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ORIGINAL ARTICLE

Age-related difference in protective effect of early post-conditioning on ischemic brain injury: possible involvement of MAP-2/Synaptophysin role

Hedayat Samandari[1,2] · Fatemeh Nabavizadeh[2] · Ghorbangol Ashabi[2]

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Abstract

Brain Ischemia/Reperfusion (I/R) injury leads to the failure of the microtubules function and neuronal death. Ischemic post-conditioning is defined as a series of rapid alternating interruptions of blood flow in the first seconds of reperfusion. In the present study, the caspase-3, Microtubule-Associated Protein-2 (MAP-2), Protein Kinase C α (PKCα), c-fos, and synaptophysin were evaluated in the hippocampus of focal I/R post-conditioning model in a time -dependent study in aged and young rats. Adult and aged rats were subjected to right MCAO for 30 min and post-conditioned (10 s) for 3 cycles. Sensory-motor tests were performed, and locomotion and anxiety-like behavior were evaluated. Molecular tests were done by detection kit, RT-PCR, and Western blotting techniques. Ninety-six hours after I/R post-conditioning, neurological signs, locomotion, anxiety-like behavior, and ischemic area were improved in young rats compared to 6 h after I/R post-conditioning ($P < 0.001$). Caspase-3 activity declined in the hippocampus and cortex of I/R post-conditioned young rats in 96 h after I/R post-conditioning compared with 6 h after I/R post-conditioning ($P < 0.001$). Also, MAP-2 mRNA, MAP-2 protein level, PKCα, c-fos and synaptophysin protein levels were enhanced during post-conditioning in young rats in 96 h after I/R post-conditioning compared with 6 h after induction of I/R post-conditioning. The results of the present study suggested that, early post-conditioning might be considered as a candidate for therapeutic methods against I/R in the adult animals not aged rats. Moreover, inhibition of cell death in post-conditioned ischemic rats was found to be regulated by some neuroprotective molecules as well as MAP-2 and c-fos in young rats.

Keywords Focal cerebral ischemia · Post-conditioning · Young · Aged · MAP-2 · Synaptophysin

Introduction

Stroke is the second prominent cause of death and the third prominent cause of disability (Johnson et al. 2016). Therapeutic drugs and medications for stroke treatment are still inadequate. Therefore, there is a serious need to find more effective therapeutic approaches for stroke recovery. Cerebral damage due to reperfusion following ischemia has been proven to be an important factor influencing the prognosis of revascularization of occluded blood vessels (Tjoumakaris et al. 2009) and finally increasing the neuronal death. Thus, researchers focus on emerging new methods or compounds to prevent brain injury caused by reperfusion. Ischemic post-conditioning is defined as a series of rapid alternating interruptions of blood flow in the first seconds of reperfusion mechanically modifying the toxic effect of reperfusion (Zhao et al. 2006). Recently, many evidences from experimental and clinical trials have shown that ischemic post-conditioning is an effective technique to suppress secondary tissue injury following recovery of blood supply (Feyzizadeh and Badalzadeh 2017).

Cytoskeleton is involved in keeping cell structure contributing in the cellular functions such as transportation and cleavage of substances, intracellular signal transduction, nucleation, and stabilization of microtubules. Brain Ischemia/Reperfusion (I/R) injury leads to the failure of the microtubules function and cell

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✉ Ghorbangol Ashabi
gh-ashabi@tums.ac.ir

[1] Electrophysiology Research Center, Neuroscience Institute, Tehran University of Medical Sciences, Tehran, Iran

[2] Department of Physiology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran

death (Li et al. 2017a, b). Microtubule-Associated Protein-2 (MAP-2) is a neuron-restricted cytoskeletal protein and is necessary for neurite outgrowth (Buee et al. 2000). Additionally, cerebral ischemia disrupts synaptic transmission, synaptic development, and neuronal plasticity (Liu et al. 2006). Evidences have displayed I/R diminishes neuronal synaptic protein (synaptophysin) and brain -derived neurotrophic factor (Glantz et al. 2007; Patkar et al. 2012).

The intracellular mechanisms of post-conditioning against ischemia toxicity are not well understood. To elucidate the alteration of neuronal cytoskeleton and plasticity during post-conditioning, the caspase-3, MAP-2, synaptophysin, Protein Kinase C (PKCα), and c-fos were evaluated in the hippocampus and cortex of the Middle Cerebral Artery Occlusion (MCAO) followed by early post-conditioning model in a time-dependent manner in aged and young rats.

Materials and methods

Animals

Six month old male adult (270-300 g) and 20 mon old aged Wistar rats (320-350 g) were obtained from Tehran University of Medical Sciences (Tehran, Iran) and habituated to animals' room in Plexiglas cages and maintained in a room with controlled light/dark cycle (12/12 h with light beginning at 7:00 a.m.), temperature (22 ± 2 °C) and food and water ad libitum. All experimental procedures were approved by the Ethics Committee of Tehran University of Medical Sciences, Tehran, Iran in accordance with international guidelines for animal experiments (IR.TUMS.VCR.REC.1396.4097).

