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Spontaneous running wheel improves neuroprotection efficacy of ischemic postconditioning in mice following ischemia/reperfusion injury

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Key words: Ischemic postconditioning, ischemia/reperfusion, running wheel, apoptosis, PI3K, STAT3

Abstract: Ischemic postconditioning (IP) has been shown to provide protection for ischemia/reperfusion (IR) injury, but its efficacy is limited. In this study we hypothesized that spontaneous running wheel (RW) could improve neuroprotection efficacy of IP for IR. We established mouse models of IR and showed that compared to Sham group, IR group had obvious brain infract and neurological dysfunction. In IR+IP group, brain infract and neurological dysfunction improved compared to IR group. However, in IR+IP+RW group brain infract and neurological dysfunction improved much better. TUNEL assay showed that IP but not RW significantly reduced the number of apoptotic cells after IR. However, the number of apoptotic cells was significantly reduced in RW+IP group. In addition, the levels of pro-apoptotic factors increased in IR group but significantly reduced in IR+IP+RW group, while the levels of anti-apoptotic factors decreased in IR group but significantly increased in IR+IP+RW group. Moreover, in IR+IP+RW group, MDA level was further decreased and SOD level was further increased compared to IR+IP group. Finally, both PI3K inhibitor and STAT3 inhibitor significantly worsened brain infract and neurological dysfunction and promoted apoptosis in IR mice. In conclusion, RW combined with IP reduces brain infract and neurological dysfunction in mice after IR, and this is associated with enhanced anti-apoptotic and anti-oxidant benefits via the activation of PI3K and STAT3 pathways.

Introduction

Ischemic brain injuries resulting from global or focal decreases in perfusion are the most common and important causes of the disability and even the death in the world (Dirnagl *et al.*, 2009). The transient and sublethal ischemia in the brain would induce a form of endogenous protection called ischemic conditioning (Dezfulian *et al.*, 2013). Ischemic conditioning involves intended application of non-injurious physiologic or pharmacologic stimuli before or after stroke (named pre- and post-conditioning, respectively) with the purpose of triggering adaptive changes in the brain to become transiently resistant to ischemic injuries (Gidday, 2015). Ischemic preconditioning has limitations because it has to be executed before the onset of stroke. In contrast, ischemic postconditioning (IP) has advantages and could provide protection against ischemia and reperfusion (I/R) injury after stroke (Zhao *et al.*, 2006).

Many studies have shown that spontaneous exercise is beneficial to brain injury by improving neuronal plasticity and cognitive function and promoting neural repair as well as behavioral rehabilitation (Griesbach *et al.*, 2009; Itoh *et al.*, 2011). Our previous study reported that spontaneous running wheel (RW) improved cognitive functions of mouse following traumatic brain injury (Bao *et al.*, 2014). Thus we hypothesized that RW could improve neuroprotection efficacy of IP for IR. In this study we established mouse models of IR and divided them into different groups subject to RW alone, IP alone or both RW and IP. We compared the efficacy of RW and IP alone or in combination on neuroprotection after IR and investigated the underlying mechanism.

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Material and Methods

Animals

All animal experiments were performed according to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (Bethesda, MD, USA). Adult male C57BL/6J mice (4-4.5 month old) were purchased from Kunming Medical University, and housed with ad libitum access to food and water in a 12-h light/dark cycle. The mice were allowed to acclimatize to the environment for 1 week and then randomly divided into five groups (7 mice in each group) as shown in Fig. 1: 1. animals that received sham operation (sham); 2. animals that received IR by transient middle cerebral artery occlusion (MCAO). Briefly, the left common carotid artery was exposed and the external carotid artery (ECA) was dissected distally. Then internal carotid artery (ICA) was isolated and a blunted suture was introduced into the ECA and then gently advanced into the ICA to block MCA blood flow. After 60 min of MCAO, cerebral blood flow was restored by suture withdrawal and the incision was closed; 3. animals that received IR+IP. After 60 min of MCAO, reperfusion was established for 30 second, after which the MCA was occluded again for 30 sec, and the procedures repeated for six cycles; 4. animals that received IR+RW. Animals received RW for 2 weeks before MCAO in the cages equipped with RW (diameter=12 cm, width=5 cm; Nalge Nunc International, Rochester, NY, USA) used for the voluntary exercise rotated freely and attached to a receiver that monitored the number of revolutions (Vital Viewer Data Acquisition System software, Mini Mitter, Sunriver, OR, USA); 5. animals that received IR+IP+RW. Animals received RW for 2 weeks before MCAO and IP. In addition, animals received pretreatment with PI3K inhibitor LY294002 and STAT3 inhibitor SH454 (all from Selleck, Houston, TX, USA) and were named IR+IP+RW-PI3K group and IR+IP+RW-STAT3 group, respectively.

