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Celecoxib Alleviates Radiation-Induced Brain Injury in Rats by Maintaining the Integrity of Blood-Brain Barrier

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Abstract

The underlying mechanisms of radiation-induced brain injury are poorly understood, although COX-2 inhibitors have been shown to reduce brain injury after irradiation. In the present study, the effect of celecoxib (a selective COX-2 inhibitor) pretreatment on radiation-induced injury to rat brain was studied by means of histopathological staining, evaluation of integrity of blood-brain barrier and detection of the expressions of inflammation-associated genes. The protective effect of celecoxib on human brain microvascular endothelial cells (HBMECs) against irradiation was examined and the potential mechanisms were explored. Colony formation assay and apoptosis assay were undertaken to evaluate the effect of celecoxib on the radiosensitivity of the HBMECs. ELISA was used to measure 6-keto-prostaglandin F1α (6-keto-PGF1α) and thromboxane B2 (TXB2) secretion. Western blot was employed to examine apoptosis-related proteins expressions. It was found that celecoxib protected rat from radiation-induced brain injury by maintaining the integrity of the blood-brain barrier and reducing inflammation in rat brain tissues. In addition, celecoxib showed a significant protective effect on HBMECs against irradiation, which involves inhibited apoptosis and decreased TXB2/6-keto-PGF1α ratio in brain vascular endothelial cells. In conclusion, celecoxib could alleviate radiation-induced brain injury in rats, which may be partially due to the protective effect on brain vascular endothelial cells from radiation-induced apoptosis.

Keywords

celecoxib, radiation-induced brain injury, blood-brain barrier, apoptosis

Introduction

Radiation-induced brain injury is a serious complication that can occur after radiation therapy for head and neck cancers and brain tumors as well as metastases in general.[1,2] Radiation-induced brain injury, particularly following stereotactic radiosurgery,[3-7] accounts for 68% of such complications, including radiation necrosis, with a frequency of 3%-24%.[4,8-10] Risk factors for radiation necrosis include the radiation dose, fractionation, the use of chemotherapy (especially concurrent chemotherapy), re-irradiation, and additional boost irradiation.[4,11]

The pathogenesis of radiation-induced brain injury is still poorly understood but is known to involve vascular endothelial cell injury, glial cell damage, and autoimmune reaction.[2] Animal studies showed that radiation decreased the microvessel density of the brain, induced decreased spinal blood flow,[12] and increased blood vessel permeability.[13] Radiation also decreases the production of endothelial progenitor cells.[14] Eventually, tissue inflammation and ischemia will promote the infiltration of macrophages and lymphocytes, which secrete interleukin (IL)-1α, IL-6, and tumor necrosis factor (TNF)-α, maintaining and enhancing the inflammatory state.[15]

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It has been reported that the content and the ratio of thromboxane A2 (TXA2) and prostaglandin I2 (PGI2) play key roles in the vascular system. TXA2 causes vasoconstriction, induces platelet aggregation and thrombosis and promotes inflammation.[16] PGI2 inhibits platelet aggregation and is a vasodilator. Under normal circumstances, the effects of PGI2 are dominant.[17] Ionizing radiations can break the balance between TXA2 and PGI2, resulting in increased secretion of TXA2 and decreased secretion of PGI2, impairing the cerebral vasculature and promoting thrombosis.[18] Thromboxane B2 (TXB2) and 6-keto-prostaglandin F1α (6-keto-PGF1α) are metabolic products of TXA2 and PGI2, which are relatively stable and easy to measure.[19,20]

Cyclooxygenase (COX), prostacyclin synthase, and thromboxane synthase participate in the synthesis of TXA2 and PGI2, among which COX is the most important.[21] COX-2 expression is increased by inflammation, and leads to increased production of TXA2 from arachidonic acid, and low levels of PGI2.[22] Celecoxib is a selective COX-2 inhibitor that has been shown to reduce the levels of inflammatory cytokines induced by radiation in rats.[23] Meloxicam, another selective COX-2 inhibitor, has been shown to reduce brain injury after irradiation, partly through the relief of vascular endothelial cell injury.[24] Hence, we supposed that inhibiting COX-2 by celecoxib might also be an appropriate approach to prevent radiation-induced brain injury.

On the other hand, radiation can stimulate the formation of ceramide, thereby reducing the stability of the mitochondrial membrane and promoting apoptosis through the c-Jun N-terminal kinase (JNK) pathway.[25,26] JNK can be activated by a variety of stimuli, such as oxidative damage, DNA and endoplasmic reticulum damage.[27] The activated form of JNK is phosphorylated JNK (p-JNK), which translocates to the cytoplasm, activating caspase-3 and the mitochondrial apoptotic pathway.[25,26] However, whether the radiation-induced brain injury involves JNK-mediated apoptosis signaling remains unclear.

The present study aims to examine the effect of celecoxib pretreatment on radiation-induced brain injury in rats and explore the related molecular mechanisms using human brain microvascular endothelial cells (HBMECs).

