Dexmedetomidine Protects Against Neurological Dysfunction in a Mouse Intracerebral Hemorrhage Model by Inhibiting Mitochondrial Dysfunction-Derived Oxidative Stress

Jing Huang, PhD, and Qiang Jiang, PhD

Background: Intracerebral hemorrhage (ICH) is a subtype of stroke with high disability and mortality. Dexmedetomidine (Dex) has been shown to provide neuroprotection in several neurological diseases. The aim of present study was to investigate the effects of Dex on ICH-induced neurological deficits and brain injury and the underlying mechanisms. *Methods:* ICH mouse model was established by intracerebral injection of autologous blood, followed by Dex or vehicle treatment. Neurological function, brain water content, neuronal activity, and oxidative parameters were determined. The protein expressions of peroxisome proliferator-activated receptor gamma coactivator 1-α (PGC-1α), uncoupling protein 2, and manganese-dependent superoxide dismutase were examined by western blotting. *Results:* Dex administration significantly inhibited ICH-induced the memory impairment, dyskinesia, brain edema, and neuron loss. In addition, ICH-induced the increase in brain oxidative stress level was markedly attenuated after Dex treatment, as evidenced by increased glutathione peroxidase and superoxide dismutase levels and reduced malondialdehyde and nitric oxide levels. Compared with vehicle-treated ICH mice, Dex-treated ICH mice showed significantly decreased intracellular reactive oxygen species (ROS) and mitochondrial ROS (mROS) production in brain, but had no effects on the increased nicotinamide-adenine dinucleotide phosphate oxidase activity. However, stimulation of mROS abrogated the inhibitory effects of Dex on neurological deficits and oxidative stress. The decrease in production of adenosine triphosphate and the expressions of PGC-1α, uncoupling protein 2, and manganese-dependent superoxide dismutase induced by ICH was restored by Dex treatment. *Conclusions:* Our results reveal that Dex improves ICH-induced neurological deficits and brain injury by inhibiting PGC-1α pathway inactivation and mitochondrial dysfunction-derived oxidative stress.

Key Words: Dexmedetomidine—Intracerebral hemorrhage—Neurological deficits—Oxidative stress—Mitochondrial dysfunction

Introduction

Intracerebral hemorrhage (ICH) is a severe subtype of stroke, which takes up for 8%-15% of all strokes in Western countries and 20%-30% in the Asian area.[1] It causes much higher mortality and disability rate than ischemia stroke.[2] Despite the remarkable progress in the understanding of ICH pathogenesis, effective treatment options are still lacking.

Increasing evidences indicate that oxidative stress that being triggered in the early period after ICH challenge is a critical factor exacerbating ICH-induced the secondary brain injury.[3,4] Oxidative stress induces damages of macromolecules, disruption of cellular signaling, and loss of

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neuronal activity that known to lead to neurological dysfunction.[5,6] Mitochondrial dysfunction is implicated in oxidative stress linked to the pathogenesis of neurological diseases.[3] The dysfunction of mitochondria impairs adenosine triphosphate (ATP) synthesis and induces excessive reactive oxygen species (ROS) production, which in turn aggravates brain injury.[5,7]

Dexmedetomidine (Dex) is a highly selective agonist of α2-adrenergic receptors with anxiolytic, analgesic, and sedative effects.[8] The neuroprotective effects of dexmedetomidine have been reported in clinical practice and basic studies. A previous study showed that Dex is a safe and effective sedation for hypertensive cerebral hemorrhage patients who received craniotomy, because it can reduce blood pressure, alleviate the cardiovascular responses, and prevent respiratory depression.[9] In addition, a pilot study also revealed that Dex appears effective in sedating severely brain-injured patients without adverse physiological effects.[10] In a mouse model of ICH, Dex attenuates memory impairment by reducing neuronal cell apoptosis.[11] Moreover, Wang et al reported that post-treatment with Dex ameliorates subarachnoid hemorrhage-induced brain injury via activation of extracellular signal-regulated kinase.[12] However, the mechanisms by which Dex prevents ICH-induced brain injury and neurological dysfunction remain to be further explored. In this study, we uncover a novel mechanism of the neuroprotective effects of Dex and provide solid theoretical evidence for the clinical use of Dex for ICH treatment.

Methods

Materials and Reagents

2′,7′-Dichlorofluorescin diacetate, MitoSOX Red, nicotinamide-adenine dinucleotide phosphate (NADPH) Assay Kit and Fluoro-Jade B (FJB) were purchased from Sigma Chemical Co. (MO). Male C57/BL6 mice (8-10 weeks) were purchased from the Chinese Academy of Sciences (Shanghai, China). All animal experiments were performed in accordance with China Animal Welfare Legislation and approved by the Institutional Animal Care and Use Committee of Nanjing Medical University.