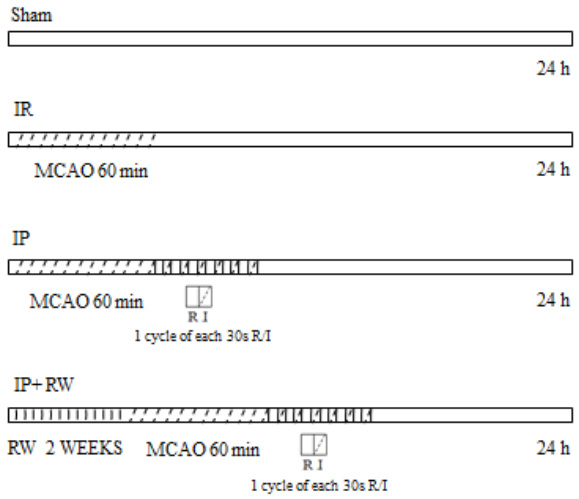

FIGURE 1. Schematic presentation of the treatment protocols.

Behavior tests

A MWM paradigm was employed to assess spatial learning of the mice and neurological score was calculated as described previously (Loane *et al.*, 2009; Gurusamy & Subramaniam, 2017).

Measurement of infarct volume

Mice from each groups were killed by cervical dislocation and decapitated. The brain was rapidly removed and infarct volume was measured as described previously (Tsubokawa *et al.*, 2007). Briefly, brains were cut into 2 mm thick coronal sections, stained with 2% 2,3,5-triphenyltetrazolium chloride (Sigma) at 37°C for 30 min, and fixed in 10% formalin overnight. The infarcted tissue was unstained as white while normal tissue was stained red. The infarct zone was analyzed by Image J software and infarct volume was calculated.

Histologic analysis

The brain was embedded in paraffin and paraffin-embedded tissues were cut into 5 μm sections. The sections were stained by using in situ terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling (TUNEL) cell death detection kit (Roche, Basel, Switzerland) following the manufacturer's instructions. TUNEL-positive (apoptotic) cells were stained brown.

Detection of malondialdehyde (MDA) and superoxide dismutase (SOD) levels

Brain tissues were homogenized. MDA and SOD levels in the homogenized tissues were measured using commercially available kits (Nanjing Jiancheng Bioengineering Institute, China) following the manufacturer's instructions.

Western blot analysis

Brain tissues were homogenized with a homogenizer in 5 volumes of buffer (20 mmol/L HEPES, 1.5 mmol/L MgCl2, 10 mmol/L KCl, 1 mmol/L EDTA,1 mmol/L EGTA, 250 mmol/L sucrose) supplemented with proteinase inhibitor cocktail. The samples were centrifuged at 750 *g* at 4°C for 15 min. The supernatant was collected which contained cytosolic/mitochondrial proteins, and further centrifuged at 16,000 *g* at 4°C for 30 min. The supernatant was used as cytosolic fraction and the pellet was used as mitochondrial fraction. The protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were blocked with 5% nonfat dry milk and incubated with primary antibodies (Abcam Inc) at 4°C overnight. The membranes were then washed and incubated with secondary antibodies (Cell Signaling, Danvers, MA, USA) for 1 h at room temperature. The membrane was developed using ECL kit (Pierce, Rockford, IL, USA) and exposed to X-ray film for quantitative analysis by Image +5.1 software.

Statistical analysis

All data were presented as mean±SE and analyzed by SPSS 14.0 software. The comparison was analyzed by ANOVA and post hoc paired t test if necessary. $P<0.05$ indicated significant difference.