Materials and Methods

Cell Culture, Grouping, and Irradiation

HBMECs were purchased from Shanghai Biological Technology Co., Ltd. (Shanghai, China) and cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA), 1% penicillin sodium and 100 μg/mL streptomycin, at 37°C in 5% CO_2 in a humidified incubator (Thermo Scientific, NC, USA). Cells in the logarithmic growth phase were divided into the control group, irradiation (IR) group, and celecoxib pretreatment before IR group (IR+C). The IR+C group was pretreated with celecoxib for 24 h at an appropriate concentration selected according to the cytotoxicity pre-experiment (see below). Irradiation of the IR group and IR+C group was carried out using a RS-2000 X-ray irradiator (Rad Source Technologies Inc., Suwanee, GA, USA) at a dose rate of 1.2 Gy/min. Field size was 20 × 30 cm and the focus-surface distance was 20 cm.

Animal and Irradiation Treatment

Two hundred and forty 8-week-old male Sprague-Dawley (SD) rats, SPF-housed in the Laboratory Animal Center of Soochow University with access to water and food *ad libitum*, were randomly divided into 3 groups, the Control group (anesthetized only), the Irradiation group (irradiated after anesthetization) and celecoxib-plus Irradiation group (celecoxib administered twice before and once after exposure for 3 consecutive days), with 80 rats in each group. Animals were subjected to cranial irradiation of 20 Gy X-rays from a linear accelerator (Siemens Mevatron MD2, Erlangen, Germany) at a dose rate of 2 Gy/min after being anesthetized by 10% chloral hydrate intraperitoneally (400 mg/kg body weight). A dose of 20 Gy was selected since it was reported that the late delayed effect of whole brain radiation, cognitive dysfunction, can occur with a dose as low as 20 Gy in adults.[28] No signs of peritonitis were observed following the administration of the 10% chloral hydrate. Celecoxib (Pfizer, New York, NY, USA), was administered intraperitoneally at a dose of 30 mg/kg/day. The permeability of the blood-brain barrier (BBB) was examined by Evans Blue (EB) assay. Briefly, 1 mL/kg EB (2% in 0.9% saline) was administered to rats by tail vein injection. After 3 hours of EB circulation, all rats were deeply intraperitoneally anesthetized by 10% chloral hydrate (400 mg/kg) and transcardially perfused with ice-cold saline to flush away the blood and EB from the blood vessels. The brain tissues were quickly harvested by decapitation and weighed, cut into small pieces and homogenized with 3 mL of formamide at 37°C for 72 h for EB extraction. After centrifuged at 15000 g for 15 min at 4°C, the supernatant was collected and diluted with 100% ethanol at a ratio of 1:3. The EB content in brain tissues was quantified at 620 nm using a spectrophotometer (BioTek Instruments, VT, USA)) and expressed as micrograms of per gram of brain tissue (μg/g). The rats were sacrificed by cervical dislocation after intraperitoneally anesthetized by 10% chloral hydrate (400 mg/kg body weight) at a series of indicated timepoints from 3 h post-irradiation to 1 month and the brain water content (BWC) and genes expression were measured. Death was verified by cessation of the heartbeat and respiration and absence of reflexes. During the study, the animals were documented by the laboratory group at least twice a week and the following endpoints were employed for humane removal from this study: the animal reached a body condition score of 2/5, it was unable to right itself within 30 s, or it had severe clinical signs including evidence of lethargy, changes in ambulation, diarrhea, or increased respiratory effort. All experiments were performed in strict accordance with the U.K. Animals (Scientific Procedures) Act and associated guidelines as well as AVMA Guidelines for the Euthanasia of Animals 2013. All animal studies were reviewed and approved by the Soochow University Institutional Animal Care and Use Committee.

Cytotoxicity of Celecoxib Examined by CCK8 Assay

Celecoxib (Sigma, St Louis, MO, USA) was dissolved in DMSO (Sigma, St Louis, MO, USA) at the storage concentration of 0.5 mol/L, and further diluted with the culture medium to achieve different working concentrations (10, 30, 40, 50, and 100 μmol/L). HBMECs in 96-well plates were exposed to different drug concentrations for 24 h. Then, 10 μL of CCK-8 solution (Sigma, St Louis, MO, USA) was added to each well. Absorbance was measured at 450 nm using a microplate reader (Synergy2; BioTek Instruments, VT, USA) 2 hours later. Three independent experiments were performed, each in sextuplicates. The IC50 was calculated from the growth inhibition curve.

Colony Formation Assay

After exposed to gradient radiation doses of 0, 2, 4, 6, 8, 10 Gy with or without celecoxib pretreatment, the cells were harvested, counted and plated into Φ60 mm dishes. Cells were fixed with 70% ethanol for 5 min and stained with crystal violet 14 days later. Colonies containing more than 50 cells were counted as survivors. At least 3 parallel dishes were scored for each treatment.

Apoptosis Rate of HBMECs Determined by Flow Cytometry

The celecoxib pretreatment and irradiation were carried out in sequence. At 24 h and 48 h after irradiation, the HBMECs were collected by centrifugation of 450 g for 5 min at room temperature. The supernatant was also collected and 300 μL of Annexin V-APC binding buffer was added, followed by 5 μL of Annexin V-APC (Keygentec, Nanjing, China). After 15 min of incubation, 5 μL of PI was added (Beyotime Institute of Biotechnology, Haimen, China) and apoptosis was detected by a flow cytometer (FC500, Beckman Coulter, Brea, CA, USA).

