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Hypoxic Postconditioning Protects Astrocytes from Hypoxia/Reoxygenation Injury Through Decreasing Gap Junction Function

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Abstract: Ischemic postconditioning was induced by brief cycles of ischemia/reperfusion (I/R) at the end of ischemia scavenge brain tissues from I/R injury in several animal models. However, the relationship between ischemic postconditioning and gap junction (GJ) yet to be explored. Here, we investigated whether the beneficial effect of hypoxic postconditioning involves in decrease of GJ function via establishing the hypoxia/reoxygenation (H/R) model with astrocytes to mimic the cerebral I/R. The primary astrocytes were exposed to 8 h hypoxia/24 h reoxygenation. Hypoxic postconditioning (HPC) was induced by 3 cycles of 10 min reoxygenation/10 min hypoxia after 8 h hypoxia. Before H/R, the retinoid acid was added for 24 h, and oleamide was applied for 1 h. Parachute dye coupling assay was used to evaluate GJ function. The viability and apoptosis of astrocytes was detected by MTT, flow cytometry and Hoechst 33258 staining, respectively. Finally, the Protein expression of Cx43, Bcl-2 and Bax was tested by western blotting, while the effect of Cx43-siRNA to H/R injury and HPC was explored by Cx43-siRNA transfection. It was found that HPC attenuated the expected increase in GJ function during reperfusion increased astrocyte viability and inhibit apoptosis. Compared with H/R group, the HPC group exhibit an increased expression of Cx43 and Bcl-2 protein, but decrease in Bax. Moreover, the pretreatment with retinoid strengthened the effect of ischemic/hypoxic postconditioning, while oleamide weakened it. We attributed these effects to the inhibited gap junctional intercellular communication (GJIC) induced by HPC through inhibition of Cx43 expression on cell surface, indicating that HPC protects astrocytes from H/R injury.

Keywords: Astrocyte, Gap Junction, Hypoxia/Reoxygenation, Hypoxic Postconditioning, Apoptosis

1. Introduction

Ischemic stroke is a global health concern that leads to lifelong disability or death [1]. The principle of clinical management of Ischemic stroke is to restore blood reperfusion as soon as possible. In practice, it is difficult to restore blood supply to ischemic areas in a short period of time. The principle of clinical management of cerebral ischemic injury is to restore blood reperfusion as soon as possible. Prolonged ischemia and reperfusion may lead to irreversible tissue injury, known as ischemia-reperfusion (I/R) injury [2]. Currently, ischemic preconditioning and ischemic postconditioning are the main measures to protect organs and tissues from I/R injury. [3, 4]. A relative novel concept in contrast to preconditioning, postconditioning was first described in model of myocardial ischemia, and the protective effects of repeated mild hypoxic postconditioning (HPC) on myocardial ischemia have been confirmed by many studies [5, 6]. In this respect, the ischemic postconditioning has not only been described in cardiac ischemia but also in focal cerebral ischemia in rats and mice [7, 8]. However, the exact mechanism of the role of ischemic postconditioning in cerebral I/R injury and cerebral protection has not been fully elucidated.

Astrocytes are the main cell type in the central nervous system, which establish glial syncytia between cells through gap junctions. Almost every cellular and tissue-level process is affected by this communication pathway, including differentiation, migration, and apoptosis [9, 10]. Consistent with this, GJ regulates key processes in the pathophysiology of cellular injury and death during ischemia–reperfusion [11, 12]. In brain, the Connexin 43 (Cx43) is the major protein constituting gap junctions. Previous studies indicated that the mitochondrial Cx43 is indispensable in cardioprotection during postconditioning [13, 14]. However, the relevant mechanism between Cx43 and postconditioning-induced brain protection is unclear. Nevertheless, we believe that the beneficial effect of HPC may involve a decrease in GJ by affecting the expression of Cx43. Incontrast, the HPC can be elicit after hypoxia/reoxygenation (H/R) in vitro, to simulate ischemic postconditioning in vivo [15-17]. This reserch reveals the protective effect of HPC against H/R injury, and we used retinoid acid (RA, GJ promoter), oleamide (olea, GJ inhibitor) and siRNA targeting Cx43 to regulate GJ function to explore its involvement.

2. Materials and Methods

2.1. Materials and Primary Astrocyte Culture

DMEM/F12 (Dulbecco's Modified Eagle's Medium: Nutrient Mixture F-12 =1:1) was purchased from Gibco Service Co (USA); fetal bovine serum (FBS) from Zhejiang Tian Hang Biological Manufacture Co. Ltd. (China). Retinoid acid, oleamide, 3-(4,5-dimethylthazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and Hoechst 33258 were purchased from Sigma Chemical Co (USA); Calcein-acetoxymethyl ester was obtained from Invitrogen (USA); Annexin V-FITC/PI Apoptosis Assay kit was obtained from BD Biosciences (USA).

Astrocytes culture were prepared as previously reported [3]. Briefly, cerebral cortices from neonatal Sprague Dawley rats were isolated and carefully dissected. The tissue was dissociated by trypsin. Dissociated cells were centrifuged, resuspended in DMEM/F12 containing 10% fetal calf serum, and preplated for 30 min to minimize fibroblast contamination, then cultured at a density of 10^5 cells/mL, and maintained at 37°C and 5% -CO_2, with a change of medium after every 2-3 days. After incubation for 7-10 days, the flasks were shaken at 250 rpm for 16 h at 37°C. After removal of microglia and oligodendrocytes, the astrocytes were trypsinized, resuspended, and subcultured. The purity of the astrocytes was >98%, as assessed by immunochemical staining with glial fibrillary acidic protein (GFAP).

2.2. Cx43-siRNA Transfection

Astrocytes transfection with either Cx43 targeting siRNA or non-targeting siRNA (50 nmol/L) using Lipofectamine 2000 (Invitrogen) was performed according to the manufacturer's instructions. Then the astrocytes were divided into three groups: the non-transfected, NC-siRNA and Cx43-siRNA groups. The non-transfected group includ astrocytes without transfection with siRNA. The NC-siRNA group was astrocytes transfected with non-targeting siRNA. The Cx43-siRNA group was astrocytes transfected with targeting siRNA. The cells were subjected tonormoxia, H/R or HPCfollowing the transfection for48h.