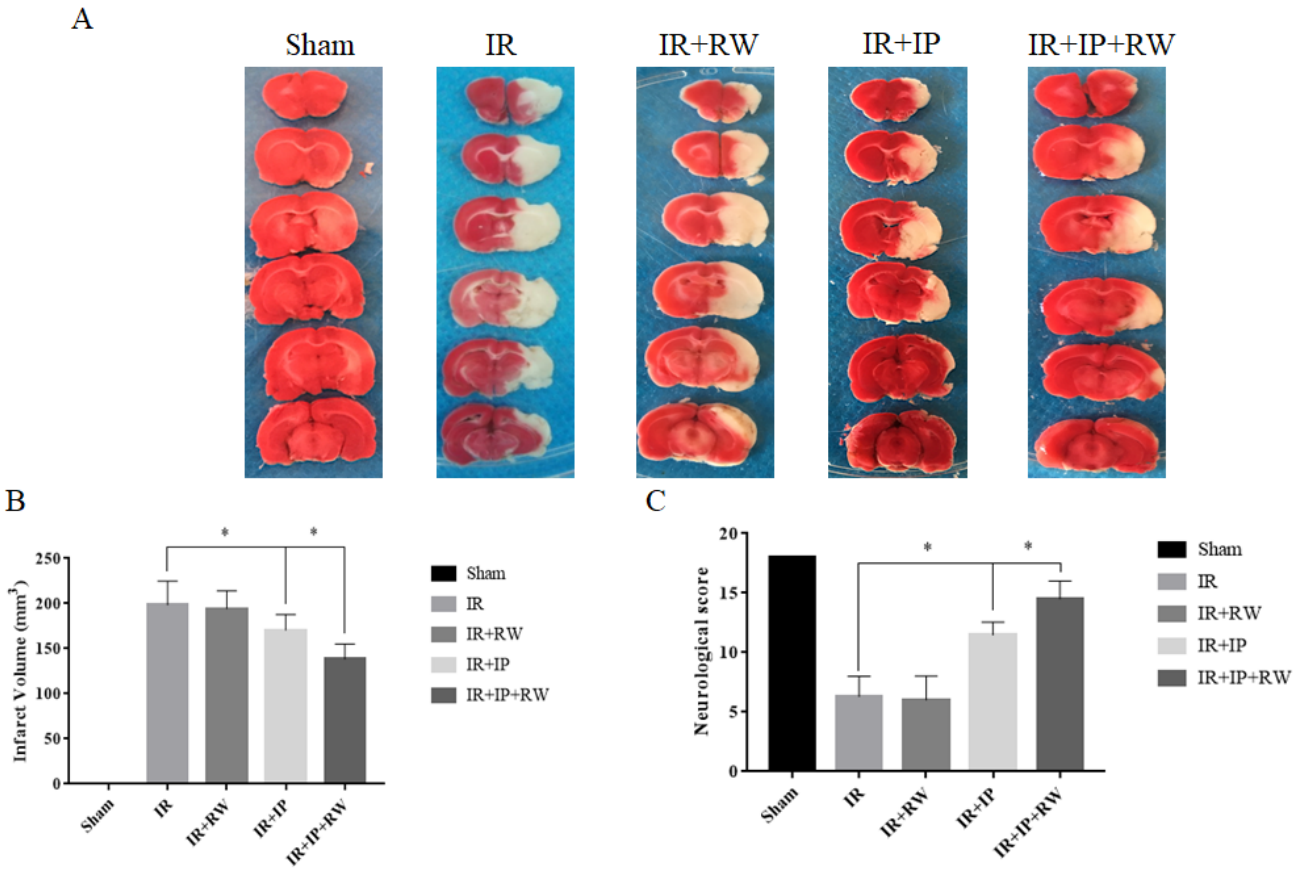

Figure 2. RW enhanced the benefits of IP on brain infract and neurological function in IR mice. A. Representative triphenyltetrazolium chloride staining of coronal sections in each group. B. Quantification of infarct volumes in each group. C. Quantification of neurologic score in each group. Data represent means±SD (n=6). *P<0.05.