ELISA of 6-Keto-PGF1α and TXB2

The celecoxib pretreatment and irradiation were carried out in sequence. At 6, 12, 24, and 48 h after irradiation, the culture media were collected and centrifuged at 2350 g for 10 min at 4°C. Then, 10 μL of supernatant was mixed with 40 μL of diluents, and ELISA was performed according to the manufacturer's instructions (Shanghai Yuanye Bio-Technology Co., Ltd., Shanghai, China).

qRT-PCR

Total RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Reverse transcription (RT) of total RNA was performed using RNA to cDNA EcoDry™ Premix Kit at 42°C for 60 min and the RT reaction was terminated by heating at 70°C for 10 min (Takara, Japan). The real-time PCR analysis was carried out using a SYBR Premix Ex Taq II Kit (Takara, Japan) in a 20 μL system and done with ABI 7500 Real-Time PCR System (Applied Biosystems, CA, USA). The program was run by initial denaturing for 2 min at 95°C before 40 cycles, each at 95°C for 15 s and 57°C for 30 s. Melting curve plotting was performed at the end of the PCR cycles to validate the correct generation of PCR product. The expression levels of genes were normalized to GAPDH and calculated by C(t) comparison method.[29] The primers are listed in Table 1.

Table 1. Primer Pairs Used for qRT-PCR.

Genes	Primer pairs	
NF-κB	Forward	5′-CCGGGATGGCTTCTATGAG-3′
	Reverse	5′-CACTGGATCCCCAGGTTCT-3′
VEGF	Forward	5′-TGATCAGACCATTGAAACCACT-3′
	Reverse	5′-GGAAGGGTAAGCCACTCACA-3′
GAPDH	Forward	5′-CCCTCTGGAAAGCTGTGG-3′
	Reverse	5′-AGTGGATGCAGGGATGATG-3′

Western Blot

The celecoxib pretreatment and irradiation were carried out in sequence. The HBMECs were collected at the indicated timepoints and lysed with RIPA buffer to extract the proteins (Invitrogen, Carlsbad, CA, USA). An amount of 30 μg of proteins was loaded in each lane for polyacrylamide gel electrophoresis. The proteins were transferred to PVDF membranes, which were then blocked at room temperature for 2 h with PBST (PBS with 0.1% Tween-20) containing 5% non-fat milk. The specific antibodies (1:1000) were incubated for 16 h at 4°C. The secondary antibodies (1:2000) were incubated for 1 h at room temperature after thorough TBST washing. The bands were revealed using an ECL reagent kit (Pierce Chemical, Dallas, TX, USA) and observed using a chemiluminescence gel imager (Carestream Health, Inc., Rochester, NY, USA). Anti-Caspase-3 (ab184787), anti-phospho-JNK (ab124956), anti-JNK (ab208035) and anti-COX-2 (ab15191) were purchased from Abcam (Cambridge, MA, USA) while anti-β-actin (4970 S) was purchased from Cell Signaling Technology (Beverly, MA, USA).

Immunohistochemistry

The hippocampus of the brain was collected for immunohistochemical analysis. Sections (3 μm in thickness) of formalin-fixed and paraffin-embedded samples were used to analyze the brain morphological changes induced by radiation and the expression of nuclear factor kappa-B (NF-κB) and vascular endothelial growth factor (VEGF). After deparaffinization, the antigen retrieval was performed in citric acid buffer (pH6.0) using an autoclave oven, then the naturally cooled sections were incubated in PBS containing 10% FBS overnight and incubated with NF-κB/VEGF polyclonal antibodies (ab16502 for NF-κB and ab1316 for VEGF, Abcam, Cambridge, MA, USA) at 4°C overnight. After thorough washing in PBS the

sections were incubated with the HRP-conjugated secondary antibodies for 30 min. Then the antibody-antigen complex was visualized using DAB reaction and hematoxylin staining for cell nuclei. The stained sections were examined under a DM2000 optical microscope (Leica, Germany) and the images were captured at a magnification of 100×.

Statistical Analysis

SPSS 19.0 (IBM, Armonk, NY, USA) was used to calculate the IC50 and to perform the statistical analyzes. Data were expressed as mean ± standard deviation (SD) and analyzed by ANOVA. Two-sided P values <0.05 were considered statistically significant.

