compliance

Eighty male Sprague-Dawley (SD) rats (8 weeks old; weight: 230–250 g) were procured from Chengdu Dashuo Biotechnological Company, Sichuan, China. All animal handling procedures were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee (IACUC) of Chengdu Medical College (Approval ID: CMCC-IACUC-2024-017). Rats were housed in groups of 3–4 per cage under controlled environmental conditions (22–25°C, 50–60% humidity, 12-hour light/dark cycle) with unrestricted access to standard chow and water.

Group assignment and experimental design

Rats were randomly assigned into four groups (n=20 per group):

Sham Group: Exposed to handling and anesthesia without radiation or RIPC.

RT Group: Received a single dose of focal cranial irradiation without further intervention.

RT + RIPC Group: Underwent cranial irradiation followed by RIPC.

RIPC-Only Group: Received RIPC without cranial irradiation.

The experimental timeline included pre-conditioning (Day 0), radiotherapy (Day 1), post-conditioning sessions (Days 1-5), behavioral testing (Days 25-30), and tissue collection (Day 31).

Radiotherapy procedure

Cranial irradiation was performed using a linear accelerator (Elekta Synergy®, Elekta AB, Sweden) operating at 6 MV. Rats were anesthetized with 3% isoflurane and positioned prone in a custom stereotaxic head holder. A collimated 2×2 cm beam was directed bilaterally at the hippocampal region, and a single dose of 15 Gy was delivered at a rate of 2 Gy/min. Non-target areas were shielded using 5 mm thick lead plates. Sham animals underwent identical preparation without beam exposure.

Remote ischemic post-conditioning (RIPC)

RIPC was initiated 2 hours post-irradiation and continued for five consecutive days. Each session involved three cycles of 10-minute ischemia followed by 10-minute reperfusion, applied to the left hind limb using a tourniquet device (internal pressure ~200 mmHg). Ischemia was confirmed by pallor and loss of pedal pulse. Reperfusion was verified by color restoration. Control animals received handling without tourniquet application.

Behavioral assessments

Morris water maze (MWM)

Spatial learning and memory were evaluated

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using the MWM on Days 25–30. The circular tank (180 cm diameter) was filled with opaque water (23° C) and divided into four quadrants. A hidden platform was submerged 1 cm below the surface in the target quadrant. Training involved four trials per day for five days. Each rat had 90 seconds to locate the platform. On Day 6, the platform was removed, and a 60-second probe trial assessed memory retention. Parameters included escape latency, time in the target quadrant, and path length (tracked by EthoVision XT software, Noldus, Netherlands).

Elevated plus maze (EPM)

On Day 20, anxiety-like behaviors were measured using the EPM consisting of two open arms (50×10 cm) and two enclosed arms (50×10×40 cm) elevated 50 cm above the floor. Each rat was placed on the central platform and allowed to explore for 5 minutes. The number of entries into open/closed arms and total time spent in open arms were recorded via overhead camera.

Novel object recognition test (NORT)

On Days 21-23, rats were subjected to the NORT in a 100×100×50 cm arena. After a habituation session (Day 1), two identical objects were introduced (Day 2). On Day 3, one familiar object was replaced with a novel one. The duration of exploration was recorded, and a Discrimination Index (DI) was calculated: DI = (Time with novel object – Time with familiar object) / (Total exploration time) × 100

Molecular analyses

qPCR

Hippocampal tissues were collected post-mortem and homogenized in TRIzol (Invitrogen, USA). RNA was extracted and reverse-transcribed using the RevertAid™ cDNA Synthesis Kit (Thermo Fisher Scientific, USA). Quantitative polymerase chain reaction (qPCR) was performed using SYBR Green on a CFX96® Real-Time System (Bio-Rad Laboratories, USA). Primers:

GAPDH: F: TATGACAATGAATATGGCTACAG |
R: CTCTTGCTCTCAGTATCCTT
Nrf2: F: AAGAGCAAGAAGCCAGAT |
R: TCACATCACAGTAGGAAGTT
SOD-2: F: GATGGATGGAGTGGTAGAG |
R: CGAATTAACAGTTGTCAGTCA
Tau: F: ATCCACAGCCTACACTAC |
R: TATTAACACCGCCTCCAT

ELISA

Protein concentrations of Nrf2, SOD-2, and P-Tau were measured using enzyme-linked immunosorbent assay (ELISA) kits (Sigma-Aldrich, USA). Absorbance was read at 450 nm on a microplate reader. Each sample was assayed in triplicate.