Experimental design

Each aged (thirty-five twenty-month old) or young (thirty-five six-month old) male rats were randomly divided into five groups ($n = 7$): 1) sham surgery group (sham): rats MCA were exposed and then the open area is stitched. The sham group rats were sacrificed 6 h after surgery, 2) I/R (6 h): aged or young rats were sacrificed 6 h after I/R, 3) I/R (96 h): aged or young rats were sacrificed 96 h after I/R, 4) I/R + PC (6 h): aged or young rats were received post-conditioning after MCAO and then were sacrificed 6 h after I/R, 5) I/R + PC (96 h): aged or young rats were received post-conditioning after MCAO and then were sacrificed 96 h after I/R. We had a time-dependent monitoring for neurological scores and open field test in 6, 12, 24, 48 and 96 h after reperfusion in I/R and I/R + PC groups. We used seven rats for all behavioral tests. Four rat's hippocampi and cortex were collected for Western blotting technique and caspase-activity measurement. Three rat's hippocampi and cortex were collected for RT-PCR (see graphical abstract).

Induction of MCAO and post-conditioning

Rats were anaesthetized with chloral hydrate (400 mg/kg, i.p, Sigma Aldrich Co. St. Louis, USA). To induce focal cerebral ischemia, rats were subjected to right Middle Cerebral Artery Occlusion (MCAO) as previously described (Longa et al. 1989) with minor modifications. Shortly, after making a 1.5 cm incision at neck, right common carotid artery (CCA) was dissected from surrounding tissues. Then the proximal end of external carotid artery (ECA) was occluded. After occlusion of the right CCA by aneurysm clips, a 4/0 nylon monofilament with silicone-coated tip was introduced through a small incision on ECA and advanced to internal carotid artery (ICA) so that it blocked the origin of MCA. After 30 min MCA occlusion, the monofilament was withdrawn.

To perform the post-conditioning, after filament withdrawal, right CCA was opened. Then, both CCAs were occluded temporarily for 10 s by tightening the sutures around CCAs followed by 30 s reperfusion. Another two cycles of 10 s occlusion and 30 s reperfusion were followed (totally 3 cycles) and a reperfusion of 6 or 96 h was allowed (Zhao et al. 2006). Finally, the neck wound was sutured and animals were allowed to recover from the anesthesia. Body temperature was monitored by a rectal probe and maintained during the surgery between 37 and 38 °C with a heating pad.

Behavioral tests

Neurological deficit evaluation

Neurological evaluations were carried out 6, 12, 24,48, and 96 h after I/R according to Bederson's exam (Bederson et al. 1986). Briefly, rats were evaluated and given deficit scores based on parameters including spontaneous activity, symmetry of movement and outstretch of limbs, circling behavior, climbing ability, body proprioception, and vibrissae touch. The Bederson's exam is scored on a scale from 0 to 3.

Open field test

The open field opaque acrylic box (50 × 50 × 40 cm) was placed in an isolated room with no objects or clues. The box floor was divided into 10 numbered squares, approximately 16 × 16 cm^2 each in order to address central or parietal localization. Twenty-four hours after reperfusion, each animal's locomotion was tested for 5 min in the box with all recorded by a video camera mounted on the ceiling of the room (Maze router, Tabriz, Iran). Scoring was performed by both maze router analysis and manually and expressed as "Moving distance (meters)" and "Time spent in the central square (s)" (Gupta et al. 2002; Pietrelli et al. 2012).

Infarct size quantification

To confirm the presence of stroke, adult and aged animals were sacrificed at 6 and 96 h post stroke and post-conditioning. Tissue infarct was determined by staining 2 mm coronal sections with 1% 3,5-triphenyltetrazolium chloride (TTC) solution at 37 °C for 20 min (Hatfield et al. 1991). Infarct size of each coronal section was quantified using Image J software as previously described and was represented as percentage of ipsilateral hemisphere infract volume.

Western blot analysis

Six and ninety-six hours after reperfusion, animals were euthanized and decapitated. The hippocampi were dissected and flash frozen in liquid nitrogen and stored at −80 °C. While ready for Western blotting, all samples were homogenized in the appropriate lysis buffer (Niimura et al. 2006) and total protein extract was prepared by centrifugation in 15,000 rpm for 5 min. The protein concentration in the supernatants was measured using the Bradford's method (Bradford 1976).

Standardized lysates equivalent to 60 μg of protein were loaded on SDS-12.5% poly acrylamide gel electrophoresis, and transferred to PVDF membrane (Chemicon Millipore Co. Temecula, USA). Then, blots were blocked in 2% Electrochemiluminescence (ECL) advanced kit blocking reagent (Amersham Bioscience Co. Piscataway, USA) and probed with MAP-2, synaptophysin (1/1000, ABCAM, Cambridge, Ma, USA), PKCα and c-fos (1/1000, Cell Signaling Technology Co. New York, USA) antibodies overnight. Membranes were then incubated with rabbit IgG-horseradish peroxidase-conjugated secondary antibody (1/3000, Cell Signaling Technology Co. New York, USA) which could be directly detectable by chemiluminescence kit reagent (Amersham Bioscience Co. Piscataway, USA). To detect β-actin as an internal control, blots were stripped in stripping buffer (pH = 6.7) and then probed with anti β-actin antibody (1/1000, Cell Signaling Technology Co. New York, USA) (Niimura et al. 2006).