Results

Protective Effects of Celecoxib on Radiation-Induced Brain Injury of Rats

A radiation-induced brain injury model was established using rats. It was found by H&E staining that there were no obvious pathological changes in the brain tissues of control group, where the cell morphology and vascular structure were intact. While in the irradiation group, a small number of cells showed cytoplasmic edema, nuclear fragmentation and pyknosis 3 hours post-irradiation. From 0.5 to 3 days after irradiation, the number of edema cells increased further, accompanied by vacuolated cytoplasm, increased steatosis, widened capillary space, and thrombosis in some vessels. On the 7th day after irradiation, the number of pathological cells peaked, and the brain tissues around vessels became disordered. 15 days after irradiation, necrosis of the cells, and fragmentation of nucleus was observed. Vascular embolism still existed, leading to infarction of some brain tissues. Thirty days after irradiation, most embolic vessels were recanalized, cell edema disappeared, and necrotic areas increased slightly. However, in the celecoxib plus irradiation group, the pathological changes of brain tissue at each indicated timepoint were similar to those in the irradiation group, but the lesion degree was significantly lighter than that in the irradiation group, especially on the 15th and 30th day. The number of necrotic cells and the disordered tissue structure areas were significantly smaller than those in the irradiation group (Figure 1A). Besides, we detected the integrity of blood-brain barrier by Evans Blue (EB) assay as well as the brain water content. As shown in Figure 1B, the permeability of blood-brain barrier was increased significantly by irradiation treatment, especially between 3 hours and 15 days post-irradiation ($P < 0.05$). However, celecoxib pretreatment decreased the EB content in brain tissues at 3 h, 12 h, and 1 day post-irradiation ($P < 0.05$). As shown in Figure 1C, the brain water content was also increased significantly at all indicated timepoints post-irradiation ($P < 0.05$), which was decreased by celecoxib significantly from 3 h through 15 days post-irradiation ($P < 0.05$).

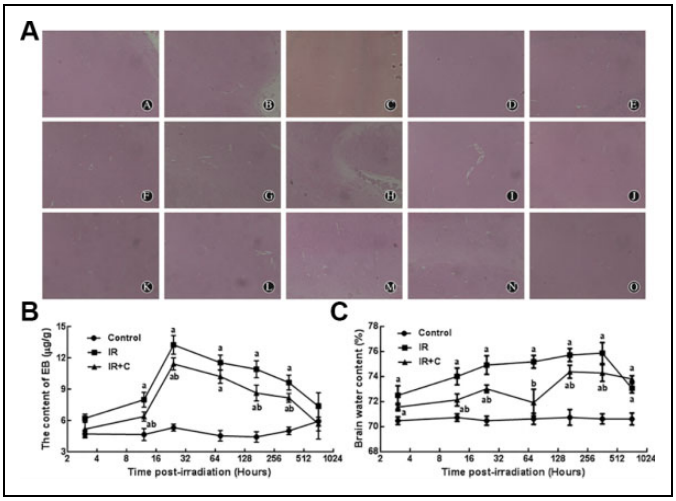

Figure 1. Celecoxib protects rat against radiation-induced brain injury. A, HE staining of pathological sections from rat brain tissues exposed to X-ray irradiation of 20 Gy. Ⓐ: HE staining of section from control group; Ⓑ-Ⓗ: HE staining of sections from irradiated groups at 3 h, 12 h, 1 d, 3 d, 7 d, 15 d and 30 d post-irradiation, respectively. Ⓘ-Ⓞ: HE staining of sections from celecoxib plus irradiation groups at 3 h, 12 h, 1 d, 3 d, 7 d, 15 d and 30 d post-irradiation, respectively. Sections were captured at 100 ×. B, Changes of Evans Blue (EB) content in rat brain tissues at indicated timepoints after 20 Gy X-rays irradiation. C, Changes in brain water content (BWC) in rat brain tissues at indicated timepoints after 20 Gy X-rays irradiation. Error bars stand for the mean ± SD derived from 3 independent experiments. a: $P < 0.05$ when compared with the control group at the same timepoint post-irradiation; b: $P < 0.05$ when compared with the IR group at the same timepoint post-irradiation.

Effects of Celecoxib Pretreatment on the Expressions of Both NF-κB and VEGF in Brain Tissues

Since NF-κB and VEGF are 2 key regulators participating in the inflammation and repair of vascular injury and repair, we investigated whether celecoxib pretreatment has a role in the expressions of both these 2 genes. Brain tissues from 3 hours through to 30 days post-irradiation were collected for both mRNA and protein detection. As shown in Figure 2A, the mRNA levels of NF-κB were up-regulated at all indicated timepoints, which peaked on the 3rd day and then reduced. However, they were still higher than that of the control group at the 30th day. Celecoxib pretreatment significantly decreased the induced NF-κB mRNA levels at all timepoints except for the 7th and the 30th day ($P < 0.05$ at the 3, 12, 24, 72, 360 hours post-irradiation). As to the VEGF mRNA, it was also up-regulated at all indicated timepoints, which peaked on the 7th day and then reduced. However, celecoxib decreased VEGF mRNA induced by irradiation from 3 hours through to 7 days post-irradiation except for at 24 hours ($P < 0.05$ at 3, 12, 72, 168 hours post-irradiation), while it didn't change VEGF mRNA significantly on the 15th or 30th day ($P > 0.05$) (Figure 2B). Western blot assay also indicated celecoxib significantly decreased both NF-κB and VEGF protein levels induced by irradiation (Figure 2C and D). To further verify that celecoxib