Histological and immunostaining procedures

Immunohistochemistry and immunofluorescence

Brains were fixed with 4% paraformaldehyde, embedded in paraffin, and sectioned at 5 μm. Sections were incubated overnight with primary antibodies (anti-Nrf2, SOD-2, LC3b, Tau, P-Tau), followed by secondary antibodies conjugated to Alexa Fluor dyes. DAPI counterstaining was used. Imaging was performed using an Olympus BX53 microscope (Olympus Corporation, Japan).

Nissl Staining

Sections were stained with 0.1% Cresyl Violet to assess neuronal density in CA1 and CA3 regions. Pyramidal neurons were counted in three randomly selected fields per section.

Statistical analysis

Data were analyzed using SPSS Statistics v22.0 (IBM Corp., USA). Results are expressed as mean ± standard deviation (SD). Intergroup differences were evaluated using one-way ANOVA followed by Tukey's post hoc test. $p<0.05$ was considered statistically significant.

RESULTS

RIPC ameliorates radiotherapy-induced cognitive dysfunction

To assess whether remote ischemic post-conditioning (RIPC) could mitigate radiotherapy-induced cognitive deficits, a battery of behavioral tests was administered at 4 and 8 weeks following cranial irradiation. In the Morris Water Maze (MWM), irradiated rats (RT group) demonstrated significantly prolonged escape latencies compared to the Sham group at both time points (4 weeks: $P<0.01$; 8 weeks: $P<0.01$), indicating impaired spatial learning (figure 1A). Rats treated with RIPC showed marked improvement: both the 3-day (RIPC3d) and 4-week (RIPC4w) post-conditioning groups exhibited significantly reduced escape latencies compared to RT rats (4 weeks: $P=0.05$; 8 weeks: $P=0.05$ for RIPC3d; 4 weeks: $P=0.05$; 8 weeks: $P=0.01$ for RIPC4w), suggesting preserved learning ability.

Consistently, platform crossing distance and frequency-measures of memory retention-were significantly lower in the RT group compared to Sham (4 weeks: $P<0.01$; 8 weeks: $P<0.05$). However, RIPC3d and RIPC4w rats exhibited significant increases in platform crossing frequency relative to RT (4 weeks: $P<0.05$ for both; 8 weeks: $P<0.05$ and $P < 0.01$, respectively), suggesting enhanced spatial memory retention (figure 1B-D).

In the Elevated Plus Maze (EPM), RT rats spent significantly less time in open arms ($P<0.01$), consistent with heightened anxiety-like behavior.

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RIPC3d rats also showed reduced open-arm duration (P<0.05), but RIPC4w rats did not differ significantly from Sham controls, and open-arm ratios in the RIPC4w group were significantly higher than RT (P<0.01), indicating an anxiolytic effect (figure 1E).

The Novel Object Recognition Test further supported the cognitive benefits of RIPC. At 4 weeks, RIPC4w rats spent significantly more time exploring novel objects compared to RT (P<0.05), while RIPC3d showed a non-significant trend. By 8 weeks, both RIPC3d (P<0.05) and RIPC4w (P<0.01) groups displayed significantly increased novel object exploration times (figure 1F). These findings suggest that RIPC enhances recognition memory and innate exploratory behavior in irradiated rats.

All behavioral assessments were conducted at standardized time points (4- and 8-weeks post-irradiation), and corresponding biochemical and histological analyses were performed at the same time points to correlate behavioral outcomes with molecular changes.