Caspase-3 activity

Caspase-3 level was measured by caspase-3 assay kit (Abcam, Cambridge, MA, USA) according to the manufacturer's protocol. The hippocampi and cortex cells were lysed using specific lysis buffer and lysate was centrifuged at 15000 rpm for 5 min at 4 °C, and the protein concentration in the supernatants was measured using the Bradford dye method (Lam et al. 2018). Caspase-3 activity was assessed by measuring the absorbance at 405 nm.

Real time PCR

We used Step One Plus Real-Time PCR System (Applied Biosystems) for real time PCR reaction. Two μl of cDNA, 2 μl of primer and SYBR Green Master Mix (Takara, Japan) were mixed according to manufacture protocol in total volume of 20 μl. The annealing temperature for all primer pairs was adjusted in 58 °C. The specificity of the PCR product was confirmed by verifying a single peak in melting curve and visualizing on 2% agarose gel with ethidium bromide in gel documentation. The quantity of target gene for each sample was calculated from the cycle at which the sample fluorescence came across a predetermined threshold (Ct) significantly beyond the background. Samples were tested in duplicate and the mean was used for further analysis. All the data of sample and control groups were normalized by the housekeeping gene (beta-actin).

Statistical analysis

Obtained data were expressed as mean ± SEM ($n = 7$). Behavioral and Western blot results were analyzed statistically by One-way ANOVA and repeated measure test followed by Tukey's multiple comparison test using SPSS software version 21. Data of gene expression experiments were analyzed by Relative Expression Software Tool (REST)-XL version 2 (Pfaffl et al. 2002) which is used to determine significant differences in relative expression levels between sample and control groups. Data are shown as fold differences of mean normalized expression values ± SEM. P value less than 0.05 is considered statistically significant.

Results

Neurological signs, locomotion and anxiety-like behavior were improved in I/R post-conditioned young not old rats

Neurological scores were assessed after I/R post-conditioning in young and old rats. Results were monitored from six till 96 h after induction of I/R and/or I/R post-conditioning (Fig. 1a and b). In young rats (Fig. 1a), twenty-four, forty-eight and ninety-six hours after induction of I/R post-conditioning, neurological signs were improved compared with the same time-points in the I/R group ($P < 0.001$). There is no significant changes on neurological scores between I/R and I/R post-conditioning in old rats (Fig. 1b, $P > 0.05$). Open field test showed locomotion and anxiety-like behavior; according to Fig. 2a, twenty-four, forty-eight and ninety-six hours after induction of I/R post-conditioning, total distance moved was increased compared with the same time-points in the I/R group in young rats ($P < 0.001$). There is no significant

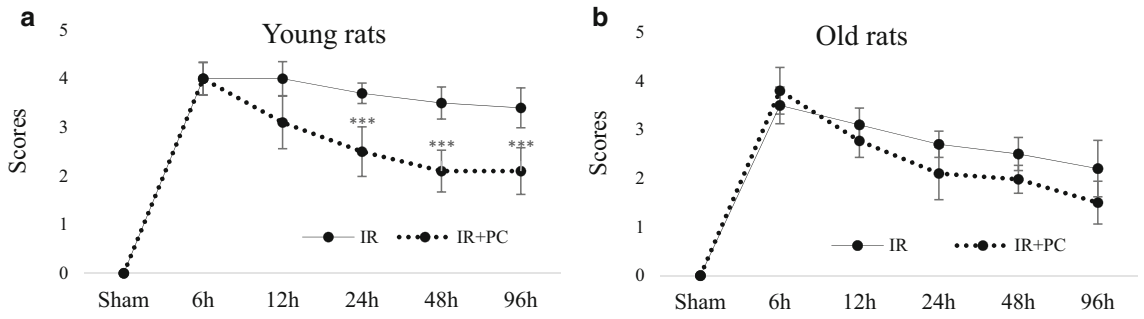

Fig. 1 Behavioral assessments. Neurological evaluation in young (**a**) and aged (**b**) rats treated with post-conditioning 6 and 96 h after MCAO ($n = 7$). Data were presented as Mean ± SEM. ***$P < 0.001$ ver. 6 h I/R post-conditioning group. I/R: ischemia/reperfusion, PC: post-conditioning

changes in total distance moved between I/R and I/R post-conditioning in old rats (Fig. 2b, $P > 0.05$). We measured "time in center" of open field as an indicator of anxiety-like behavior; in Fig. 2c, twelve, twenty-four, forty-eight and ninety-six hours after I/R post-conditioning, time spend in center of open field was increased compared with the same time-points in the I/R group in young rats ($P < 0.001$). There is no significant changes in "time in center" between I/R and I/R post-conditioning in old rats (Fig. 2b, $P > 0.05$).

