SPANDIDOS PUBLICATIONS

# Corilagin inhibits the double strand break-triggered NF-κB pathway in irradiated microglial cells

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**Abstract.** Microglia, the resident immune cells of the central nervous system (CNS), are activated by various stimuli. Resting microglia are the basis of normal neurogenesis, while activated microglia may inhibit neurogenesis through the production of pro-inflammatory mediators and cytokines. Recent research suggests that microglia are activated by irradiation. This may play a role in radiation-induced brain injury (RIBI). DNA double-strand breaks (DSBs), the most deleterious form of DNA damage after ionizing radiation, may rapidly trigger the activation of the NF-κB pathway via p53-induced protein leading to the release of pro-inflammatory mediators and cytokines. Thus, a negative regulator of the NF-κB pathway that inhibits radiation-induced microglia activation could be used to treat RIBI. Corilagin, a member of the tannin family, inhibits NF-κB pathway activation. In the present study, we examined the inhibitory effects of corilagin on radiation-induced microglia activation using a variety of techniques. Our data suggest that corilagin inhibits radiation-induced microglia activation via suppression of the NF-κB pathway and the compound is a potential treatment for RIBI.

## Introduction

Microglial cells are the resident immune cells of the central nervous system (CNS) parenchyma and play a critical role in the development of the inflammatory response within the brain. Microglia cells react to a variety of stimuli, including lipopolysaccharides (LPS), interferon-γ, and ß-amyloid. It was reported recently that microglia are activated post-irradiation (1). Upon activation *in vitro*, microglia produce a variety of pro-inflammatory mediators and cytokines, such as IL-1ß, IL-6, and TNF-α, reactive oxygen, nitric oxide (NO), and prostaglandin E ($PGE_2$), which are thought to be responsible for inflammation-related diseases, including radiation-induced brain injury (RIBI), trauma, ischemia, Alzheimer's disease, and neural death (2). Brain irradiation leads to a marked increase in microglia activation and release of pro-inflammatory cytokines associated with inhibitory of neurogenesis in hippocampus *in vivo* (3,4). Small molecule inhibitors of these pro-inflammatory mediators and cytokines are needed for treatment of RIBI and other diseases.

DNA double-strand breaks (DSBs) are the most deleterious form of DNA damage and numerous *in vitro* studies have analyzed the DSB repair system that is activated after exposure to ionizing radiation. DSBs rapidly trigger the activation of NF-κB pathway via NEMO (5,6). The death-domain protein PIDD was originally identified as an early p53-inducible gene and is implicated in p53-induced apoptosis (5). PIDD is a mediator of the DNA-damage-activated stress response and is involved in genotoxic stress-induced NF-κB activation (6,7). PIDD expression enhances genotoxic-stress-induced NF-κB activation through augmented sumoylation and ubiquitination of NEMO (7).

Corilagin (ß-1-*O*-galloyl-3,6-(*R*)-hexahydroxydiphenoyl-D-glucose) is a member of the tannin family and has been isolated from medicinal plants, such as the *Phyllanthus* species (8). The molecular formula of corilagin is $C_{27}H_{22}O_{18}$ (9). The structure of corilagin is given in Fig. 1A. Corilagin has anti-oxidative, -atherogenic, and -hypertensive effects in various models (8-11). A preliminary *in vitro* study suggested that corilagin has anti-inflammatory activity (12). The activation of microglia and release of pro-inflammatory cytokines post-irradiation are regarded as the key effectors of RIBI. The purpose of this study was to investigate whether corilagin administration inhibits microglia activation by blocking NF-κB pathway activation.