2.3. Hypoxic Postconditioning and Experimental Protocols

Astrocytes were cultured in DMEM/F12 supplemented with 10% fetal calf serum and maintained at normal-atmospheric oxygen (with 5% CO_2) for 24 h before experimentation. A known number of astrocytes were randomly and homogeneously distributed into different experimental groups namely: (1) control group: astrocytes were kept in normoxic culture for 8 h. (2) H/R group: the DMEM/F12 was replaced with low glucose DMEM prior to hypoxia induction. Astrocytes were placed into the hypoxia chamber with 94%N_2 + 1%O_2 + 5%CO_2 for 8 h. After this phase, the low glucose DMEM was replaced with DMEM/F12, followed by 24 h in normoxic culture (for reoxygenation) [18]. (3) HPC group: astrocytes were directly exposed to hypoxia for 8 h followed by 3 cycles of brief reoxygenation (10 min) and hypoxia (10 min). Then astrocytes were incubated in normoxic culture for 24 h to induce sustained reoxygenation. (4) RA + HPC group: astrocytes were preincubated with RA (10μM) for 24 h before HPC. (5) olea + HPC group: astrocytes were preincubated with olea (25μM) for 1 h before HPC. (Figure 1).

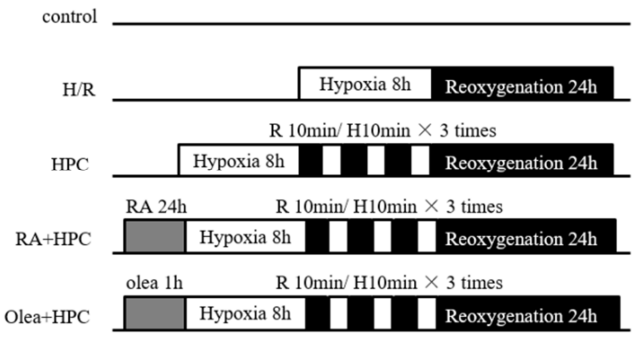


Figure 1. *Description of experimental groups.*

2.4. MTT assay

The viability of astrocytes were determined by MTT assay [19]. Briefly, astrocytes were cultured in a 96-well plate at a density of 5×10^4 cells/mL. Following each group treatment, cells were incubated with 20 μL MTT (5 mg/mL) at 37°C in culture medium for 4 h. Afterward 100 μL DMSO was added to each well at 37°C for 30 min and the absorbance was recorded by using microplate reader (iEMS, Labsystem, Turku, Finland) at 490 nm. All experiments were performed in triplicates.

2.5. Apoptosis Assay

The cellular apoptosis was detected by flow cytometry analysis [20]. The astrocytes were cultured to confluence in

12-well plate. Following each group treatment, cells were collected, washed twice with ice-cold PBS and stained with the Annexin V-FITC/PI apoptosis detection kit at room temperature for 15 min in dark. Next, the cells were analyzed by flow cytometry (Becton Dickinson, United States).

2.6. Hoechst 33258 Staining Assay

Cells were cultured in 6-well plate at a density of 2×10^5 cells/mL and incubated for 24 h. Following each group treatment, cells were fixed with paraformaldehyde for 20 min, washed with PBS twice for 5 min and incubated with Hoechst 33258 (50 ng/mL) for 30 minutes at 37°C in darkness. Next, the cells was washed with PBS for 5 min to quench extra fluoresce. Finally, the apoptotic cells were observed by fluorescence microscopy. The late apoptotic rate was calculated as the ratio of apoptotic cells to total cells counted × 100% (A minimum of 500 cells were counted for each treatment).

2.7. 'Parachute' Dye-Coupling Assay

The Parachute Dye-Coupling assay for GJ function was performed as described by Goldberg, et al [21] and Koreen, et al [22]. Briefly, the donor cells and recipient cells were grown to confluencein 12-well plates. Then the donor cells from one well were incubated with a freshly made solution of calcein-AM (5μM) in growth medium for 30 min at 37°C. The Calcein-AM converted into intracellular GJ-permeable Calcein dye Next, donor cells were trypsinized, seeded onto the recipient cells at a 1:200 (donor: receiver) and allowed to attach to the monolayer of recipient cells for 4 h at 37°C, pH 7.4 to form GJ, and examined with a fluorescence microscope. The average number of receiver cells containing calcein per donor cell was considered as a measure of the degree of GJIC.

2.8. Immunofluorescence Assay

The cells were cultured to confluence in 12-well plates, following each group treatment. The plates were fixed in 4% paraformaldehyde for 15 min at room temperature, washed with PBS twice for 5 min. Then fixed cells were incubated with phosphate buffered solution (PBS) containing 5% bovine serum albumin for 2 h and subsequently with anti-Cx43 antibody (Sigma) and FITC coupled anti-mouse IgG. The cells were then stained with DAPI (5 μg/mL) and incubated for 5 minutes at room temperature and washed with PBS. Finally, the immunofluorescence images were captured by using inverted epifluorescent microscope.

2.9. Western Blot Analysis

Cells were cultured in six-well plates at a density of 2×10^5 cells/mL and incubated for 24 h. Following each group treatment, astrocytes were washed three times with ice-cold PBS, then added lysis buffer containing protease inhibitors and incubated for 30 min in an ice bath. Next, the cell lysate was centrifuged at 12,000 rpm for 30 min at 4°C to collect the total proteins. The DC protein assay kit was used to determine Protein concentration (Bio-Rad Co., Hercules, CA, USA) in each sample. After that, 50 μg of protein was extracted from each sample and separated on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were then transferred onto PVDF membrane, which was incubated in blocking solution containing 5% skim milk powder at 37 °C for 2 h and rinsed with phosphate bu er solution containing Tween 20 (PBST) 3-times. Subsequently, immunoblotting was performed using the following antibodies: mouse anti-Cx43 IgG (1:10000), rabbit anti-Bcl-2 (1:200), rabbit anti-Bax (1:200), and mouse anti-β-actin (1:10000). The secondary antibodies used were goat anti-rabbit IgG (1:10000) and goat anti-mouse IgG (1:10000). Finally, immunopositive bands were visualized by Amersham ECL™ Plus Western Blotting Detection Kit (GE Healthcare, Piscataway, NJ, USA).