Early I/R post-conditioning decreased ischemic area in young not old rats

Figure 3 represented the infarct volume of rat's brain which treated with 1% TTC. As shown in Fig. 3 graph, densitometry analysis showed 96 h after I/R the percentage of infarct volume was decreased in the young post-conditioned group compared with young I/R group ($P < 0.001$). The percentage of infarct volume didn't change between I/R and I/R post-conditioning in old rats (Fig. 3, $P > 0.05$).

Caspase-3 activity was declined in the hippocampus and cortex of I/R post-conditioned young rats

Caspase-3 activity in the hippocampus and cortex was measured; as shown in Fig. 4a, in the hippocampus, caspase-3 activity was increased in the I/R rats compared to the sham group (in both old and young rats) ($P < 0.001$). Also, caspase-3 activity was reduced after 96 h I/R post-conditioning compared with 6 h I/R post-conditioning in young rats in the hippocampus ($P < 0.001$). The caspase-3 activity was measured in the cortex in Fig. 4b; caspase-3 activity was amplified in the I/R rats compared to the sham group (in both old and young rats) ($P < 0.001$). After 96 h I/R post-conditioning, caspase-3 activity was decreased compared with 6 h I/R post-conditioning in young rats in the cortex ($P < 0.001$). There is no significant changes in caspase-3 activity between I/R and I/R post-conditioning in old rats in both 6 h and 96 h after ischemia in the hippocampus and cortex (Fig. 4a, b, $P > 0.05$).

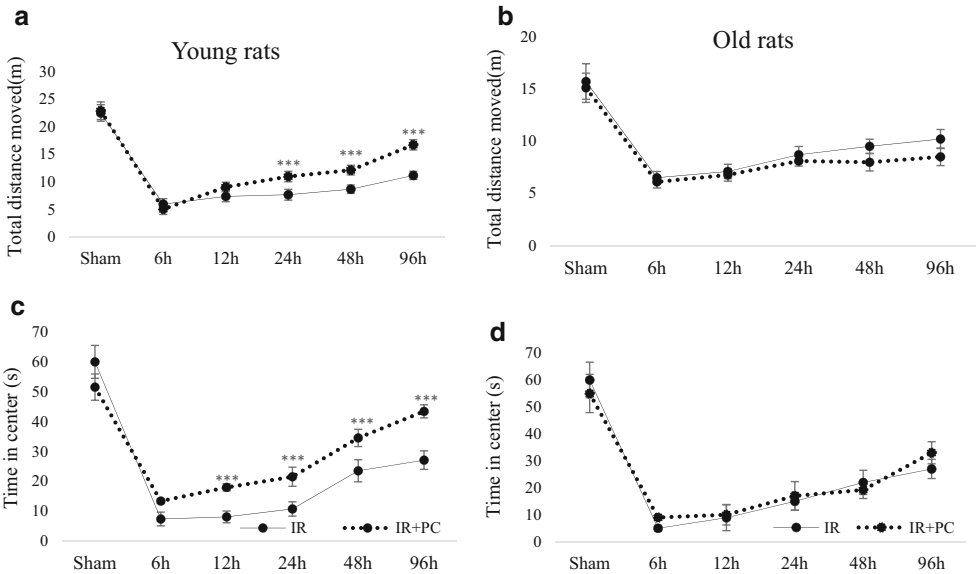

Fig. 2 Open field test showed total distance moved (meters) in young (**a**) and old (**b**) rats and Total time in center (sec) in young (**c**) and old (**d**) rats ($n = 7$). Data were presented as Mean ± SEM. ***$P < 0.001$ ver. 6 h I/R post-conditioning group. I/R: ischemia/reperfusion, PC: post-conditioning