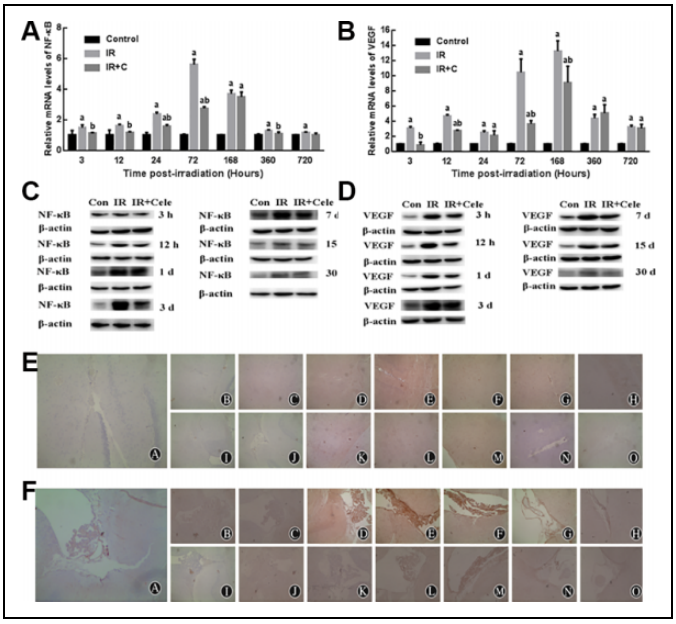

Figure 2. Changes of NF-κB and VEGF expressions in rat brain tissues after 20 Gy X-rays irradiation. A, The mRNA levels of NF-κB in rat brain tissues at indicated timepoints post-irradiation. B, The mRNA levels of VEGF in rat brain tissues at indicated timepoints post-irradiation. Error bars denote the mean ± SD derived from 3 independent experiments. a: $P < 0.05$ when compared with the control group at the same timepoint post-irradiation; b: $P < 0.05$ when compared with the IR group at the same timepoint post-irradiation. C and D, The protein levels of NF-κB and VEGF in rat brain tissues at indicated timepoints post-irradiation. E and F, The expressions of NF-κB and VEGF in rat brain tissues at indicated timepoints post-irradiation as detected by immnohistochemistry. Ⓐ: Immunohistochemical staining of either NF-κB or VEGF of sections from the control group; Ⓑ-Ⓗ: Immunohistochemical staining of NF-κB or VEGF on sections from the irradiated groups at 3 h, 12 h, 1 d, 3 d, 7 d, 15 d and 30 d post-irradiation, respectively. ①-⓪: Immunohistochemical staining of NF-κB or VEGF on sections from the celecoxib plus irradiation groups at 3 h, 12 h, 1 d, 3 d, 7 d, 15 d and 30 d post-irradiation, respectively. Sections were captured at 100 ×.

inhibited the up-regulation of NF-κB and VEGF, immunohistochemistry was carried out to analyze the expressions of both genes in brain tissues. As shown in Figure 2E and F, irradiation increased the expressions of the 2 proteins in the brain tissues, while celecoxib decreased them. Besides, disorder of the brain histological structure induced by irradiation showed pronounced alleviation with celecoxib.

Effects of Celecoxib on the Radiosensitivity of HBMECs

Based on the above achievements, it was found that serious BBB damage was caused by irradiation. As is known, human brain microvascular endothelial cells, the major component of the BBB, play an essential role in the maintenance of the integrity of the BBB and comprise the primary limitation to passage of both soluble and cellular substances from the blood into the brain. Thus we want to know whether celecoxib affects the radiosensitivity of this kind of cells. HBMECs were employed

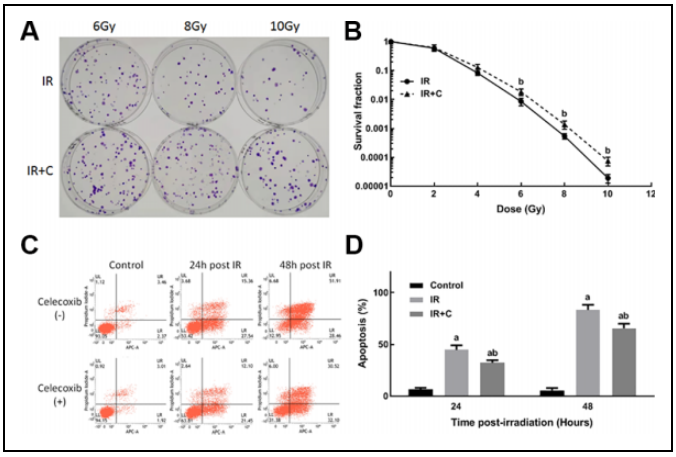

Figure 3. Celecoxib pretreatment reduced the radiosensitivity of HBMECs. A, The representative images of colony formation assay of HBMECs exposed to X-rays irradiation. B, The survival fractions of HBMECs exposed to X-rays irradiation of indicated doses. C, The representative images of apoptosis assay of HBMECs exposed to X-rays irradiation. D, The apoptosis rates of HBMECs exposed to 8 Gy X-rays irradiation. Error bars indicate the mean ± SD derived from 3 independent experiments. a: $P < 0.05$ when compared with the control group at the same timepoint post-irradiation; b: $P < 0.05$ when compared with the IR group at the same timepoint post-irradiation.