## Materials and methods

*Materials*. Corilagin standard substance (purity 99%) was obtained from the China National Institute for the Control of Pharmaceutical and Biological Products. RPMI-1640 was from Gibco (Grand Island, NY, USA). 3-(4,5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) was obtained from Sigma (St. Louis, MO, USA). The anti-Iba-1 rabbit antibody was from Wako Chemical (Osaka, Japan). NEMO and IκB-α primary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The anti-γ-H2AX mouse monoclonal antibody was purchased from Abcam (Cambridge, MA, USA). AlexaFluor-488 conjugated

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goat anti-mouse, donkey anti-goat secondary antibody, and AlexaFluor-568 conjugated goat anti-rabbit secondary antibody were purchased from Invitrogen (Carlsbad, CA, USA). Vectaschield mounting medium with 4',6-diamidino-2-phenylindole (DAPI) was purchased from Vector Laboratories (Burlingame, CA, USA). Mouse IL-1ß, TNF-α, and IL-6 Quantikine ELISA kits were purchased from R&D Systems (Minneapolis, MN, USA). The Griess reagent nitric oxide assay kit was from Beyotime Biotech (Jiangsu, China). Trizol was obtained from Invitrogen. M-MLV reverse transcriptase and SYBR Green I were purchased from Toyobo Company (Osaka, Japan). The oligo dT and primers were synthesized by Shanghai Invitrogen (Shanghai, China). The dNTP and Taq DNA polymerase were obtained from Fermentas International (Burlington, Canada). Nuclear and Cytoplasmic Protein Extraction Kits were purchased from Bio-Rad Laboratories (Hercules, CA, USA). The ECL Western blotting detection system was obtained from Millipore (Bedford, MA, USA). p65 rabbit polyclonal antibody was purchased from Santa Cruz Biotechnology and the antibody against ß-actin was from Sigma.

*Cell culture*. Immortalized BV-2 murine cells were obtained from the Central Laboratory of Wuhan Union Hospital (Wuhan, China). Cells were maintained in RPMI-1640 (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, and 100 g/ml streptomycin and were grown at 37˚C in a humidified atmosphere of 5% $CO_2$.

*MTT assay*. The cytotoxicity of corilagin was determined using the MTT assay. Cells ($1x10^5$/well) were plated in 100 $\mu$l of medium/well in 96-well plates. After incubation overnight, the medium from each well was discarded and replaced with fresh medium containing corilagin at concentrations ranging from 1.0 to 250.0 $\mu$g/ml. BV-2 cells not treated with corilagin were used as controls. After treatment with corilagin for 24 h, 20 $\mu$l of 5 mg/ml MTT (pH 4.7) was added to each well. After 4 h, the supernatant was removed, 100 $\mu$l/well DMSO was added, and samples were shaken for 15 min. The optical density (OD) at 570 nm was measured with a microplate reader (Bio-Rad, Richmond, CA, USA) using wells without cells as blanks. All experiments were performed in triplicate.

*Irradiation*. Cells were irradiated using a $^{137}$Cs irradiator (Siemens, Germany). The irradiation parameters were as follows: beam energy, 6 MV photons; dose-rate, 2.0 Gy/min; source skin distance (SSD), 99 cm; irradiation size, 30x30 $cm^2$. Sham-exposed cells were used as controls. All irradiations were performed at room temperature.

*Expression of ionized calcium binding (Iba)-1 in BV-2 cells*. After BV-2 cells were irradiated for 0.5, 3, 6, 12, 24, or 48 h in the presence or absence of 5.0 $\mu$g/ml corilagin, sections were incubated with anti-Iba-1 antibody (1:200) followed by AlexaFluor-568 conjugated secondary antibodies (1:200). Expression levels were determined using confocal microscopy for comparison to control, untreated cells.

*Analysis of IL-1ß, TNF-α and IL-6 in supernatant*. After irradiation in presence or absence of corilagin, BV-2 cell supernatants were assayed for IL-1ß, TNF-α, and IL-6 by ELISA as per the manufacturer's instructions. The absorbance at 450 nm was determined using a microplate reader.