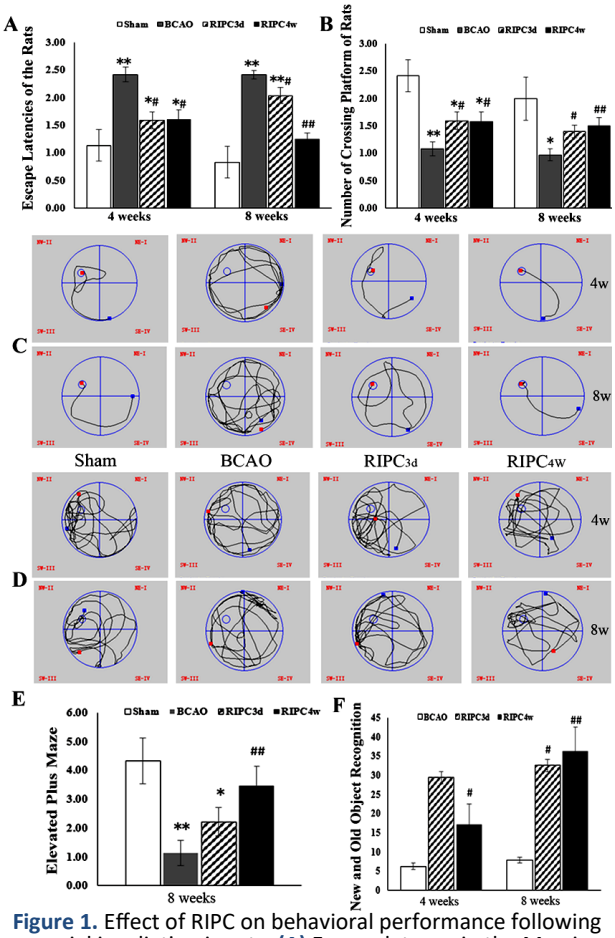


Figure 1. Effect of RIPC on behavioral performance following cranial irradiation in rats. **(A)** Escape latency in the Morris water maze (MWM) at 4 and 8 weeks. **(B)** Platform crossing frequency during probe trials. **(C)** Representative swimming trajectories showing search paths. **(D)** Platform crossing distance. **(E)** Anxiety-like behavior in the elevated plus maze (EPM) showing time spent in open arms. **(F)** Recognition memory in the novel object recognition test (NORT). Groups: Sham, RT, RIPC3d, RIPC4w. Symbols: *P < 0.05, P < 0.01 vs Sham; #P < 0.05, ##P < 0.01 vs RT. Abbreviations: RT - Radiotherapy; RIPC - Remote ischemic post-conditioning; MWM - Morris Water Maze; EPM - Elevated Plus Maze; NORT - Novel Object Recognition Test. Software: EthoVision XT (Noldus, Netherlands).

RIPC participated in the regulation of Nrf2-ARE signal

Q-PCR analysis showed the expression levels of Nrf2 and its downstream targets SOD-2 and HO-1. RIPC significantly upregulated the mRNA level of Nrf2 compared with Sham (P<0.05), and 3d of RIPC exerts the highest observation (P<0.01). Meanwhile, RIPC3d significantly increased Rnc2 mRNA levels compared with BCAO (P<0.05, figure 2A).

After BCAO, SOD-2 expression was significantly elevated in RIPC3d (P<0.01) and RIPC4w (P<0.05) groups compared to the Sham group, and SOD-2 levels appeared to be significantly increased in the RIPC3d than in the BCAO groups (P<0.05, figure 2B). In addition, HO-1 expression in the RIPC4w group was significantly elevated compared than Sham (P<0.01) and BCAO (P<0.05) groups (figure 2C), indicating a possible role of RIPC in the modulation of antioxidant defense system.

Furthermore, ELISA was employed to ascertain the optical density (OD) values of Nrf2 and SOD-2, and a standard curve was constructed. The correlation coefficient of the standard curve surpassed 0.99, affirming enhanced detection precision. Compared to the BCAO group (figure 2D), Nrf2 expression was significantly elevated in both the RIPC3d and RIPC4w groups (P < 0.01); however, the increase observed in the RIPC3d group did not reach statistical significance. Likewise, SOD-2 protein expression in the RIPC3d group exhibited a similar pattern to Nrf2, showing a significant increase (P<0.05, figure 2E), while the RIPC4w group displayed only a slight upward trend that did not achieve statistical significance.