2.10. Statistical Analysis of Data

All of the experiments had a minimum of three determinations. The statistical analysis between groups was performed by unpaired student's t-test. Statistical analyses were carried out using one-way ANOVA. All results are presented as mean ± SEM. All p-values ≤0.05 were considered statistically significant. Data were analyzed with GraphPad (GraphPad, Software, San Diego, CA, USA).

3. Results

3.1. The Viability of Astrocytes Was Increased Through the Downregulation of GJ by HPC

The cultured astrocytes were first identified by positive immunofluorescence staining with cytoskeletal protein GFAP. The purity of the astrocytes was > 98% (Figure 2A).

We first examined the number of dyed cells diffusing from the donor to the recipient with "parachute" dye coupling assay to determine the relationship of GJ to postconditioning. The data showed that HPC significantly inhibitedthe diffusion of dye from donor cells to recipient cells compared with the H/R group ($p<0.01$, Figure 2B&C). We further studied the effect of GJ of HPC by using RA, olea to regulate GJ function. Compared with HPC group, the dye-coupling in RA + HPC group was increased, while decreased in olea + HPC group ($p<0.01$). Concomitantly, cell survival was assessed by MTT assay, cell survival was significantly increased in the presence of HPC compared with the H/R group ($p<0.05$, Figure 2D).

3.2. Role of GJ on Apoptosis of Astrocytes During HPC and Effects of HPC on Expression of Bcl-2, Bax and Caspase-3

The early-stage apoptosis was assessed by Annexin V-FITC/PI staining assay, and the late-stage apoptosis was assessed by Hoechst 33342 staining. The apoptosis rate of early and late-stage in HPC group was significantly lower than that in H/R group ($p<0.01$). It is suggested that the HPC inhibited astrocytes apoptosis by increasing GJ function. Additionally, compared with H/R group the number of apoptotic astrocytes identified by Annexin V-FITC ($p<0.01$,

Figure 3A&B) and Hoechst 33342 staining ($p<0.01$, Figure 3C&D) was significantly lower in HPC group. These results indicated that GJ is involved in HPC protection against H/R-induced astrocytes injury.

Bcl-2 and Bax were involved in mitochondrial apoptosis pathway, and caspase-3 is the downstream target of apoptosis signaling [23]. Therefore, we investigated the role of Bcl-2 and caspase-3 in HPC. Western blotting demonstrated that the Bax/Bcl-2 expression ratio andlevel of cleaved caspase-3 was increased significantly in the H/R group, as compared to control group ($p<0.01$, Figure 3E-H). HPC significantly inhibited H/R-induced increase of Bax/Bcl-2 expression ratio ($p<0.01$) along with cleavage of caspase-3 ($p<0.01$). Importantly, compared with HPC group, the ratio of Bax/Bcl-2 and the expression of cleaved caspase-3 were increased in the RA + HPC group ($p<0.05$) and decreased in the olea + HPC group ($p<0.05$, Figure 3E-H).

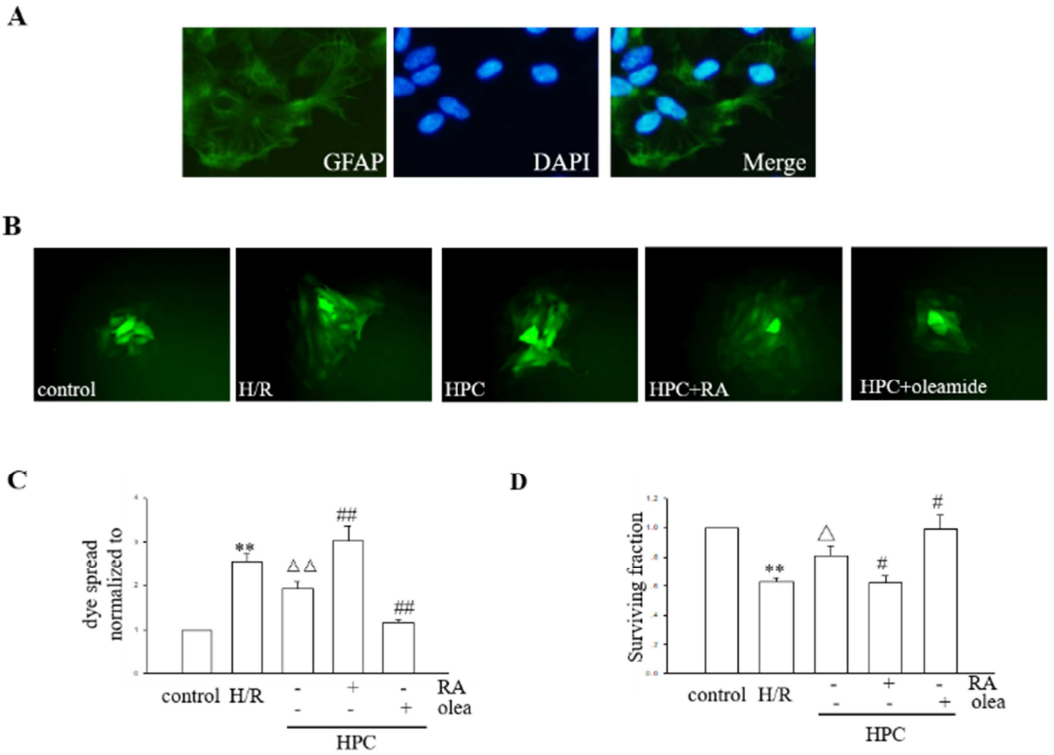
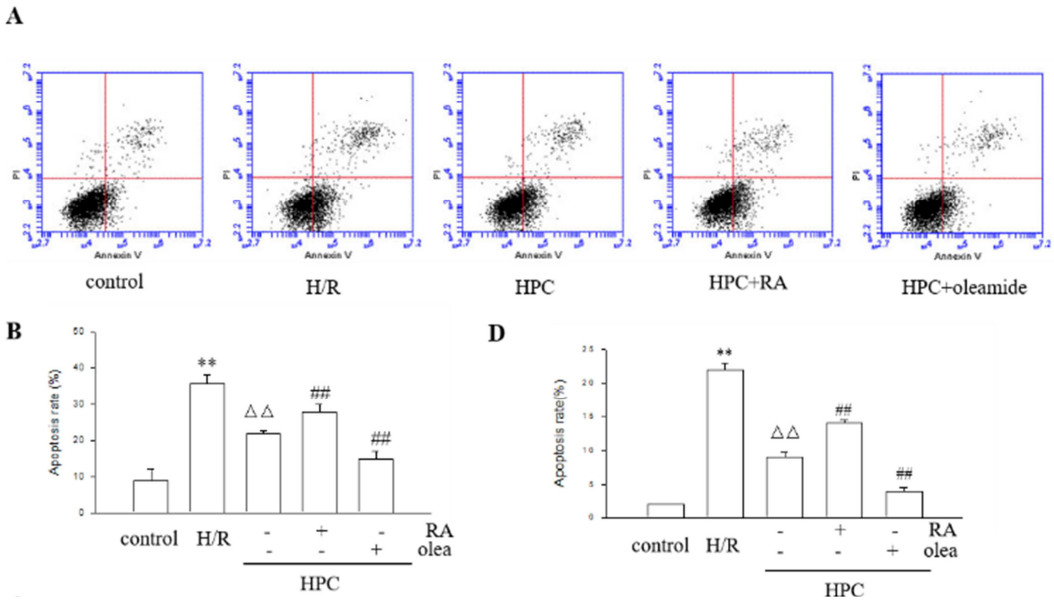