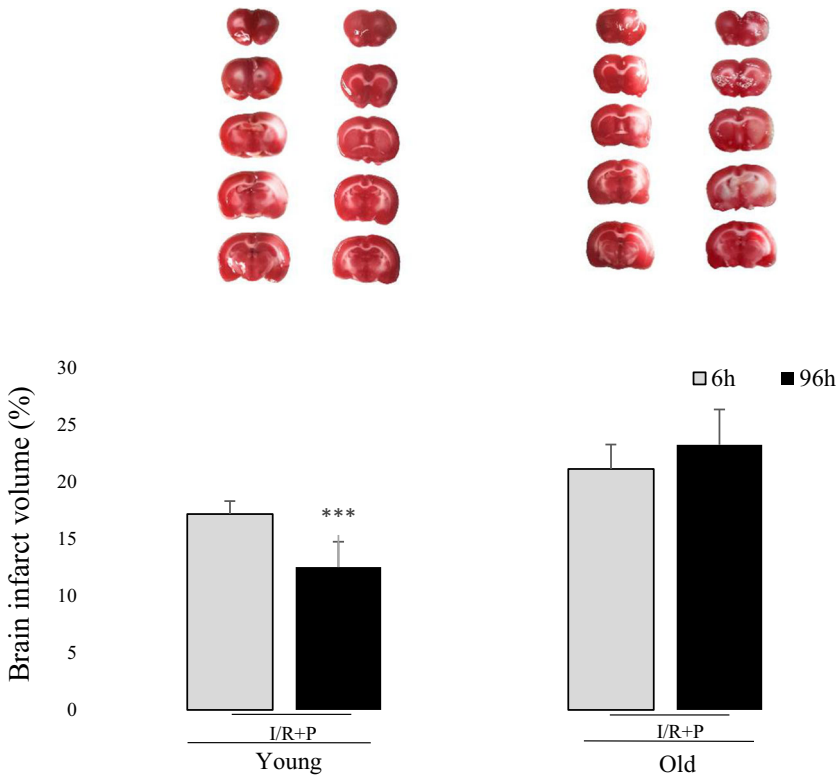

Fig. 3 Determination of ischemic area. TTC staining showed ischemic sites in experimental groups. Densitometry analysis of percentage of ischemic area in whole brain ($n = 5$). Data were presented as Mean ± SEM. ***$P < 0.001$ ver. 6 h I/R post-conditioning group. I/R: ischemia/reperfusion, PC: post-conditioning

MAP-2 gene and protein and synaptophysin protein level were improved during post-conditioning in young rats

MAP-2 mRNA expression, MAP-2 and synaptophysin protein level in the hippocampus were showed in Fig. 5. MAP-2 expression was reduced after I/R in the young and old rats in comparison to sham group (Fig. 5a and b, $P < 0.001$). MAP-2 expression was increased after 96 h I/R post-conditioning compared with 6 h I/R post-conditioning in young rats in the hippocampus (Fig. 5a, $P < 0.001$). As shown in Fig. 5b, MAP-2 expression was increased after 96 h I/R post-conditioning compared with 6 h I/R post-conditioning young rats in the cortex (Fig. 5b, $P < 0.001$). Figure 5c showed the represent blot for MAP-2 and synaptophysin. MAP-2 protein level was decreased after I/R in the young and old rats in comparison to sham group (Fig. 5d, $P < 0.001$). MAP-2 protein level was enhanced after 96 h I/R post-conditioning compared with 6 h I/R post-conditioning young rats (Fig. 5d, $P < 0.001$). There is no significant changes in MAP-2 mRNA expression and protein level between I/R and I/R post-conditioning in old rats in both 6 h and 96 h after ischemia (Fig. 5a and d, $P > 0.05$). Synaptophysin protein level was reduced after I/R in the young and old rats compared with the sham group (Fig. 5e, $P < 0.001$). Synaptophysin protein level was enhanced after 96 h I/R post-conditioning compared with 6 h I/R post-conditioning young rats (Fig. 5e, $P < 0.001$). There is no significant change in synaptophysin protein level between I/R and I/R post-conditioning in old rats in both 6 h and 96 h after I/R (Fig. 5e, $P > 0.05$).

PKCα and c-fos protein levels were enhanced during early post-conditioning in young not aged rats

Figure 6a showed the represented blots of PKCα and c-fos in the hippocampus. Figure 6a represent a sample blot for PKCα and c-fos. PKCα and c-fos protein level were enhanced after 96 h I/R post-conditioning compared with 6 h I/R post-conditioning young rats (Fig. 6b and c, $P < 0.001$). Level of c-fos was increased in I/R-96 h group compared with I/R-6 h group ($P < 0.001$). There is no significant change in PKCα and c-fos protein level between I/R and I/R post-conditioning in old rats in both 6 h and 96 h after ischemia (Fig. 6b and c, $P > 0.05$).

Discussion

Cerebral ischemia post-conditioning has been established as a potential cure for ischemic stroke in animal models (Zhao et al. 2006); clinical studies are limited, because