to conduct colony formation assay and apoptosis assay after irradiation. The growth inhibition rates of HBMECs by celecoxib at concentrations of 10, 30, 40, 50, and 100 μmol/L were 4.52% ± 0.13%, 6.31% ± 0.07%, 9.01% ± 0.10%, 15.26% ± 0.70%, and 70.65% ± 089%, respectively (Supplementary Figure 1), based on which the IC50 was calculated as 81.50 ± 0.75 μmol/L. A concentration of 30 μmol/L was used in the *in vitro* experiments. As shown in Figure 3A and B, compared with the irradiation group, celecoxib pretreatment resulted in a significantly higher survival rate of HBMECs exposed to X-rays of 6 Gy, 8 Gy and 10 Gy ($P < 0.05$), while no appreciable radioprotective effects were observed when 2 Gy or 4 Gy was used ($P > 0.05$). The apoptosis assay found that 8 Gy X-ray irradiation caused severe apoptosis in HBMECs ($P < 0.05$), approaching an apoptosis rate of 91.28% ± 4.95% 48 hours post-irradiation. However, celecoxib pretreatment decreased this rate to 78.58% ± 4.36% ($P < 0.05$). At 24 h post-irradiation, celecoxib pretreatment also inhibited the radiation-induced apoptosis (Figure 3C and D, $P < 0.05$). These results suggested that celecoxib considerably decreased the radiosensitivity of HBMECs.

Celecoxib Pretreatment Decreased the Secretion of TXB2 by HBMECs After Irradiation

Since TXA2 promotes thrombosis by inducing platelet aggregation, maintains a specific balance with its antagonist PGI2 and plays a vital role in radiation-induced brain injury. We detected TXB2 (a metabolite of TXA2) and 6-keto-PGF1α (a metabolite of PGI2) secreted by the irradiated HBMECs. As shown in Figure 4A, 8 Gy X-ray irradiation increased the

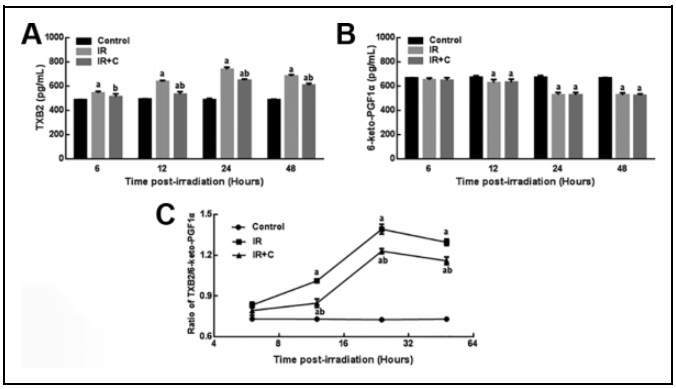

Figure 4. Celecoxib pretreatment decreased the secretion of TXB2 by HBMECs exposed to irradiation. A, The concentrations of TXB2 in the culture medium of HBMECs at indicated timepoints post 8 Gy X-rays irradiation were measured with ELISA. B, The concentrations of 6-keto-PGF1α in the culture medium of HBMECs at indicated timepoints post 8 Gy X-rays irradiation were measured with ELISA. C, The ratios of TXB2/6-keto-PGF1α at indicated timepoints post 8 Gy X-rays irradiation were calculated. Error bars denote the mean ± SD derived from 3 independent experiments. a: $P < 0.05$ when compared with the control group at the same timepoint post-irradiation; b: $P < 0.05$ when compared with the IR group at the same timepoint post-irradiation.

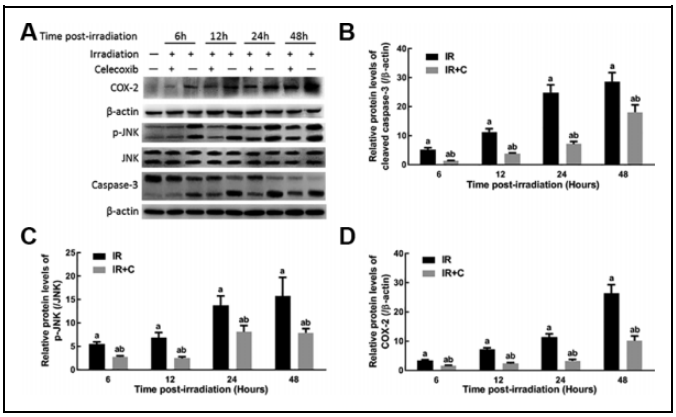

Figure 5. Celecoxib pretreatment decreased p-JNK, COX-2 as well as cleaved caspase-3 in HBMECs after irradiation. A, Western blot analysis of p-JNK, JNK, COX-2 as well as Caspase-3 in HBMECs at different timepoints post-irradiation, and the representative images were shown. B-D, Grayscale analysis of the expressions of p-JNK, COX-2 as well as cleaved caspase-3 in HBMECs at different timepoints post-irradiation. The expressions of COX-2 and cleaved caspase-3 were normalized to β-actin while the expression of p-JNK was normalized to JNK. Error bars denote the mean ± SD derived from 3 independent experiments. a: $P < 0.05$ when compared with the control group at the same timepoint post-irradiation; b: $P < 0.05$ when compared with the IR group at the same timepoint post-irradiation.