Figure 2. *The viability of astrocytes was increased through the downregulation of GJ by HPC. (A) GFAP staining of astrocytes. (B) The dye coupling through GJ in astrocytes as shown by parachute dye coupling assay (200X). (C) Relative quantity of dye spread. Bar chart presentation of the number of dye receiver cells shown in A. Bars are means ± SEM., n=3, **p<0.01, vs H/R group and HPC group. (D) Cell viability was detected by MTT assay. Bars are means ± SEM. n=4. **p<0.01, *p<0.05 vs control group; $^{\triangle}$p<0.05 vs H/R group; $^{\#}$p<0.05 vs HPC group.*



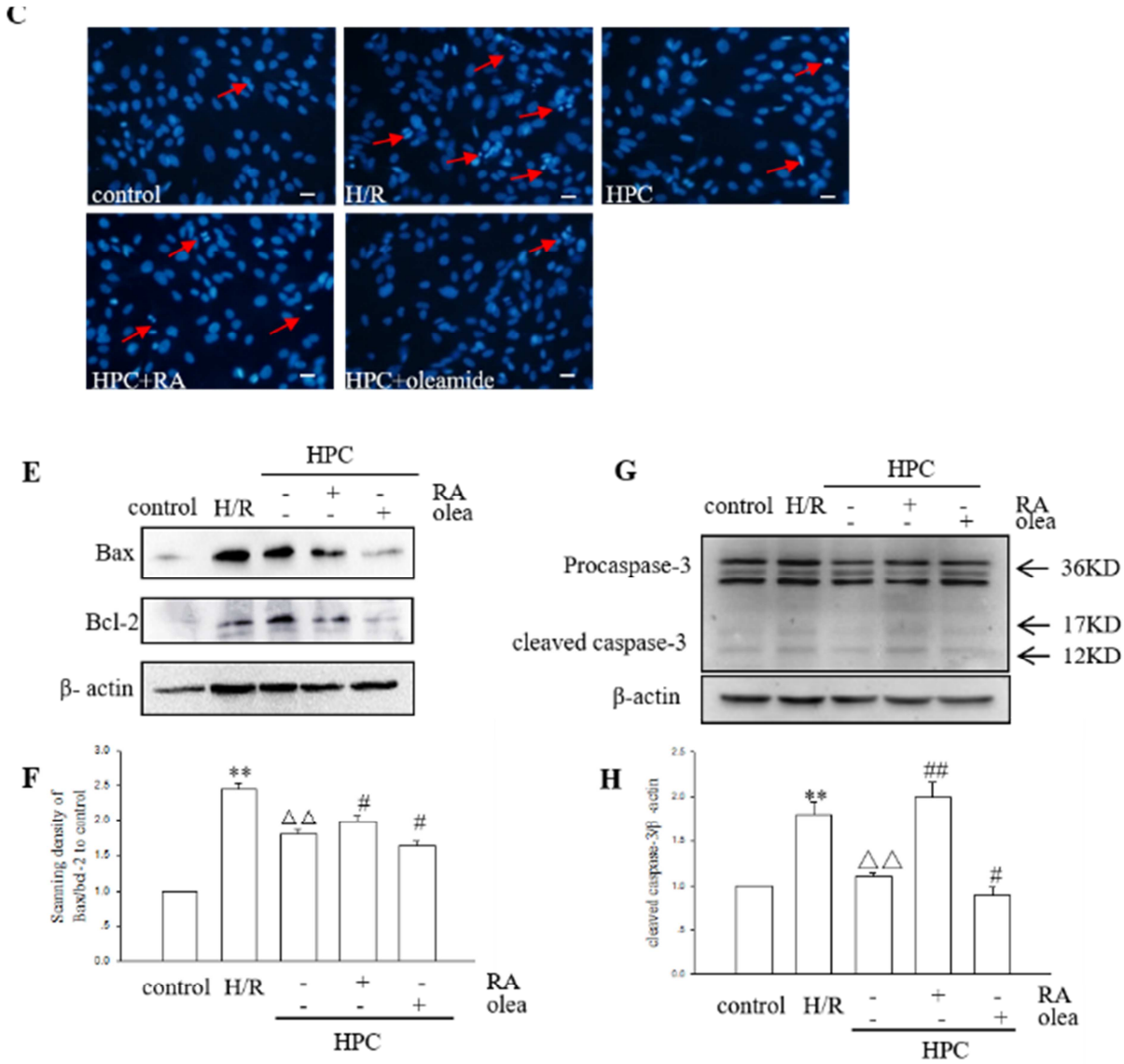


Figure 3. *Role of GJ on apoptosis of astrocytes during HPC and effects of HPC on expression of Bcl-2, Bax and caspase-3. (A) Distribution of viable, apoptotic, and necrotic astrocytes measured by the Annexin V/PI double staining method using flow cytometry. (B) Relative quantity of early-stage apoptosis ratio. Bars are means ± SEM. n=3, **p<0.01, vs control group. ##p<0.01, vs HPC group. ΔΔp<0.01, vs H/R group. (C) Late-stage apoptosis induced by HPC was shown by Hoechst 33258 staining assay (magnification, x200). (D) Relative quantity of late-stage apoptosis ratio. Bars are means ± SEM. n=3. **p<0.01 vs control group; ΔΔp<0.01 vs H/R group; ##p<0.01 vs HPC group. The arrows indicate apoptotic cells. (E) Representative bands of Bcl-2 and Bax. (F) Relative quantity of Bcl-2 and Bax. (G) Representative bands of cleaved caspase-3. (H) Relative quantity of cleaved caspase-3. Bars are means ± SEM. n=3, **p<0.01, vs control group. ##p<0.01, #p<0.05, vs HPC group. ΔΔ p<0.01, vs H/R group.*

3.3. The H/R-Induced Apoptosis Was Reduced Through the Downregulation of Cx43 by HPC

HPC reduced the ratio of Bax/Bcl-2 and the expression of cleaved caspase-3 in H/R-induced apoptosis. HPC can reduce H/r-induced apoptosis by downregulating GJ channels. Cx43 is the major gap junction protein expressed by astrocytes, responsible to form functional gap junctions, so Cx43 could play an important role in regulating gap junction communication [24]. Here, we investigated the role of Cx43 in HPC by effecting the function of GJ with the RA or olea, and investigating the effect of Cx43 on astrocytes by reducing the expression of Cx43 by siRNA, experimental groups are shown in Figure 5A. Compared with H/R, pretreatment with RA or olea increased and reduced the expression of endogenous Cx43 ($p<0.05$, Figure 4A&B) and green fluorescence of Cx43 protein on cell surface (Figure 4C), respectively. Compared with control, expression of endogenous Cx43 is significantly reduced after transfection of astrocytes with Cx43-siRNA ($p<0.01$, Figure 5B&C). Similarly, compared with the control group and the NC-siRNA group, the fluorescence intensity of Cx43 protein on the cell surface (Figure 5D) was also reduced significantly, confirmed that the expression of Cx43 protein on cell membrane was decreased ($p<0.01$).