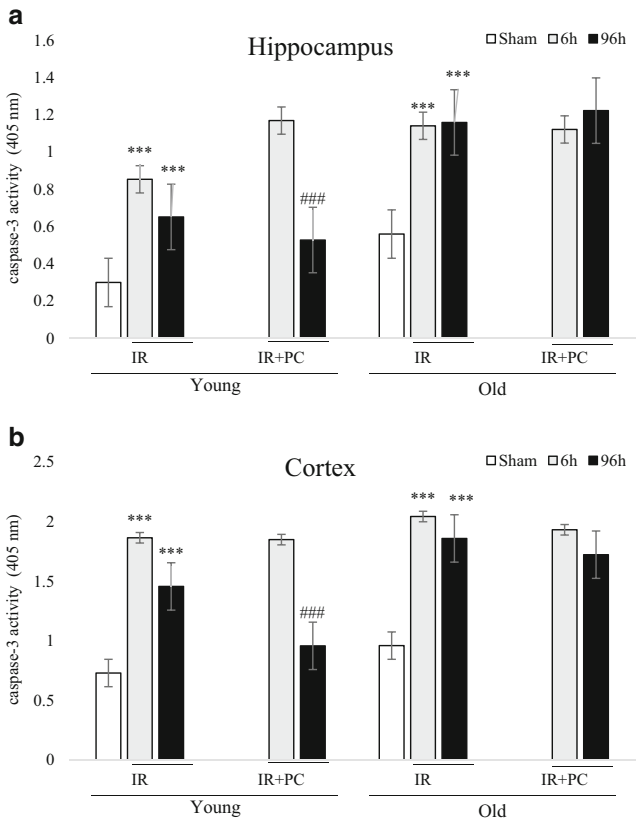

Fig. 4 Hippocampal (**a**) and cortex (**b**) caspase-3 activity in 6 and 96 h after reperfusion in I/R and I/R plus post-conditioning groups in experimental groups ($n = 4$). The hippocampal and cortex caspase3 activity was measured in 405 nm wave length (technical repeat number is 3). Data were presented as Mean ± SEM. ***$P < 0.001$ ver. Sham group in young or old. ###$P < 0.001$ ver. I/R-PC-6 h young rats. I/R: ischemia/reperfusion, PC: post-conditioning

of lack of knowledge about neuroprotective mechanisms of ischemia post-conditioning (Wei et al. 2016). The present study underlines the importance of early ischemic post-conditioning regarding attenuation of apoptosis and maintenance of cytoskeleton associations in young animals but not old ones.

Ageing increases brain's susceptibility to ischemic injury and boosts infarct size (Rosen et al. 2005; Dong et al. 2014). In the current study, the ischemia and/or ischemia post-conditioning was induced in the old and young rats and their behaviors (neurological scores, locomotion, and anxiety-related behavior) were monitored 6 h until 96 h after reperfusion. Ischemia was found to reduce the neurological scores, locomotion, and anxiety -like behavior in aged and young rats. Our results are consistent with other studies (Rosen et al. 2005; Dong et al. 2014), indicating that both aged and young rat brains are more susceptible to ischemic injury. In addition, post-conditioning could not improve the behavioral deficits in old rats, but behavioral deficits were enhanced in young rats 96 h after ischemia. A time-dependent study showed behavior deficits were improved in young rats 18 mon after reperfusion (Kiryk et al. 2011); herein, 24 h after ischemia post-conditioning, behavioral deficit began to improve in young rats. The mechanism by which post-conditioning exerts its effects on the ischemic brain injury has remained unclear. Ischemia seems to induce neuronal apoptosis and reducing the neuroprotective signaling pathways (Dinapoli et al. 2010). Early post-conditioning or low -oxygen treatment might enhance neurogenesis, angiogenesis, and some protective signaling pathways (Doeppner et al. 2018; Pietrogrande et al. 2018), but current data showed that, post-conditioning has no power to improve these protective factors in the aged cases.

In the following of this study, two time points [6 and 96 h after induction of ischemia and/or post-conditioning] were chosen to evaluate the molecular changes in the hippocampus and cortex. Firstly, the infarct size of animals was evaluated in the experimental groups. Ninty-six hours after induction of ischemia post-conditioning, the infarct size reduced in young rats; however, no change was observed in the infarct size of old rats. Caspase-3 activity was assessed in the hippocampus and cortex 6 and 96 h after ischemia so that, the neuronal apoptosis can be somehow confirmed, but more evaluations such as assessment of Bax and Bcl-2 levels are needed to establish it firmly. Reduction of active caspase-3 after post-conditioning represents the protective role of post-conditioning in the adults. Studies showed that, caspase-3, as an apoptotic marker decreased after early post-conditioning (Li et al. 2017a, b; Zhao et al. 2017). Furthermore, there is no significant data in 6 and 96 h after I/R in young animals. Kiryk et al. (2011) reported that, young animal's brain need 3 to 18 mon for recovery, nevertheless neurons of old rats could not be recovered after 18 mon (Kiryk et al. 2011). In the current study, the recovery period was detected 96 h after ischemia post-conditioning; therefore, post-conditioning might reduce the recovery period.

Movement disorders have been reported to be associated with the neuronal plasticity. In the current study, some molecules involved in the synaptic plasticity were evaluated. MAP-2 is a neuron-specific phosphoprotein which can be phosphorylated by multiple protein kinases and dephosphorylated by several protein phosphatases (Ainsztein and Purich 1994). MAP-2 is measured as a marker of ischemic injury following cerebral ischemia (Dawson and Hallenbeck 1996). Synaptophysin is a calcium-binding synaptic vesicle membrane glycoprotein and it has been shown to contribute in the synaptic