Immunohistochemistry results indicated that in the sham group, Nrf2 (figure 2G) and SOD-2 (figure 2H) positive cells were predominantly situated in both the cytoplasm and nucleus, in contrast to the Sham and BCAO groups. Furthermore, red fluorescence labelling Nrf2-positive cells demonstrated a notable enhancement in immunofluorescence signals within the cerebral cortex of rats subjected to RIPC treatment (figure 2F). These findings indicate that RIPC markedly enhances the expression of Nrf2 and its downstream elements, SOD-2 and HO-1, hence reinforcing the involvement of RIPC in the activation of the Nrf2-ARE signaling pathway.

RIPC reduced death of hippocampal neurons

Immunofluorescence, as shown in figure 3A, revealed that the count of positive NeuN cells significantly decreased after BCAO but increased notably in the $RIPC_{3d}$ and $RIPC_{4w}$ groups. Nissl staining (figure 3B) showed intact nuclei and a high density of Nissl bodies in the sham group. Conversely, post-BCAO, the cell number in the hippocampal CA1 was significantly reduced with the neurons present showing features of abnormal morphology. Additionally, the density of Nissl bodies

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was significantly diminished.

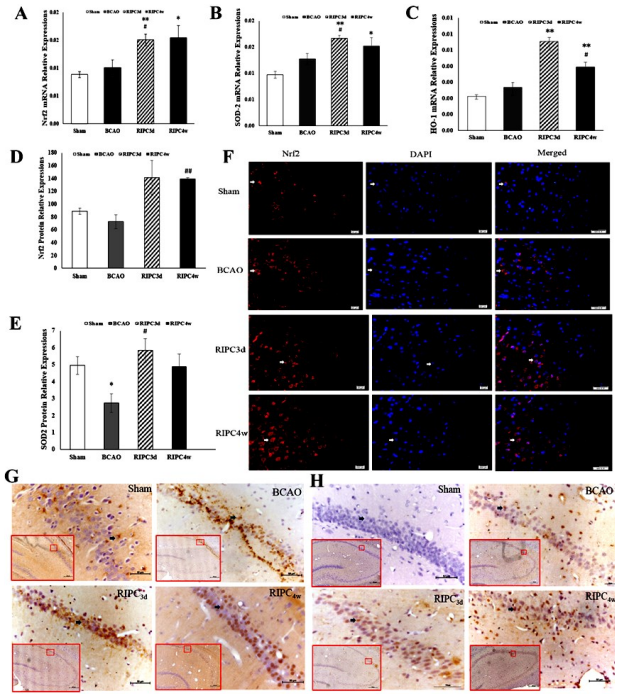


Figure 2. Activation of Nrf2-ARE signaling pathway by RIPC in the hippocampus. **(A–C)** mRNA expression of Nrf2, SOD2, and HO-1 by qPCR. **(D–E)** Protein levels of Nrf2 and SOD2 by ELISA. **(F)** Nrf2 immunofluorescence in the cortex. **(G-H)** Nrf2 and SOD2 immunohistochemistry in the CA1 region of the hippocampus. Symbols: *P < 0.05, **P < 0.01 vs Sham; #P < 0.05, ##P < 0.01 vs RT. Abbreviations: Nrf2 - Nuclear factor erythroid 2-related factor 2; ARE - Antioxidant response element; SOD2 - Superoxide dismutase 2; HO-1 - Heme oxygenase 1; RT - Radiotherapy; RIPC - Remote ischemic post-conditioning. Magnification: Immunohistochemistry images at ×400; scale bar = 50 μm.

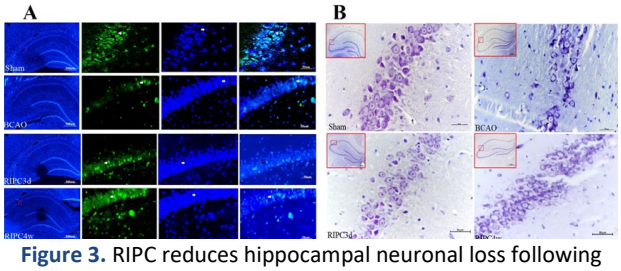


Figure 3. RIPC reduces hippocampal neuronal loss following RT. **(A)** Immunofluorescence staining for NeuN-positive neurons in CA1 region. **(B)** Nissl staining showing neuronal density and morphology. White arrows indicate surviving neurons. Abbreviations: NeuN - Neuronal nuclear antigen; RT - Radiotherapy; RIPC - Remote ischemic post-conditioning. Magnification: ×400 for both panels; scale bar = 50 μm.