*Analysis of NO*. Levels of the NO derivative nitrite were determined using the Griess reaction. The nitrite detection assay was preformed according to instructions provided by the manufacturer. The samples were assayed in triplicate and a standard curve using $NaNO_2$ was generated for each experiment for quantification. Briefly, 100 $\mu$l of medium or $NaNO_2$ standard was mixed with 100 $\mu$l of Griess reagent in a 96-well plate. After 15 min, OD was read in a microplate reader at 540 nm.

*RNA isolation and real-time qPCR*. Total RNA was purified from cultured cells using Trizol reagent according to the manufacturer's protocol. Primer sequences were designed using Beacon Designer software (Bio-Rad) and were as follows: mouse TNF-α 5' primer TTC TCA TTC CTG CTT GTG G and 3' primer CTT GGT GGT TTG CTA CGA C; mouse IL-1ß 5' primer AAA TCT CGC AGC AGC ACA T and 3' primer CAC ACA CCA GCA GGT TAT CA; mouse IFN-γ 5' primer TAA CTC AAG TGG CAT AGA TGT G and 3' primer GAA GAA GGT AGT AAT CAG GTG TG; mouse IL-6 5' primer TTG CCT TCT TGG GAC TGA T and 3' primer TTG CCA TTG CAC AAC TCT T; mouse COX-2 5' primer GAG TGG GGT GAT GAG CAA and 3' primer GCA ATG CGG TTC TGA TAC T; mouse PIDD 5' primer GGT CCA CCT GCC ACT GAT and 3' primer AAT AGC CTG GGC ATT TCT G; GAPDH 5' primer TCA CCA CCA TGG AGA AGG C and 3' primer GCT AAG CAG TTG GTG GTG CA. Real-time RT-PCR was performed using the Stratagene MX3000P QPCR System (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions. The $2^{-\Delta\Delta Ct}$ method was used to analyze the results. The relative expression levels of each mRNA were calculated using the ΔΔCt method normalizing to GAPDH and relative to the control samples. Briefly, the Ct of GAPDH was subtracted from the Ct of the gene of interest to yield ΔCt. The ΔCt value of sham-irradiated sample was then subtracted from the ΔCt of treatment sample to yield the ΔΔCt value. Fold differences compared to sham-irradiated sample were obtained by calculating $2^{-\Delta\Delta Ct}$ for each treatment group. Data represent the mean ±SEM of three independent experiments.

*Western blot analysis*. Cells were seeded in six-well plates and pre-treated with or without 5.0 $\mu$g/ml corilagin before 32-Gy irradiation for 6 h. Cells were then lysed. To determine the levels of NF-κB, nuclear extracts were prepared from irradiated cells (13) and then resolved on 15% sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE). Proteins were transferred to a nitrocellulose membrane for 12-16 h at 30 V, blocked in 5% nonfat skim milk in Tris-buffered saline with 0.1% Tween 20 (TBST) for 1 h at room temperature and then incubated overnight with primary antibody (1:200) diluted in 2% BSA in TBST at 4˚C. The following day, proteins were visualized using the ECL detection system after incubation with the respective HRP-conjugated secondary antibody (1:1.000). Complexes of the primary and secondary antibodies were visualized by using a chemiluminescent substrate kit and

o medical X-ray film. The intensity of the blots was l with a gel-image analyzer (JS380; Peiqing Science and Technology, Shanghai, China).

*Immunofluorescence staining and confocal microscopy*. BV-2 cells were plated onto polylysine-coated cover glass and treated with irradiation. Samples were fixed in methanol, permeabilized in acetone, and then blocked in 1% goat serum. Anti-NEMO, IκB-α primary antibody (1:200), or γ-H2AX primary antibody (1:800) was applied after blocking overnight at 4˚C, followed by the AlexaFluor-488 conjugated goat anti-rabbit and donkey anti-goat secondary antibodies (1:200). Samples were mounted in Vectashield mounting medium with 4,6-diamidino-2-phenylindole. Cells were observed using a fluorescence microscope (Olympus, Tokyo, Japan) for green, red, and blue fluorescence, then observed using confocal laser microscopy (FV500, Olympus, Tokyo, Japan) at x400 and x1,000 magnification.