TXB2 secretion of HBMECs markedly at all indicated timepoints when compared with the control group (all $P < 0.05$), while celecoxib decreased its secretion (all $P < 0.05$) (at 6, 12, 24 and 48 h post-irradiation: control group, 487 ± 2, 488 ± 10, 486 ± 6 and 487 ± 2 pg/mL, respectively; IR group, 543 ± 14, 635 ± 11, 736 ± 19 and 682 ± 12 pg/mL, respectively; IR+C group, 513 ± 23, 535 ± 20, 648 ± 10 and 607 ± 14 pg/ml, respectively). Although irradiation reduced the secretion of 6-keto-PGF1α at 12 h, 24 h and 48 h post-irradiation significantly compared with the control group ($P < 0.05$), celecoxib pretreatment did not noticeably change the 6-keto-PGF1α secretion when compared with the IR group (Figure 4B, all $P > 0.05$) (at 6, 12, 24 and 48 h post-irradiation: control group, 666 ± 4, 672 ± 13, 672 ± 14 and 666 ± 5 pg/ml, respectively; IR group: 651 ± 16, 628 ± 26, 529 ± 17 and 527 ± 18 pg/ml, respectively; IR+C group: 647 ± 22, 632 ± 24, 627 ± 20 and 524 ± 12 pg/ml, respectively). However, the ratio of TXB2/6-keto-PGF1α was increased in the IR group compared with the control group at all indicated timepoints (all $P < 0.05$) while it was decreased in the IR+C group (all $P < 0.05$, compared with IR group) (Figure 4C).

Celecoxib Pretreatment Decreased p-JNK and COX-2 in HBMECs After Irradiation

As an essential member of MAPK family, JNK plays a key role in regulating intracellular and extracellular stress response, and its phosphorylated form could activate the mitochondrial apoptotic pathway. As shown in Figure 5, the protein amounts of phosphorylated JNK, cleaved caspase-3 and COX-2 were increased substantially after 8 Gy X-ray irradiation at all indicated timepoints ($P < 0.05$), and the expression of both cleaved caspase-3 and COX-2 increased from 6 h through to 48 h post-irradiation. Compared with the IR group, the amounts of phosphorylated JNK and cleaved caspase-3 in the IR+C group was decreased significantly (all $P < 0.05$) with celecoxib pretreatment, which also reduced the protein expression of COX-2 induced by irradiation (all $P < 0.05$). These results are in accordance with the apoptosis assay.

Discussion

The mechanisms of radiation-induced brain injury are poorly understood. It has been reported that COX-2 inhibition can reduce radiation-induced brain necrosis and edema.[30] This study aimed to examine the effect of celecoxib on radiation-induced injury both *in vivo* and *in vitro* and to explore the potential mechanisms. The results showed that celecoxib might help protect both brain tissues and HBMECs from irradiation.

Previously research indicated that vascular endothelial cells are sensitive to X-rays and that after irradiation, endothelial cells shed from the basement membrane present cytoplasmic vacuolization and apoptosis, leading to damage to the blood-brain barrier, vascular wall remodeling, lumens stenosis, and thrombosis.[31,32] Radiation-induced endothelial cell apoptosis is a critical factor in radiation-induced brain injury.[33] The present study confirms that irradiation increased the apoptosis rate of HBMECs and caused damage to the BBB.

Recent investigation revealed that COX-2 inhibition could protect against radiation-induced damage.[34,35] Indeed, Khayyal et al[23] showed that celecoxib, a selective COX-2 inhibitor, reduced the damaging effects of radiation in animals. Han et al[24] demonstrated that meloxicam reduces brain injury after

irradiation, partly through relief of vascular endothelial cell injury. Yang et al[36] showed that celecoxib can reduce the degree of brain edema in rats and glial cell injury after irradiation, which plays a protective role against radiation-induced brain injury. The present study showed that celecoxib suppressed the radiation-induced brain injury by alleviation of radiation-induced BBB damage, which may involve the apoptosis inhibition of brain microvascular endothelial cells, suggesting a potential protective role of celecoxib against radiation-induced brain injury and that celecoxib decreases COX-2 expression in addition to the selective inhibition. However, celecoxib was shown to inhibit HBMECs growth at higher concentrations as shown in Supplementary Figure 1, which suggests that celecoxib at higher concentrations may present toxicity on both HBMECs and the integrity of BBB. Thus the protective role of celecoxib should be measured at a relatively safe concentration.