RIPC enhances autophagy and reduces Tau and phospho-Tau accumulation

Since proteinopathy and impaired protein clearance contribute to RICD, we evaluated autophagic activity and Tau protein levels in hippocampal tissue. qPCR showed that LC3b mRNA, a marker of autophagosome formation, was significantly upregulated in the RIPC3d group compared to both Sham and RT (P<0.05), while no significant difference was observed between RT and sham (figure 4A). These results suggest that RIPC, but not radiation alone, activates autophagy.

Tau mRNA expression was significantly elevated in RT rats relative to sham (P<0.01), consistent with Tau accumulation. RIPC significantly reduced Tau expression in both RIPC3d and RIPC4w groups (P<0.05 vs RT) (figure 4B). Immunohistochemistry further confirmed elevated Tau and phospho-Tau (P-Tau) immunoreactivity in the hippocampal CA1 and cortical regions of RT rats, which was markedly diminished in RIPC-treated rats (figure 4C-D).

Immunohistochemical staining for LC3b also showed increased autophagic activity in the hippocampus following RIPC (figure 4C). These results indicate that RIPC enhances neuronal autophagy, which may facilitate the degradation of pathological Tau proteins, thereby protecting against radiation-induced cognitive impairment.

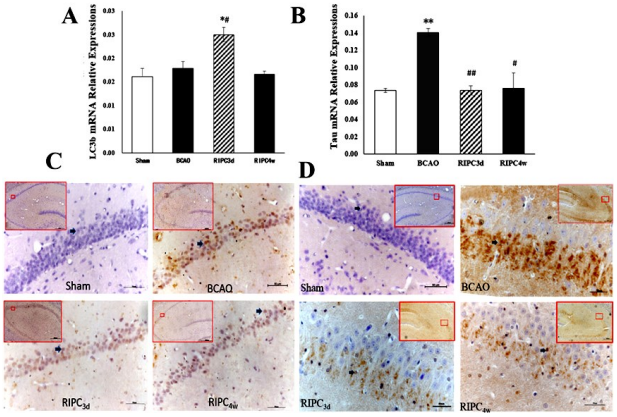


Figure 4. RIPC promotes autophagy and reduces Tau accumulation after RT. **(A)** LC3b mRNA levels (marker of autophagy). **(B)** Tau mRNA expression. **(C-D)** Immunohistochemistry for Tau and phospho-Tau (P-Tau) in hippocampal CA1. Black arrows indicate Tau/P-Tau-positive neurons. Symbols: *P < 0.05, **P < 0.01 vs Sham; #P < 0.05, ##P < 0.01 vs RT. Abbreviations: LC3b - Microtubule-associated protein light chain 3 beta; P-Tau - Phosphorylated Tau; RT - Radiotherapy; RIPC - Remote ischemic post-conditioning. Magnification: ×400; scale bar = 50 μm.

DISCUSSION

The increasing global incidence of aging populations and changes in dietary patterns have highlighted chronic cerebral hypoperfusion (CCH) as a critical pathological condition contributing to neurodegenerative diseases, such as vascular dementia (VD) and Alzheimer's disease (AD) [17, 18]. In parallel, cranial radiotherapy (RT) remains a primary treatment for patients with brain tumors and metastases [19]. However, despite its therapeutic success, RT frequently induces long-term cognitive dysfunction, particularly affecting memory and spatial learning, which shares overlapping mechanisms with CCH, including oxidative stress,

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neuronal loss, and protein aggregation [3].