*Statistical analysis*. Each test was repeated three times. Data are presented as mean ±SD. Comparisons of the measurement data among multiple groups were performed with one-way ANOVA test. Rates were compared with Chi-square test. Results were considered statistically significant when $p<0.05$. The statistical analysis was performed with SPSS12.0 software.

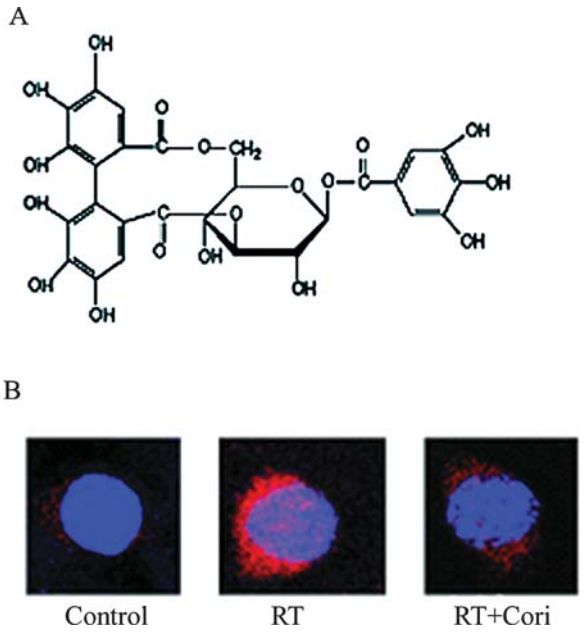


Figure 1. A. Structure of corilagin. B. Iba-1 expression in BV-2 cells monitored by immunofluorescence staining. BV-2 cell sham-irradiation showed very low expression of Iba-1 (control), whereas irradiated BV-2 cells showed significantly increased expression of Iba-1 (RT). The expression of Iba-1 was inhibited by pre-treatment with corilagin (RT+Cori).

## Results

*Effects of corilagin on in BV-2 cells*. Iba-1 is upregulated during the activation of microglial cells. Under normal conditions, Iba-1 was expressed at low levels in BV-2 cells (Fig. 1B). The expression of Iba-1 was significantly increased post-irradiation with 32 Gy, demonstrating that microglia cells are activated by irradiation. The expression of Iba-1 was inhibited by pre-treatment with corilagin, which indicated that the activation of microglial cells was repressed. As shown in Fig. 2A, the concentrations of corilagin used here (1.0 to 250.0 μg/ml) had no effect on the viability of BV-2 cells.

We investigated corilagin's effect on the expression of pro-inflammatory cytokines and mediators, including IL-1ß, TNF-α, IL-6, PIDD, and COX-2. BV-2 microglia cells were incubated with corilagin and then cells were irradiated (32 Gy). Expression of pro-inflammatory cytokines, with the exception of IL-6, was significantly decreased in cells pre-treated with corilagin compared to untreated control cells as determined by RT-PCR (Fig. 2B). IL-1ß, TNF-α, and IL-6 protein levels in cell supernatant were increased post-irradiation (Fig. 2C-E). Corilagin pre-treatment significantly inhibited the production of these cytokines ($p<0.05$). As shown in Fig. 2F, after 32-Gy irradiation, secretion of NO was significantly decreased by treatment with corilagin in comparison to untreated cells ($p<0.05$).

*Corilagin inhibits signaling through the NF-κB pathway*. Expression of p65 protein increased significantly in the nucleus after irradiation with 32 Gy at every time point (data not shown). When pre-treated with 5.0 μg/ml corilagin, the level of p65 protein in the nucleus was significantly reduced (Fig. 3) at 6 h post-irradiation. This implies that corilagin inhibited the radiation-induced NF-κB pathway activation.