Results

RW enhanced the benefits of IP on brain infract and neurological dysfunction in mice after IR

Compared to Sham group, IR group showed obvious brain infract and neurological dysfunction. In IR+IP group, brain infract and neurological dysfunction improved compared to IR group. However, in IR+IP+RW group, brain infract and neurological dysfunction improved much better (Figs. 2A-C). These data suggest that RW enhanced the benefits of IP on brain infract and neurological dysfunction in mice after IR.

RW enhanced the effects of IP to inhibit apoptosis in mice brain after IR

TUNEL assay showed that the number of apoptotic cells in the brain was the highest in IR group. IP but not RW significantly reduced the number of apoptotic cells after IR. However, the number of apoptotic cells was significantly reduced in RW+IP group (Fig. 3A). In addition, we performed Western blot analysis to detect the levels of apoptosis related factors in brain tissues of each group. The results showed that the levels of pro-apoptotic factors increased in IR group but significantly reduced in IR+IP+RW group, while the levels of anti-apoptotic factors decreased in IR group but significantly increased in IR+IP+RW group (Fig. 3B). Taken together, these data indicate that RW enhanced the anti-apoptotic effects of IP in the brain of mice after IR.

RW enhanced the anti-oxidant benefits of IP in mice brain after IR

Compared to Sham group, the levels of MDA was much higher in IR group. In IR+IP group, MDA level was significantly lower than in IR group. Moreover, in IR+IP+RW group, MDA level was further decreased (Fig. 4A). In contrast, compared to Sham group, the levels of SOD was much lower in IR group. In IR+IP group, SOD level was significantly higher than in IR group. Moreover, in IR+IP+RW group, SOD level was further increased (Fig. 4B). These results demonstrate that RW enhanced the anti-oxidant benefits of IP in mice brain after IR.

RW activated PI3K/mTOR and STAT3 pathways in mice brain after IR

IP is known to activate PI3K/mTOR and STAT3 (signal transducer and activator of transcription 3) pathways to confer cardioprotection (Xue *et al.*, 2016). To understand the mechanisms by which RW enhances the benefits of IP on neuroprotection, we detected the levels of PI3K, mTOR and STAT3 in brain tissues. We found that RW increased protein levels of PI3K, mTOR and STAT3 (Fig. 5A).

To confirm that PI3K and STAT3 pathways mediate the benefits of RW, we treated mice with PI3k and STAT3 inhibitors. While brain infract and neurological dysfunction improved in IR+IP+RW group compared to IR+IP group, both PI3K inhibitor and STAT3 inhibitor significantly

worsened brain infract and neurological dysfunction (Figs. 5B-D). These data suggest that RW enhanced the benefits of IP on brain infract and neurological dysfunction in mice after IR via the activation of PI3K and STAT3 pathways.

RW Enhanced Anti-Apoptotic Effects of IP in Mice Brain After IR via the Activation of PI3K and STAT3 Pathways

TUNEL assay showed that while RW significantly reduced the number of apoptotic cells after IR, the number of apoptotic cells was significantly increased if PI3K and STAT3 were inhibited by specific inhibiters (Fig. 6A). In addition, Western blot analysis showed that the levels of pro-apoptotic factors significantly decreased in IR+IP+RW group compared to IR+IP group, but their levels significantly increased if PI3K and STAT3 were inhibited by specific inhibiters. In contrast, the levels of anti-apoptotic factors significantly increased in IR+IP+RW group compared to IR+IP group, but their levels significantly decreased if PI3K and STAT3 were inhibited by specific inhibiters (Fig. 6B). Taken together, these data indicate that RW activated PI3K and STAT3 pathways to enhance anti-apoptotic effects of IP in the brain of mice after IR.