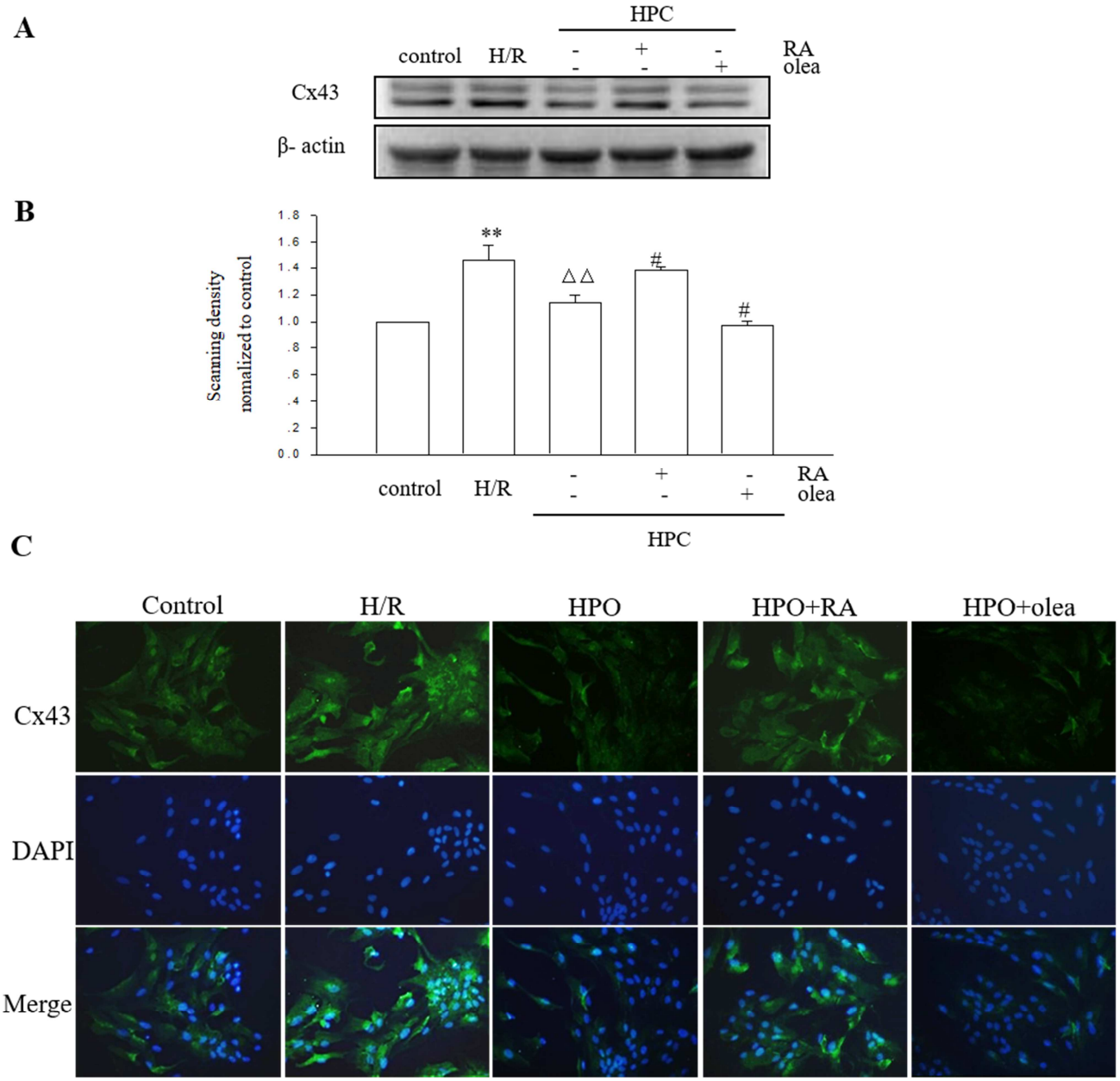


Figure 4. *Effect of HPC on the expression of Cx43 in astrocytes. (A) The expression of Cx43 determined by western blotting. (B) Relative quantity of Cx43 expression. Bars are means±SEM. n=3. **p<0.01, vs control group. #p<0.05, vs HPC group.△△ p<0.01, vs H/R group. (C) Immunofluorescence assay was used to detect the expression of Cx43 on the membrane (200X).*

In line with this, the Parachute dye coupling assay was used to evaluate GJ function. The data in Figure 5E&F showed that HPC markedly inhibit the dye spread from donor cells to receiver cells as compared to the H/R group. Moreover, compared with HPC group, the dye-coupling of the Cx43-siRNA group was notably decreased.

Meanwhile, the number of apoptotic astrocytes identified by MTT, Annexin V-FITC and Hoechst 33342 staining in H/R group was significantly lower than that in Cx43-siRNA + HPC group ($p<0.01$, Figure 6A-E), implied that HPC down-regulates the GJ channel composed of Cx43, which can reduce H/R-induced apoptosis.

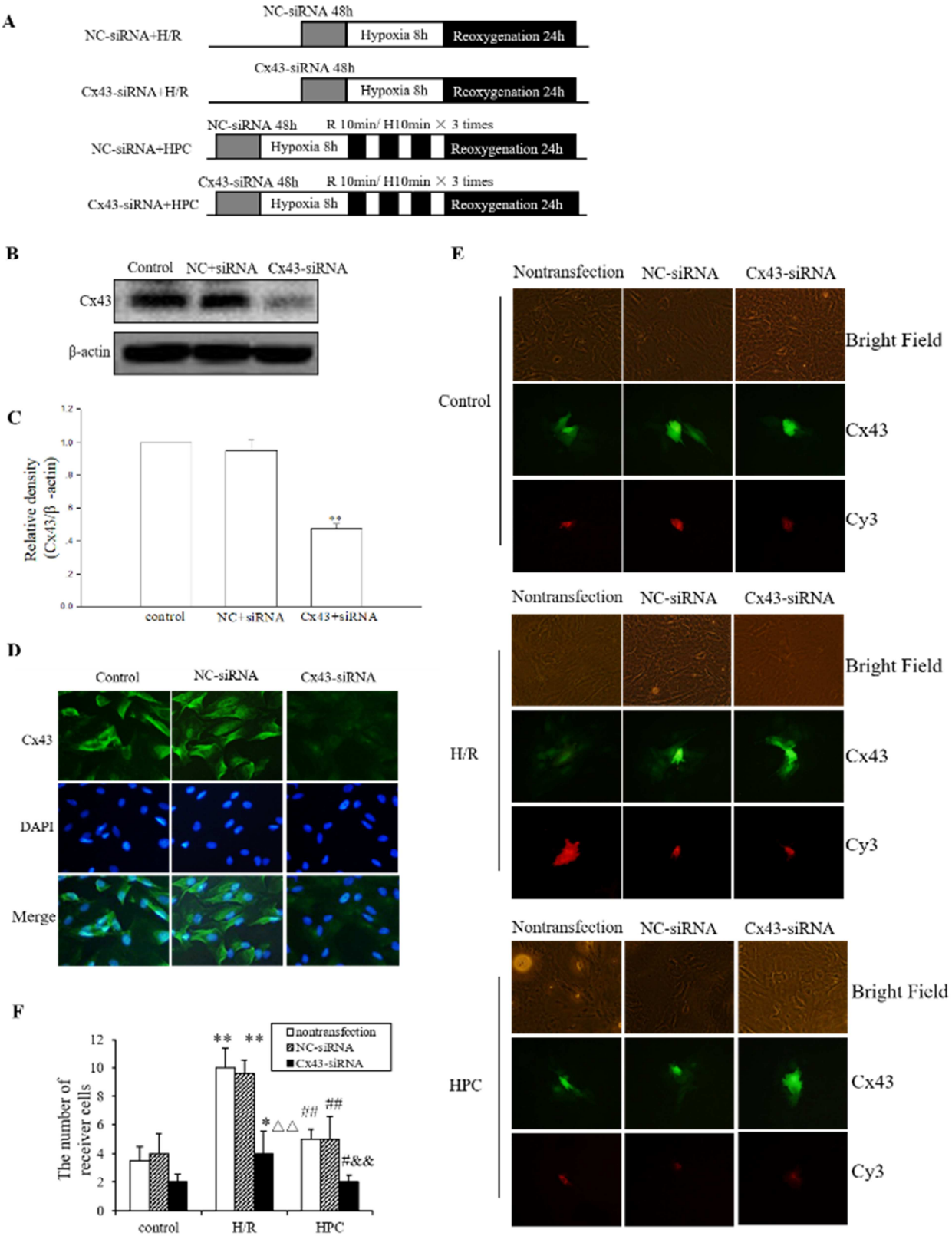


Figure 5. *Effect of Cx43 knockdown in H/R and HPC. (A) Description of Cx43 knockdown experimental groups. (B) Western blot was performed for the detection of Cx43 knockdown. (C) Relative quantity of Cx43 expression. Bars are means±SEM. n=3, ** p<0.01, vs control group. (D) The expression of Cx43 located on the membrane of astrocytes treated with Cx43-siRNA (200x), n=3, the scale bars represent 50 μm. (E) The dye coupling through GJ in astrocytes as shown by parachute dye coupling assay (magnification, x200). (F) Relative quantity of dye spread. Bars are means ± SEM. n=3, **p<0.01, ##p<0.01; ΔΔp< 0.01, vsnontransfection H/R group; &&p< 0.01, vsnontransfection HPC group, the scale bars represent 50 μm.*