*Effect of corilagin on the expression of NEMO and IκB-α*. The DSB repair kinetics in BV-2 cells was evaluated by analysing γ-H2AX foci at indicated time points (0.5-48 h) post irradiation. Non-irradiated microglia were predominantly negative for γ-H2AX; a homogeneous pattern of discrete nuclear γ-H2AX foci was observed at 0.5 h post-irradiation (Fig. 4A). BV-2 cells displayed only low levels of residual damage, as indicated by the number of foci at 48 h post-irradiation.

Immunofluorescence analysis (Fig. 4B) showed that in untreated, non-irradiated BV-2 cells, IκB-α was expressed indicating that NF-κB was inactivated. After 32-Gy irradiation, the expression of IκB-α was inhibited, which indicated that NF-κB was activated and IκB-α was dissociated and degraded. After pre-treatment with 5.0 μg/ml corilagin for 12 h, the levels of IκB-α expression were higher than in the non-irradiated cells.

Fig. 4C shows the immunofluorescence staining of NEMO in the BV-2 cells analysed 12 h after 32-Gy irradiation. Cells that were not irradiated were almost completely negative for NEMO. The degree of NEMO staining clearly increased after irradiation. The expression of NEMO was inhibited when cells were pre-treated with 5.0 μg/ml corilagin. This indicated that corilagin inhibited NEMO activation, resulting in the observed inhibition of p65 expression.

## Discussion

Microglia play a major role in pathological events leading to inflammatory-related diseases such as radiation-induced brain injury, Alzheimer's disease, Parkinson's disease, and multiple sclerosis. Recent studies indicate that irradiation induces microglia activation *in vitro*, which leads to a marked