Our *in vivo* experiments confirmed a significant increase of NF-κB and VEGF. It has been reported that radiation-induced COX-2 can promote prostaglandin synthesis, which facilitates the secretion of various kinds of inflammatory cytokines, such as TNF-α, IL-1β, IL-6, ICAM-1. Additionally, COX-2 could promote the expression of the inflammatory cytokines through activation of AP-1 and NF-κB. The up-regulated inflammatory cytokines will play important roles in the late stage of radiation-induced brain injury.[37] Besides, radiation-induced up-regulation of COX-2 will increase the expression of VEGF, which will cause increased capillary permeability, resulting in increased BBB permeability, vascular endothelial dysfunction, vasodilation, wall thickening, decreased vascular density and length, as well as increased vascular permeability.[38] As shown by the results, celecoxib treatment significantly reduced the radiation-induced up-regulation of NF-κB and VEGF on both mRNA and protein levels. Thus it can be concluded that the alleviation of radiation-induced BBB damage by celecoxib is partially caused by the inhibition of NF-κB and VEGF signaling, which play key roles in radiation-induced brain injury.

Radiation stimulates the formation of ceramide, thereby reducing the stability of the mitochondrial membrane and promoting apoptosis through the JNK pathway.[25,26] JNK can be activated by various stimuli, controls the intracellular and extracellular stress reactions of cells, which is called the stress-activated protein kinase (SAPK), and it plays a very important role in regulating cell apoptosis.[39,40] In the present study, irradiation increased the p-JNK and cleaved caspase-3 expression in HBMECs, while celecoxib partially reversed these increases. The results further suggested that the protective effects of celecoxib are associated with the regulation of apoptosis.

The content and ratio of TXA2 and PGI2 play crucial roles in thrombosis and inflammation.[41] Ionizing radiations can break the balance between TXA2 and PGI2 by inducing COX-2 expression while inactivating prostacyclin synthase, resulting in decreased PGI2 production while increasing TXA2 production, impairing the cerebral vasculature and promoting thrombosis as well as inducing apoptosis.[18,42,43] The present study showed that irradiation increased the ratio of TXB2 to 6-keto-PGF1α, which were stable metabolic products of TXA2 and PGI2. COX-2 is involved in the synthesis of TXA2 and PGI2.[44] However, compared with the IR group, there was no significant reduction of 6-keto-PGF1α in the IR+C group. Thus the decrease in the ratio of TXB2/6-keto-PGF1α may be due to the reduction of TXA2, which can induce an antagonistic effect on PGI2 secretion, then increase the secretion of PGI2 through the prostaglandin synthesis pathway.[45] As shown in Figure 6, celecoxib pretreatment decreased the amount of TXB2 after irradiation by inhibiting COX-2, which suggests a reduction in PGH2 as well as TXA2, leading to the inhibited apoptosis of endothelial cells. Furthermore, inhibited production of PGH2 resulted in decreased VEGF and NF-κB production, leading to the remission in increased BBB permeability and vascular endothelial dysfunction, finally alleviated radiation-induced brain injury.

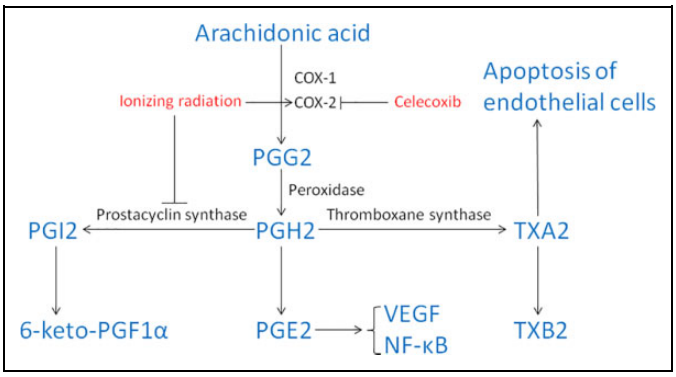

Figure 6. Schematic model delineating the pathway showing that celecoxib alleviates radiation-induced brain injury. Irradiation induced COX-2 expression while inactivated prostacyclin synthase, caused increased production of PGG2 and PGH2, further leading to the increased TXA2, VEGF and NF-κB, which in turn induced apoptosis of endothelial cells, increased BBB permeability and vascular endothelial dysfunction. Celecoxib pretreatment decreased the amount of TXA2 after irradiation by inhibiting COX-2, leading to inhibited apoptosis of endothelial cells. In parallel, suppressed production of PGH2 resulted in decreased VEGF and NF-κB production, leading to the remission in increased BBB permeability and vascular endothelial dysfunction, which eventually alleviated radiation-induced brain injury.

In conclusion, the results from this study demonstrated that celecoxib helps alleviate radiation-induced brain injury by maintaining the integrity of BBB and reducing the inflammation in the rat brain tissues, and the mechanisms involve the inhibited apoptosis and decreased TXB2/6-keto-PGF1α ratio in brain vascular endothelial cells. However, we just focused on mitigation effect of celecoxib on BBB damage in the present study by measuring certain cellular, biochemical and histopathological parameters. As is known, neurogenesis, neurons and neural functions are important to the radiation-induced cognitive impairment, thus additional experimental studies are required to determine the protective effect of celecoxib on the neurocognitive functions in the future.

Authors' Note

Xiaoting Xu, Hao Huang and Yu Tu contributed equally to this paper. XTX, HH, YT, JXS, YZX and CYM conducted the experiments and SBQ performed data analysis. JYZ and WTH designed the research and wrote the paper. All authors contributed substantially to this research and reviewed this manuscript.

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Supplemental Material

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