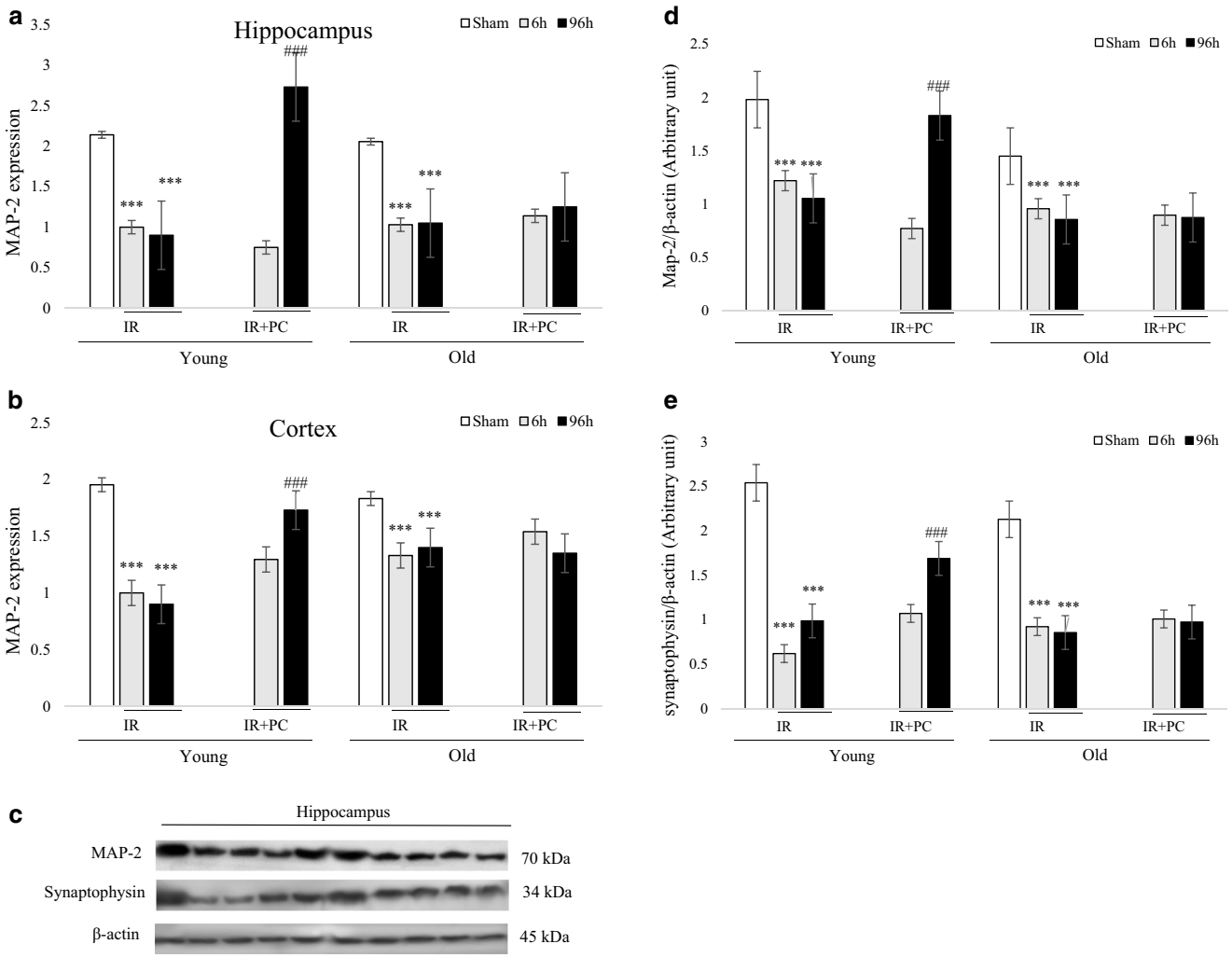

Fig. 5 Hippocampal and cortical MAP-2 gene expression ($n = 3$) and hippocampal Western blot analysis ($n = 4$) of MAP-2 and synaptophysin in 6 and 96 h after reperfusion in experimental groups. Expression of MAP-2 in 6 and 96 h after reperfusion in I/R and I/R plus post-conditioning groups in experimental groups in the hippocampus (**a**) and cortex (**b**). A represented blot showed MAP-2 and synaptophysin protein level (**c**) and the density of hippocampal MAP-2 bands (**d**) was measured and their ratio was calculated (technical repeat number is 3). The density of hippocampal synaptophysin bands (**e**) was measured and their ratio was calculated (technical repeat number is 3). Data were presented as Mean ± SEM. ***$P < 0.001$ ver. Sham group in young or old. ###$P < 0.001$ ver. I/R-PC-6 h young rats. I/R: ischemia/reperfusion, PC: post-conditioning

development and plasticity (Liu et al. 2005). Down-regulation of MAP-2 and synaptophysin can cause an abnormal microtubule assembly and neurotransmission, respectively, abolishing the synaptic activity (Lin et al. 2010). Our study results are in agreement with the results of the study by Liu et al., represented that MAP-2 and synaptophysin decreased during cerebral ischemia (Lin et al. 2010), besides, post-conditioning following cerebral ischemia was found to reverse the toxic effect of ischemia through MAP-2 and synaptophysin, which consequently enhanced the microtubules stability. Current study evaluated the hippocampal and cortical neurons. The changes of hippocampal caspase-3 activity and MAP-2 expression is in parallel with cortical neurons. On the other hand, hippocampus can modulate anxiety-like behavior and also, hippocampal neurons are very susceptible to post-conditioning in the acute phase (Miao et al. 2016; Parfitt et al. 2017), so, we assessed hippocampal neuronal signaling in the following of study.