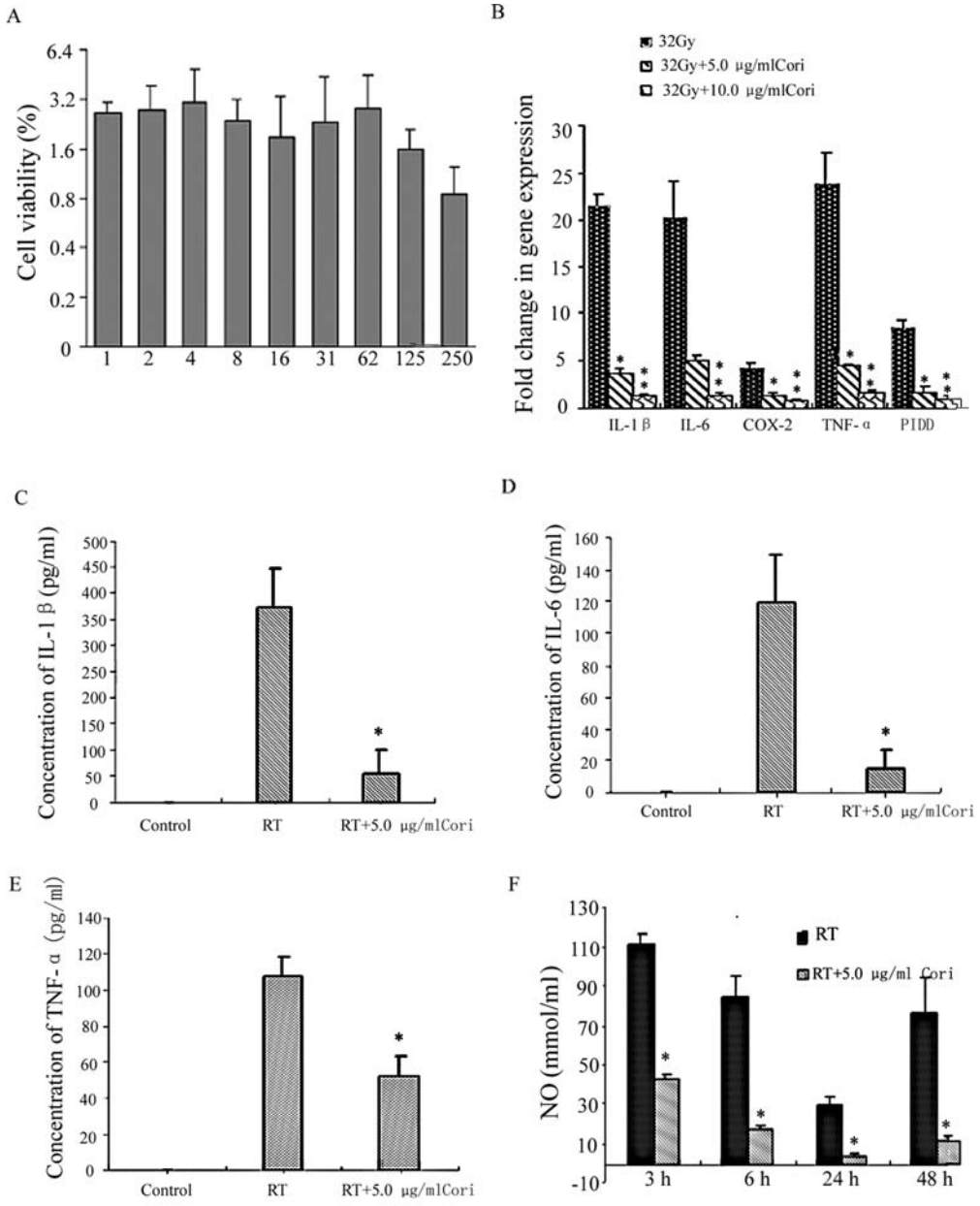


Figure 2. A. Effect of corilagin on cell viability of BV2 microglia. BV-2 cells were seeded into culture plates and treated with corilagin at the indicated concentration for 24 h, then cell viability was determined by MTT assay. Data are expressed as mean ±SD of three measurements. B. Effect of corilagin on pro-inflammatory cytokines in irradiation-stimulated microglia. Cells were pre-treated with corilagin for 12 h before 32-Gy irradiation. Expression of inflammatory cytokines was analyzed after 24 h by real-time PCR. $P<0.05$, cells pre-treated with 5.0 $\mu$g/ml corilagin vs. untreated cells. $P<0.05$, cells pre-treated with 10.0 $\mu$g/ml corilagin vs. untreated cells. Original magnification x1,000. C. Effects of corilagin on radiation-induced release of IL-1ß. D. Effects of corilagin on radiation-induced release of TNF-α. E. Effects of corilagin on radiation-induced release of IL-6. BV-2 cells were pre-treated with 5.0 $\mu$g/ml corilagin for 12 h followed by irradiation. *$P<0.05$, corilagin treated vs. untreated cells. F. Influence of corilagin on NO production. BV-2 cells were treated with 32-Gy irradiation in the presence or absence of corilagin. $P<0.05$, corilagin treated vs. untreated cells.

increase level of pro-inflammatory cytokines (1,14,15). Radiation-induced microglia activation may play a role in RIBI since pro-inflammatory cytokines inhibit neurogenesis. In our study, we evaluated the effect of the tannin family member corilagin on activated microglial cells.

Iba-1 is highly and specifically expressed in monocytic cell lines and cultured microglia. Iba1 protein levels are upregulated in activated microglia (6). Our study showed that Iba-1 expression was upregulated upon irradiation. Pre-treatment with corilagin attenuated this upregulation of Iba-1. COX-2 and NO are the key mediators and regulators of inflammation. COX-2 and NO are induced by inflammatory

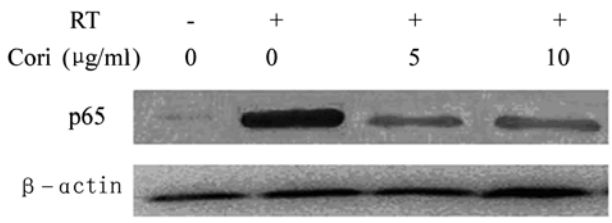


Figure 3. Effects of corilagin on radiation-induced NF-κB activation in BV-2 cells. Cells were pre-treated for 12 h with or without 5.0 $\mu$g/ml corilagin and then irradiated with 32 Gy. After 6 h, nuclear extract (NE) was isolated and analyzed. The level of p65 protein in nuclear was determined by Western blotting.