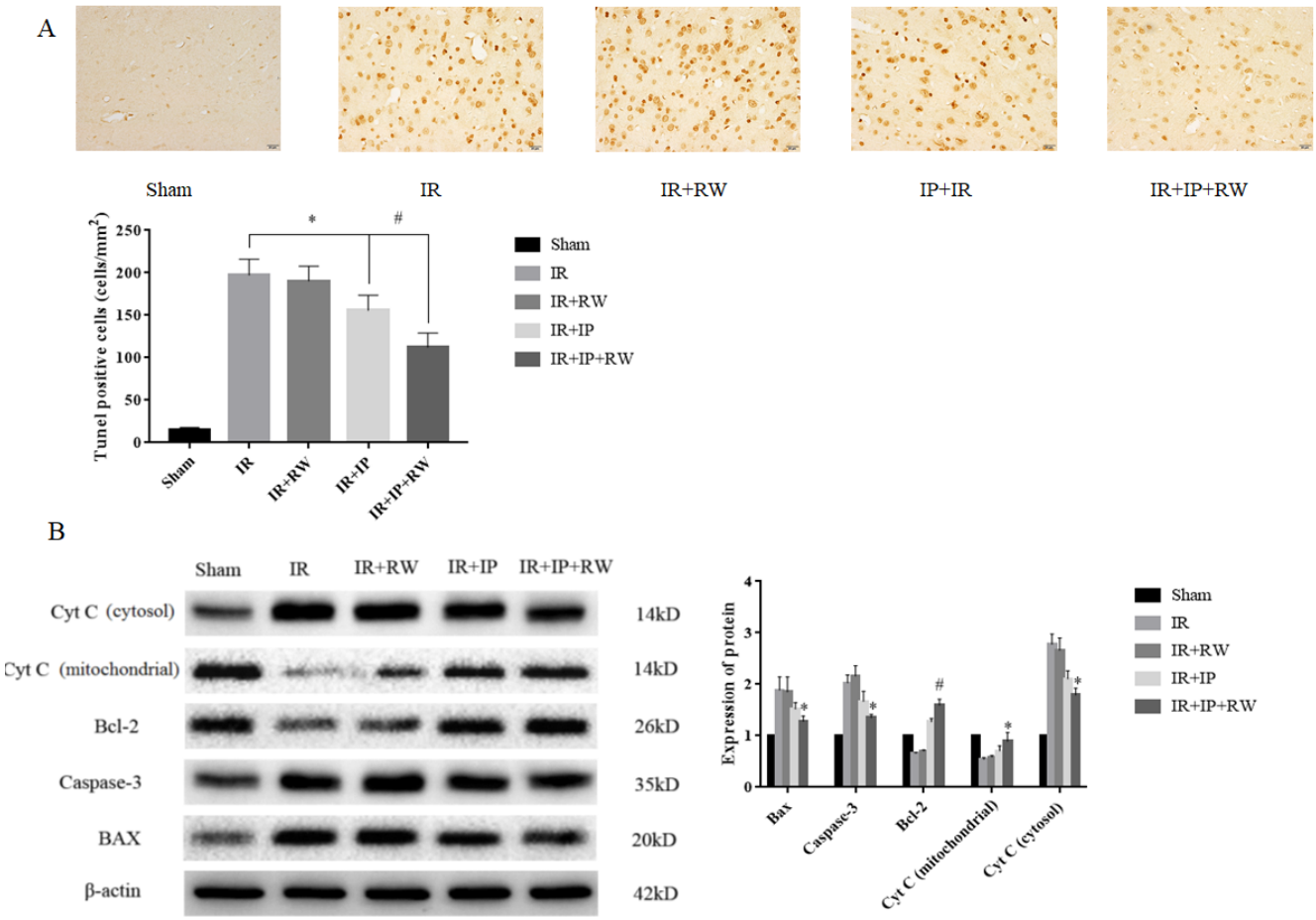

FIGURE 3. RW enhanced anti-apoptosis benefits of IP in mice brain after IR. A. Representative TUNEL staining of coronal sections in each group, and the quantification of TUNEL positive cells. B. Western blot analysis of apoptosis related factors in brain tissues of each group. Data represent means±SD (n=6). **P*<0.05, #*P*<0.01 compared to IR group.