Synaptic protein MAP-2 and synaptophysin increased neuronal plasticity. Moreover, we evaluated PKCα and c-fos protein level; it has been shown PKC could induce c-fos (Zhang et al. 2014; Beckhauser et al. 2016) and c-fos is a potent transcription factor for neuronal plasticity (Wang and Zhuo 2012). Evidences showed PKCγ and PKCδ inhibits c-fos via Mitogen activated kinases (Yokoyama et al.

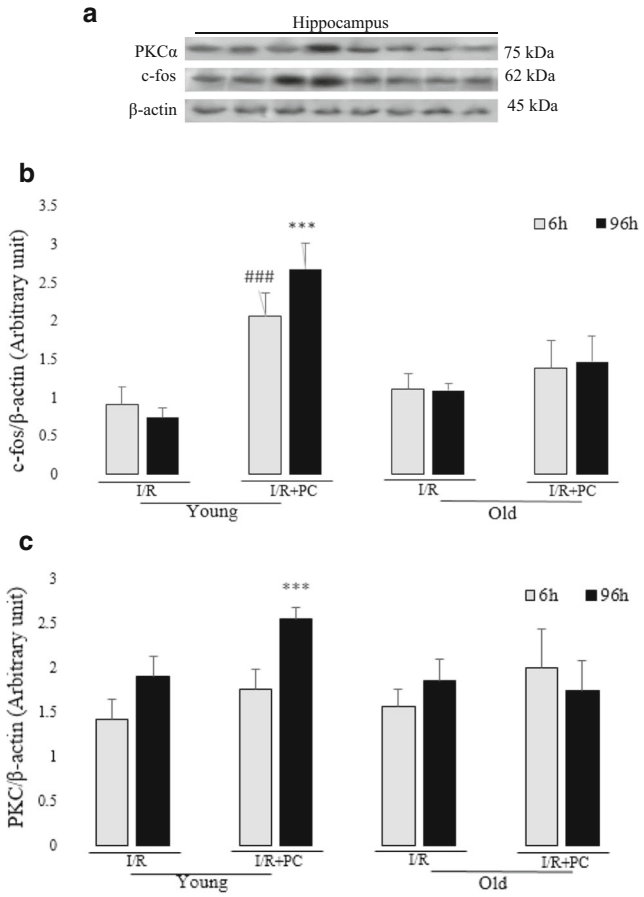

Fig. 6 Hippocampal PKCα and c-fos in 6 and 96 h after reperfusion in I/R and I/R plus post-conditioning groups in experimental groups ($n = 4$). A represented blot showed PKCα and c-fos levels (**a**). The density of hippocampal PKCα (**b**) and the density of hippocampal c-fos (**c**) were measured and their ratio was calculated (technical repeat number is 3). Data were presented as Mean ± SEM. ***$P < 0.001$ ver. 6 h I/R post-conditioning group; ###$P < 0.001$ ver. 6 h I/R group. I/R: ischemia/reperfusion, PC: post-conditioning

2013), however, Kuriyama and Mayor reported PKCα has inhibitoy role on c-fos in *Xenopus* neurons (Kuriyama and Mayor 2009) but we showed PKCα could alleviate c-fos expression in ischemic neurons after induction of post-conditioning in rodents. On the other hand, PKCα and c-fos are signaling molecules which activates several pathways involved in synaptogenesis, neuroprotection and neuronal plasticity (Dahlqvist et al. 2003; Alkon et al. 2007). In parallel to the current data, studies have suggested that, the cerebral ischemia decreased PKCα and c-fos (Tsai et al. 2011; Ashabi et al. 2017; Zhao et al. 2018), further, our data suggested that, these proteins increased by post-conditioning treatment in adults not aged rats showing that, the post-conditioning might enhance the neuronal plasticity factors,consequently leading to the amendment of movement and psychological deficits after ischemia. Previous studies showed that, ischemia post-conditioning increased c-fos 6 h after reperfusion; and c-fos was considered as an immediate early gene expressed after 6 h in the MCAO model (Cho et al. 2001).

Macri et al. suggested that, the hippocampus has susceptibility to the hypoxia on cerebral ischemia,and also old age boosts this increment in the susceptibility (Macri et al. 2010) conceivably through reduction of antioxidant pathways (Sarkar et al. 2013). Interestingly, 96 h after induction of ischemia or ischemia post-conditioning, old rats showed a recovery in behavioral deficits, perhaps long-term study might display improvement in old rat's movement disorders. However, it still is not clear that, why post-conditioning has no role on aged animals; considering the enlargement of infarct size in old rats compared to the young ones, herein, it was proposed that, the disruption of neuronal cytoskeleton and attenuation of neurogenesis caused a weakness in the post-conditioning protective function.

To sum, the results of the present study suggested that, early post-conditioning might be considered as a candidate for therapeutic methods against I/R in the adult animals. Post-conditioning enhanced the sensory-motor function, movement disorders,and anxiety-like behavior through attenuation of cell death in infarct area (cortex) and hippocampus; and the inhibition of cell death was regulated by some neuroprotective signaling molecules as well as MAP-2 and c-fos in young not old rats (graphical abstract).

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