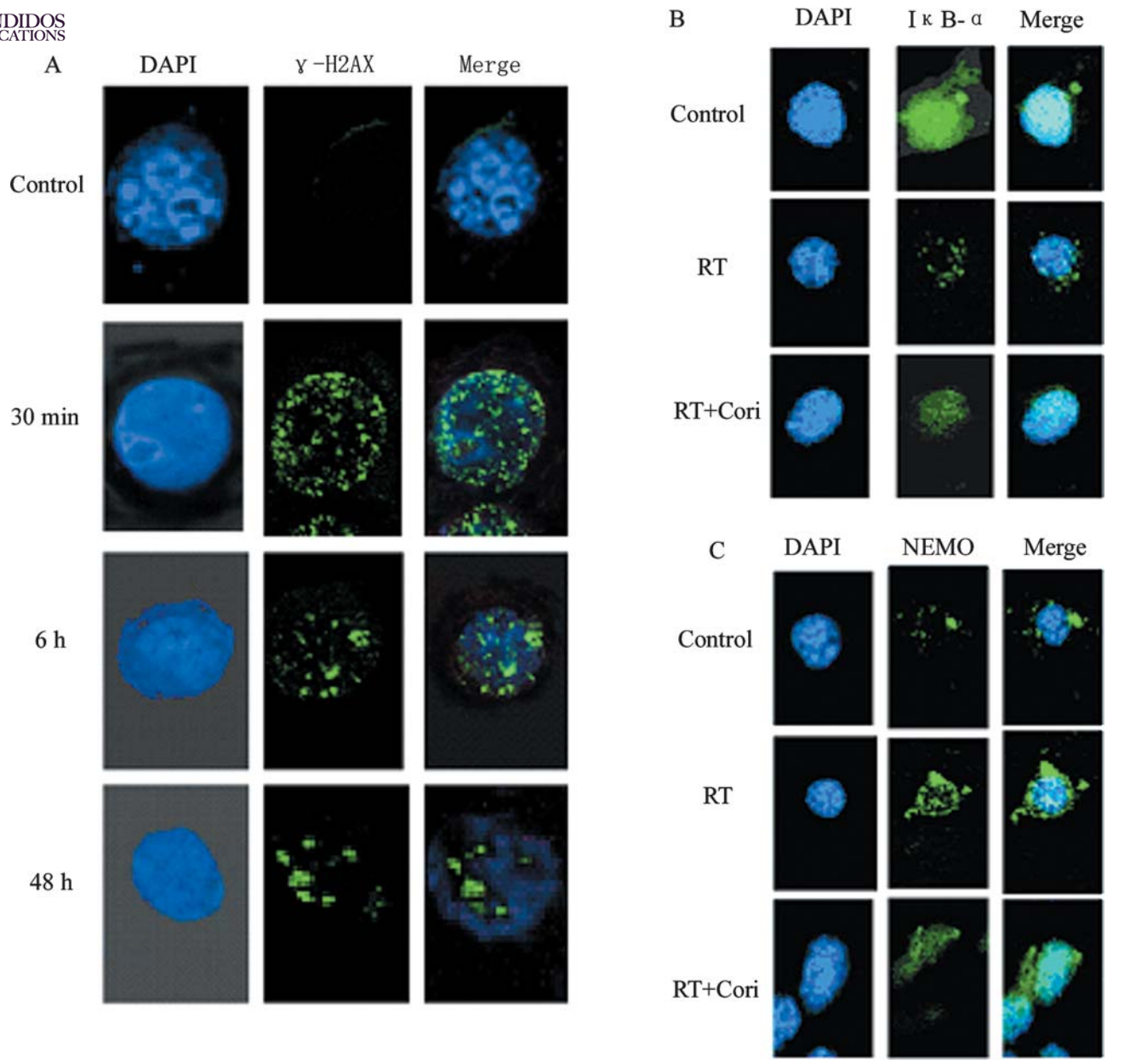


Figure 4. A. Immunofluorescence staining of γ-H2AX in BV-2 cells at 0.5, 6, and 48 h after irradiation with 32 Gy compared to non-irradiated control cells. Non-irradiated normal cells were predominantly negative for γ-H2AX, whereas a homogeneous pattern of discrete nuclear γ-H2AX foci was observed after 0.5 h in irradiated cells. DNA repair is rapid in BV-2 cells and only low levels of residual damage were observed at 48 h post-irradiation. Original magnification x1,000. B. Confocal images of IκB-α (green) immunofluorescence staining in BV-2 cells analyzed at 12 h after irradiation with 32 Gy. DNA was counterstained with DAPI (blue). Shown are control cells, irradiated cells (RT), and cells treated with 5.0 μg/ml corilagin prior to irradiation (RT+Coril). Original magnification x1,000. C. Confocal images of NEMO (green) immunofluorescence staining in BV-2 cells analyzed at 12 h after irradiation with 32 Gy. DNA was counterstained with DAPI (blue). Original magnification x1,000.

stimuli (16,17). Corilagin treatment significantly reduced the levels of these mediators in irradiated BV-2 cells. These data indicated that corilagin inhibited activation of microglial cells.

DSBs are considered the most deleterious DNA lesions. If left unrepaired, this damage severely threatens not only the integrity of the genome but also the survival of the organism. Within minutes following the induction of DSBs, the histone H2AX is phosphorylated. The phosphorylated form, called γ-H2AX, recruits DNA damage response factors to sites of DNA damage (18,19). Using a fluorescent antibody specific for the γ-H2AX, discrete nuclear foci can be visualized at sites of DSBs. Numbers of γ-H2AX foci are correlated with the extent of radiation-induced DSBs *in vitro* and *in vivo* (20,21). Irradiation induced significant DNA damage in B7-2 cells.