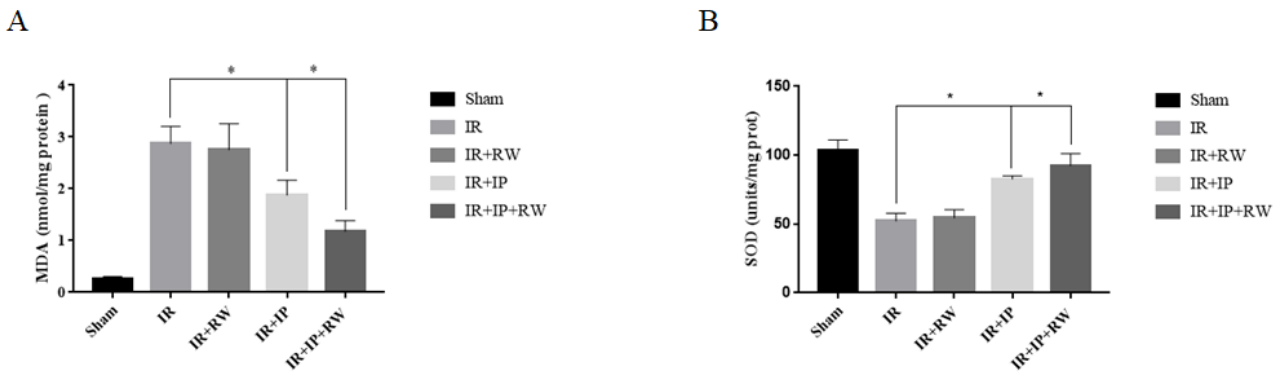

FIGURE 4. RW enhanced the anti-oxidant benefits of IP in mice brain after IR. A. Quantification of MDA levels in brain tissues of each group. B. Quantification of SOD levels in brain tissues of each group. Data represent means±SD (n=6). **P*<0.05.