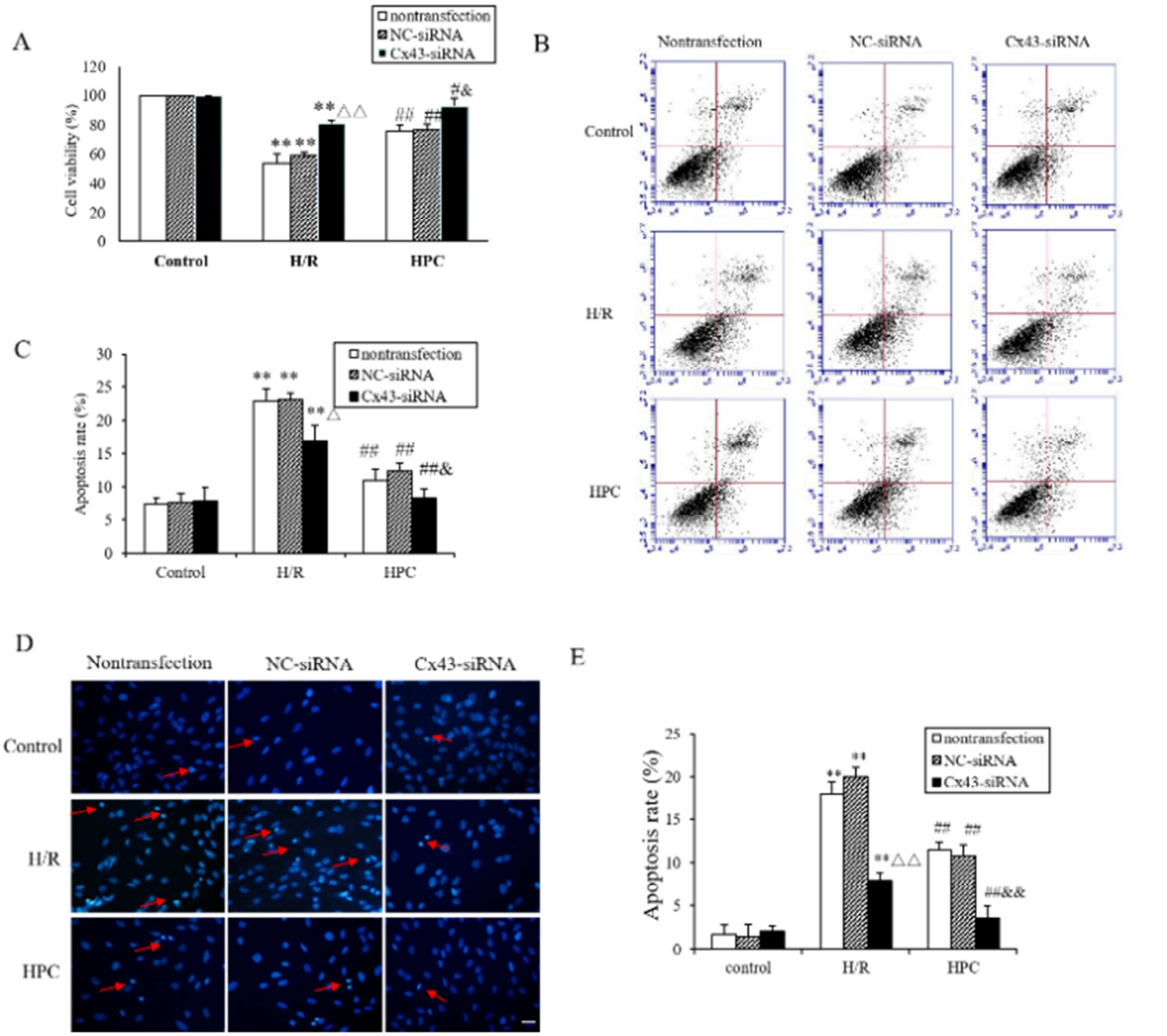


Figure 6. *Effect of Cx43 knockdown on viability and apoptosis of astrocytes during HPC. (A) Cell viability detected by MTT assay. Bars are means±SEM. n=4. **p<0.01 vs control group; ##p<0.01, #p<0.05, vs H/R group; ΔΔp< 0.01, vsnontransfection H/R group; &p< 0.05, vsnontransfection HPC group. (B) Distribution of viable, apoptotic, and necrotic astrocytes measured by the Annexin V/PI double staining method using flow cytometry. (C) Relative quantity of early-stage apoptosis ratio. Bars are means±SEM. n=4. **p<0.01, vs control group; ##p<0.01, vs H/R group; Δp<0.05, vsnontransfection H/R group; &p<0.05, vsnontransfection HPC group. (D) Late-stage apoptosis induced by HPC was shown by Hoechst 33258 staining assay (200X). (E) Relative quantity of late-stage apoptosis ratio. Bars are means±SEM. n=3, **p<0.01, vs control group. ##p<0.01, vs HPC group. ΔΔp<0.01, vs H/R group, the scale bars represent 50 μm. The arrows indicate apoptotic cells.*

4. Discussion

In 2006, Zhao H *et al* [25] first introduced the concept of ischemic postconditioning in which brief intermittent repetitive interruptions to reperfusion after a prolonged period of focal ischemia significantly reduced infarct size in rats. It is well known that reperfusion injury is a complex process involved various mechanisms and postconditioning can attenuate the multiple manifestations of reperfusion injury by endogenous mechanisms. Although the neuroprotective mechanism of HPC has not been completely understood, we demonstrated that GJ channels composed of Cx43 may be involved in HPC protection resulted in the reduction of astrocyte apoptosis and necrosis via regulation of Bcl-2, Bax, caspase-3.