NF-κB is activated in response to DSBs (22-24). The NF-κB signaling pathway mediates a variety of important cellular functions by regulating immune and inflammatory responses. In unstimulated cells, NF-κB is in the form of a heterodimer of p65/p50 and binds to the inhibitor protein, IκB. After stimulation, p65/p50 is released from the IκB-α, IκB-α is degraded, and p65 translocates into the nucleus to regulate gene transcription. Central to the activation of NF-κB by stimuli is the IκB kinase (IKK) complex that is composed of IKKα/1 and IKKß/2 and NF-κB essential modulator (NEMO/IKKγ). The death-domain protein PIDD, originally

identified as an early p53-inducible gene that is implicated in p53-induced apoptosis (5), is a mediator of the DNA-damage-activated stress response (6,7). PIDD expression enhances genotoxic-stress-induced NF-κB activation through augmented sumoylation and ubiquitination of NEMO (7). Pre-treatment with corilagin prior to irradiation increased IκB-α levels and inhibited expression of NEMO when compared to levels in untreated microglial cells.

Our studies demonstrated that corilagin exhibited anti-inflammatory activity in irradiated B7-2 cells by suppressing the release of pro-inflammatory cytokines and mediators. Corilagin suppressed the transcription of pro-inflammatory cytokine genes through effects on the DSB-triggered NF-κB signaling pathway. Microglia cells play a critical role in the development of an inflammatory response within the brain. Microglia cells are activated in response to ionizing radiation, resulting in induction of pro-inflammatory cytokines. Pre-treatment of the microglia cells with corilagin inhibited pro-inflammatory responses. This compound may be used for the treatment of RIBI.

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