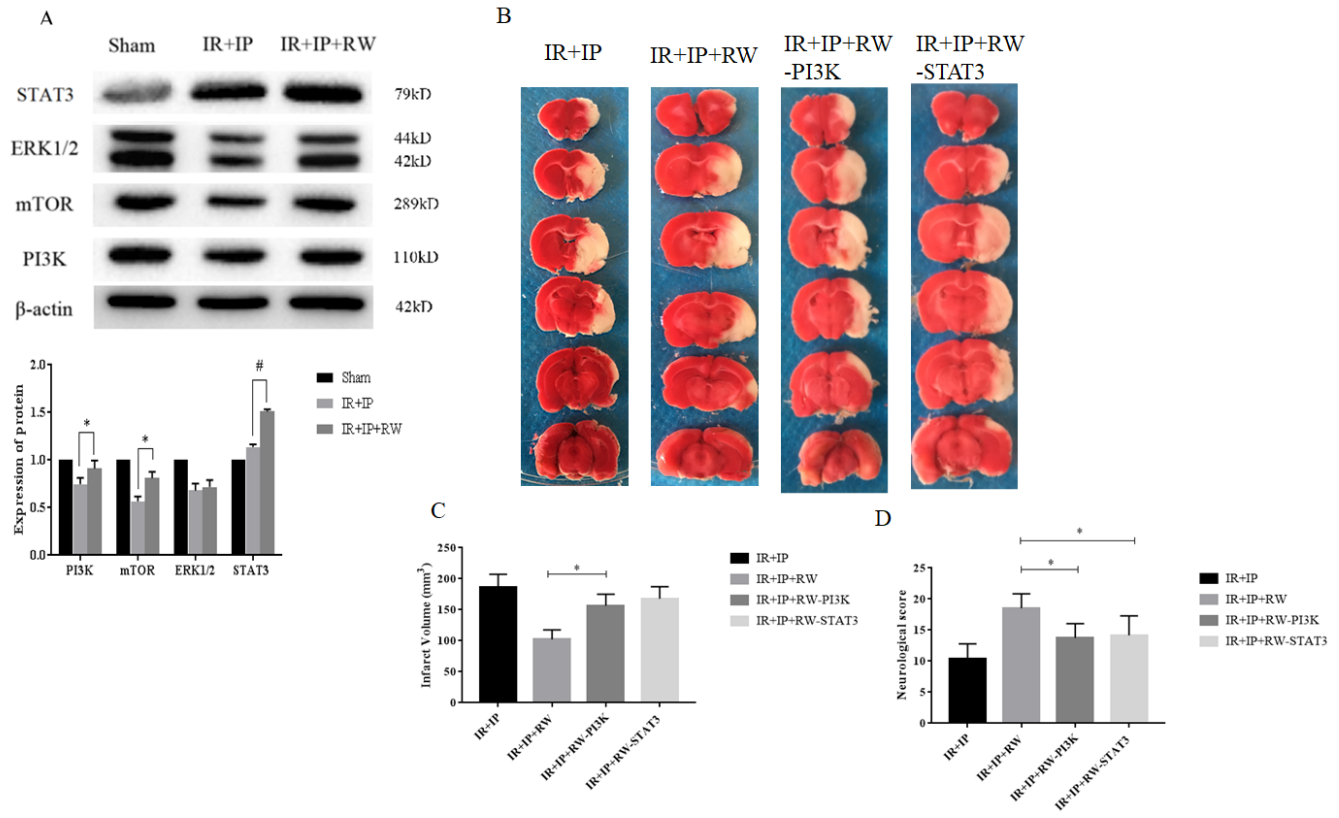

FIGURE 5. PI3K/mTOR and STAT3 pathways were involved in neuroprotective effects of RW in mice brain after IR. A. Western blot analysis of the levels of PI3K, mTOR, ERK1/2 and STAT3 in brain tissues. B. Representative triphenyltetrazolium chloride staining of coronal sections in each group. C. Quantification of infarct volumes in each group. D. Quantification of neurologic score in each group. Data represent means±SD (n=6). **P*<0.05, #*P*<0.01.

Discussion

In this study we present a series of evidence that RW exercise enhanced the effects of IP to improve brain infract and cognitive deficits after IR and this was associated with the inhibition of oxidative stress and apoptosis and the activation of PI3K and STAT3 pathways in the brain.

The reperfusion is regarded as one important factor in the pathogenesis of postischemic injury. Ischemic postconditioning (IP) could reduce cerebral IR injury (Zhao *et al.*, 2006). In this study we confirmed that IR caused significant damage to the neurological behavior of mice and IP improved the damage to the neurological behavior of mice. RW exercise alone did not improve the damage to the neurological behavior of mice, but it enhanced the benefits of IP to ameliorate the cognitive deficits in the mice following IR. Our results are consistent with previous reports that neurological dysfunction was associated with brain injury, which could be improved by timely rehabilitation (Andelic *et al.*, 2012; Wilde *et al.*, 2007; Kobayashi *et al.*, 2017). Exercise has been shown to promote neurogenesis and increase the number of new neurons, in particular, exercise supports brain plasticity via the upregulation of the expression of neurotrophic factors (Cotman & Berchtold, 2002; Koo *et al.*, 2013). Further studies are needed to reveal how RW exercise improves the efficacy of IP on the treatment of IR injury.

To elucidate the mechanisms by which RW enhances the benefits of IP on IR brain injury, we focused on PI3K and STAT3 pathways because they are involved in mediating neuroprotective effects of various treatments (Gao *et al.*, 2017; Arunsundar *et al.*, 2015). Notably, the results showed that RW unregulated protein expression of PI3K, mTOR and STAT3. Furthermore, we treated IR mice with PI3K and STAT3 inhibitors, respectively, and found that the benefits of RW and IP on brain infract and neurological dysfunction in IR mice were abolished. Taken together, these results suggest that RW enhanced neuroprotective effects of IP in IR mice via the activation of PI3K and STAT3 pathways.

Interestingly, PI3K and STAT3 pathways have been shown to provide resistance to apoptosis in endothelial cells and cardiomyocytes exposed to oxidative stress (Lu *et al.*, 2008; Xie *et al.*, 2018; Qin *et al.*, 2015). IR is known to induce oxidative stress in the brain and in this study we confirmed that IR increased MDA level while decreased SOD level in brain tissue, accompanied by high number of apoptotic cells and changed expression of apoptosis related proteins. As expected, RW+IP significantly decreased MDA level and increased SOD level, accompanied by low number of apoptotic cells and the modulation of the expression of apoptosis related proteins. Moreover, all of these effects of RW+IP were attenuated by PI3K and STAT3 inhibitors. Collectively, these findings suggest that RW+IP activated PI3K and STAT3 pathways to provide resistance to oxidative stress induced apoptosis in the brain exposed to IR.