We showed that HPC instigate viability and the reduction of apoptosis in astrocytes that subjected to H/R. These data indicated thatpostconditioning has a protective effect on I/R injury [26, 27]. Both H/R and I/R injury can cause cell death, while, apoptosis is the main mechanism of cell death, and mediated by a relative balance of pro- and anti-apoptotic signaling pathways, determine cellular fate (Figure 7). In this respect, Bcl-2 and Bax were involved in mitochondrial apoptosis pathway, and caspase-3 is the downstream target of apoptosis signaling [28-30]. Moreover, Bcl-2 can prevent

apoptosis by interfering with caspase-3 signaling cascade [31]. In addition, Bax binds and antagonizes Bcl-2 protein. The ratios of pro- to anti- apoptotic molecules, such as Bax/Bcl-2, determine response to death signal. In this study, we evaluatedthe expression of Bcl-2 family members following the treatment of HPC. Furthermore we identified that HPC inhibit apoptosis by decreasing the ratio of Bax/Bcl-2 and the expression of cleaved caspase-3, could be responsible for protective effect of HPC in H/R injury. These results suggested that (i) HPC protected the astrocytes against H/R-induced apoptosis by increasing Bcl-2 and decreasing Bax, caspase-3 levels. (ii) RA treatment inhibited the antiapoptotic effects of HPC by decreasing Bcl-2 and increasing Bax, caspase-3 levels, and (iii) olea treatment promoted the antiapoptotic effects of HPC by increasing Bcl-2 and decreasing Bax, caspase-3 levels. Furthermore, during cell apoptosis, some neurotoxic metabolites can be transmitted to neighboring cells to aggravate tissue damage [32, 33]. Zhang L *et al* confirmed that blocking gap junction with CBX can attenuate brain injury after cerebral I/R by partially contributing to amelioration of apoptosis [34], infered that inhibiting the GJ function may attenuate brain injury after astrocytes H/R.

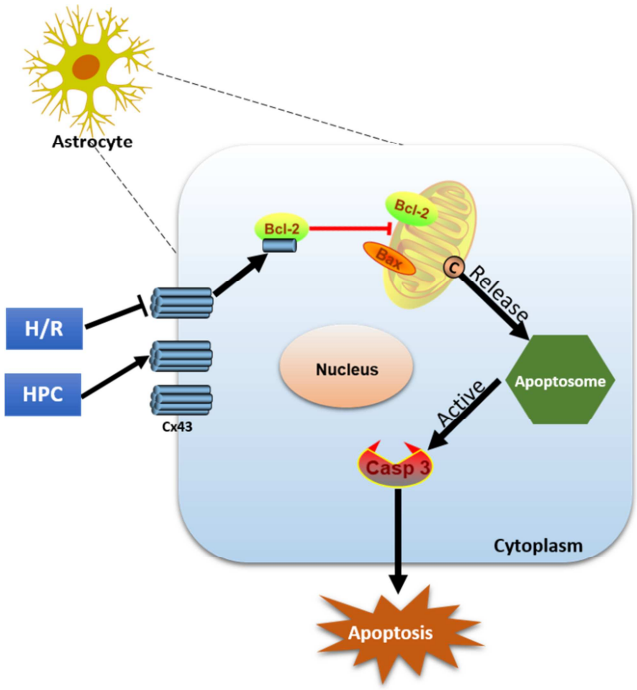


Figure 7. *Signal transduction of apoptosis in H/R and HPC.*

To investigate the relationship between the protective effect of postconditiong and the GJ function, we used RA for 24 h and olea for 1 h before HPC. After using RA or olea before HPC, the Cx43-formed GJ was increased and decreased compared with postconditioning alone, meanwhile, the apoptosis of astrocytes was also increasd and decreased respectively. GJ channels formed by connexin (Cx), Cx43 is thought to be the main functional connexin in cultured astrocytes [35]. Thus, the Cx43 expression could affect the function of GJ. From our findings, the Cx43 expression of plasma membrane was decreased after postconditioning compared with H/R, and then the GJ function was significantly increased after H/R, while, HPC markedly inhibited the H/R-induced increase in GJ function. Furthermore, knockdown of Cx43 canceled the protective effect induced by HPC. Therefore, HPC may protect astrocytes from hypoxia/reoxygenation injury through decreasing the GJ function, and protective effect of HPC could be reversed by regulating the GJ function.

Collectively, our data represent that anti-apoptotic effect and GJ are the central aspects of HPC protection. The protective kinases may play an important role in cellular mechanisms and reduce some pro-apoptotic elements. In particular, decreasing the GJ function may be associated with protective mechanisms by inhibiting the transfer of “death factor”. Thus, the H/R-induced injury could be limited by HPC.

5. Conclusions

In summary, our results demonstrated that HPC reduced H/R-induced apoptosis through downregulating the ratio of Bax/Bcl-2 and expression ofcleaved caspase-3. HPCblocked GJIC through inhibition of Cx43 expression on cell surface. These findings highlight HPC as a potential therapeutic technique specifically for hypoxia/reoxygenation injury. Manipulating astrocytic networks by pharmacologically targeting GJ channels may provide a novel strategy for therapeutic intervention.

Data Availability Statement

The datasets generated for this study are available on request to the corresponding author.

Ethics Statement

The animal study was reviewed and approved by Ethical Committee of Bengbu Medical College.

Author Contributions

SD and XT designed the research. YG performed the research. ZL and YS analyzed the data. ZL wrote the article, and all authors had access to the study data, and have reviewed and approved the final manuscript.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Footnotes

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