Remote ischemic post-conditioning (RIPC) is an emerging non-invasive strategy, originally developed in cardiology, which involves repetitive brief ischemia-reperfusion cycles in a remote organ (e.g., limb) to activate protective responses in target tissues [20, 21]. Recent studies suggest its potential for neurological protection by enhancing antioxidant defense, anti-apoptotic signaling, and promoting repair mechanisms [22]. Our study demonstrates that RIPC can mitigate RT-induced cognitive decline, reduce oxidative stress, and preserve neuronal viability, particularly in the hippocampus, a radiosensitive region associated with learning and memory. This has led to the development of hippocampal-sparing radiation techniques, including hybrid IMRT–VMAT plans, which aim to reduce radiation burden on cognitive centers while maintaining tumor control [23].

The Nrf2-antioxidant response element (ARE) pathway, a master regulator of redox homeostasis, plays a central role in defending against RT- and ischemia-induced oxidative injury [12, 13]. In our model, RIPC significantly upregulated Nrf2, SOD2, and HO-1 at both the mRNA and protein levels, suggesting enhanced antioxidant defense. These results align with prior research showing that Nrf2 activation is critical for neuroprotection in models of ischemic stroke and AD [24, 25]. The increase in Nrf2-positive cells observed via immunostaining confirms that RIPC facilitates Nrf2 nuclear translocation in the irradiated hippocampus.

In addition to redox regulation, our findings demonstrate that RIPC enhances autophagic flux, evidenced by elevated LC3b expression, a key marker of autophagosome formation. This mechanism may contribute to the clearance of hyperphosphorylated Tau (P-Tau), thereby reducing the neurotoxic effects of protein aggregation. These observations are consistent with earlier studies showing that autophagy activation helps degrade pathological Tau and improves cognitive function in neurodegenerative diseases [26, 27].

Importantly, our study is among the first to explore the link between RIPC, Nrf2 activation, and autophagy in the context of RT-induced hippocampal injury. We showed that RIPC downregulated Tau and P-Tau expression, both at mRNA and histological levels, which are hallmarks of radiation-induced proteinopathy [28, 29]. These molecular effects corresponded to behavioral improvements, as seen in Morris water maze (MWM) and novel object recognition (NORT) testing, where RIPC-treated rats showed improved spatial learning and memory.

Limitations of this study include its short-term follow-up (up to 8 weeks), which may not fully capture the long-term cognitive trajectory after RT. Previous findings suggest that early vascular injury in the hippocampus may serve as a surrogate marker for delayed neurocognitive impairment, and incorporating such endpoints could strengthen future studies [30]. Additionally, only male rats were used, which limits generalizability, as sex-specific responses to RT and RIPC have been observed in other models. Furthermore, the exact molecular intermediaries linking Nrf2 activation to enhanced autophagy and reduced Tau burden remain to be elucidated, suggesting avenues for future mechanistic investigations. Finally, translation to clinical settings requires further studies in large animal models and ultimately in human subjects.

Overall, our findings suggest that RIPC represents a promising adjunctive therapy for patients undergoing cranial RT, particularly in reducing delayed cognitive toxicity via Nrf2-ARE-mediated mechanisms. Incorporating RIPC into radiotherapy protocols may offer a non-invasive, cost-effective method for preserving cognitive function in brain tumor survivors, warranting further translational exploration.

CONCLUSION

This study demonstrates that remote ischemic post-conditioning (RIPC) protects against radiotherapy-induced cognitive decline by activating the Nrf2-ARE pathway, enhancing autophagy, and reducing Tau pathology in the hippocampus. These findings support RIPC as a potential non-invasive adjunct to cranial radiotherapy for preserving cognitive function.

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Conflict of Interest: The authors declare no conflict of interest related to this work. No financial, personal, or professional affiliations influenced the design, execution, or reporting of this study.

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Ethical Approval: All experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of Chengdu Medical College (Approval ID: CMCC-IACUC-2024-017). All animal procedures complied with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

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Author contributions: F.Y.; designed the study, conducted the experiments, and drafted the

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manuscript. W.Q.; supervised the overall project. F.Z., S.L., M.Z., Y.Z., H.Y., and X.H.; contributed to data collection, analysis, technical support, and manuscript revision. All authors reviewed and approved the final version of the manuscript.

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