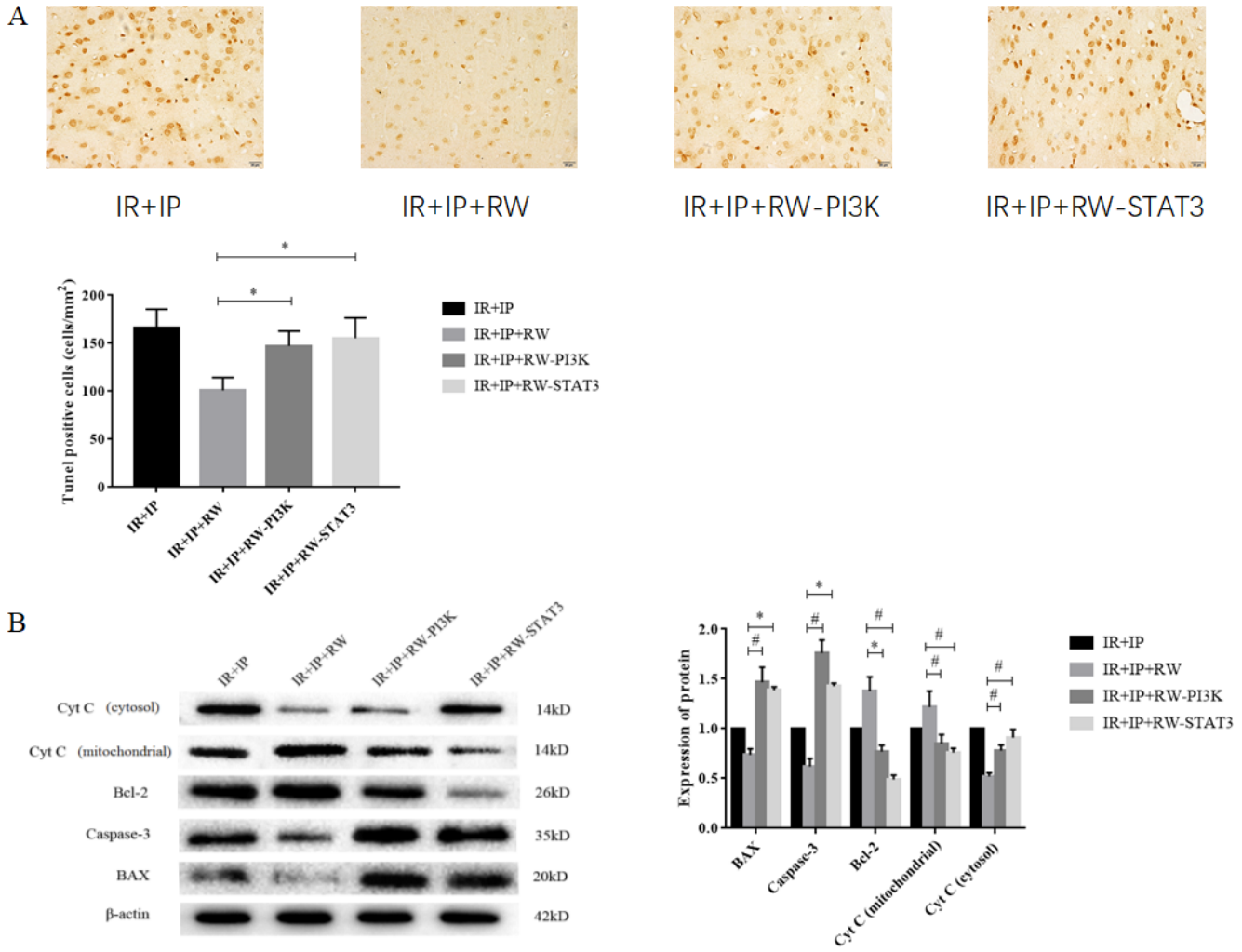

FIGURE 6. PI3K/mTOR and STAT3 pathways were involved in anti-apoptosis effects of RW in mice brain after IR. A. Representative TUNEL staining of coronal sections in each group, and the quantification of TUNEL positive cells. B. Western blot analysis of apoptosis related factors in brain tissues of each group. Data represent means±SD (n=6). *P<0.05, #P<0.01.

In conclusion, we proposed that RW exercise combined with IP reduces brain infract and neurological dysfunction in mice after IR, and this is associated with enhanced anti-apoptotic and anti-oxidant benefits via the activation of PI3K and STAT3 pathways. The use of RW and IP in the clinical may provide novel strategy to improve the recovery from IR injury in stroke patients.

Acknowledgement

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Disclosure

No competing financial interests exist.

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