ICH Model Construction

ICH model was established as previously described.[13] In brief, mice were anesthetized by intraperitoneal injection with chloral hydrate (400 mg/kg) and positioned in a mouse stereotaxic frame. Blood was collected from femoral artery catheter. The skull was drilled by a .5-mm burr hole (.2 mm anterior, 3.5 mm ventral, and 2.5 mm lateral to the bregma), and then 30 μL of autologous blood was injected straightly into the right basal ganglia at a rate of 2 μL/min using a microinfusion pump (TJ-1A, LongerPump, China). The microsyringe was remained in position for an additional 10 minutes and then carefully removed. The hole was sealed with bone wax and the skin incision was closed. The sham mice were processed with same procedure without autologous blood infusion.

Experimental Groups

Animals (n = 136) were randomly divided into the following groups: sham + vehicle (n = 26), sham + Dex (n = 26), sham + Dex + rotenone (n = 16), ICH + vehicle (n = 26), ICH + Dex (n = 26), and ICH + Dex + rotenone (n = 16). After sham or ICH surgery, each moues was intraperitoneally injected with Dex (50 μg/kg) or the same volume (200 μL) immediately. In the sham + Dex + rotenone group and ICH + Dex + rotenone group, rotenone (500 μg/kg) was intraperitoneally injected 24 hours after ICH surgery. No mice were died during the experimental period. Neurobehavioral testing or sacrifice for brain tissues collection were performed 48 hours after ICH surgery.

Morris Water Maze Test

The degree of learning ability and spatial memory was measured by Morris water maze test as previously described.[14] The experimental apparatus (Guangzhou Feidi Biology Technology Co., Ltd., Guangzhou, China) was divided into 4 quadrants that filled with nontoxic white pigments. A round and black platform was placed 2 cm under the water in the center of the fourth quadrant. Each mouse was randomly placed in varying quadrants and allotted 90 seconds to find the platform. Mice were trained 4 times per day for 4 consecutive days before experiment.

Rota-Rod Test

The motor function was evaluated using an automated Rota-rod (Ugo Basile, Comerio, Italy). Mice were trained before surgery at a constant speed of 40 rpm for 4 consecutive days (4 times per day). The average latency to fall from the rod was recorded and the maximum cutoff time was set as 180 s.

Pole Test

Pole test was performed as previously described.[6] Each mouse was placed on a ball (diameter: 2.5 cm) that glued on the top of a wooden pole (length: 50 cm, diameter: 1cm). The time spent in climbing down the pole was measured. The maximum cut-off time was set as 100 seconds. The average time of 4 tests was calculated.

Traction Test

The degree of limb impairment was examined by the traction test. A stainless steel bar (50 cm length, 2 mm diameter) was connected with 2 vertical supports and elevated 37 cm above a flat surface. Each mouse was placed on the middle of the bar by forepaws and observed for 30

seconds in 4 trials. Mice were scored according to the following system: 3, hung on the bar with 2 hind paws; 2, hung on the bar with one hind paw; 1, hung on the bar with two forepaws; 0, fell off.

Brain Water Content

After euthanization and decapitation, the brains were isolated from the skull and then divided into 3-mm coronal sections in the portion around the puncture point. The brain tissue samples from the ipsilateral basal ganglia were instantly weighed on an electronic analytical balance (BSAl24SCW, Sartorius Scientific instrument, Beijing, China), to obtain the wet weight. Afterward, the tissues were dried in an oven at 100°C for 24 hours to obtain the dry weight. The brain water content (%) was calculated with the following formula: (wet weight − dry weight)/wet weight × 100%.

Nissl Staining

The brain tissues of the perihematoma area was fixed in 4% paraformaldehyde at 4°C overnight, and then dehydrated in 30% sucrose solution before being embedded in optimal cutting temperature compound (Sakura, Tokyo, Japan). The 10-μm coronal sections from the rostral to the caudal portion of the damaged brain areas, which were spaced 200 μm apart, were washed with phosphate-buffered saline (PBS) and incubated with Nissl staining solution (Beyotime Institute of Biotechnology, Shanghai, China) at 40°C for 10 minutes. The sections were then washed with 95% ethyl alcohol, 70% ethyl alcohol in secession. Images were captured by a light microscope and quantitative analysis was performed by ImageJ software (NIH, MD).

FJB Staining

The number of degenerating neurons was determined by FJB staining. After washing with PBS twice, the sections of the brains in the range of 200 μm around the point of the puncture were immersed in .0004% FJB working solution for 10 minutes and then observed under a fluorescence microscope (IX71, Olympus Corporation, Tokyo, Japan). Quantitative analysis was performed blindly by counting the number of FJB-positive cells in the basal ganglia surrounding the hematoma with ImageJ software.

Oxidative Stress Measurement

Perihematoma basal ganglia were collected from each group and homogenized on ice and then centrifuged for 5 minutes at 5000 × g, 4°C. The concentrations of glutathione peroxidase (GPx), superoxide dismutase (SOD), malondialdehyde (MDA), and nitric oxide (NO) in the homogenate were determined by commercially available kits (Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's protocols. The activity of NADPH oxidase was measured by NADPH Assay Kit. For the determination of total ROS generation, brain sections were incubated with 2′,7′-Dichlorofluorescin diacetate (10 μmol/L) for 30 minutes at 37°C in dark. After washing with PBS, the images were taken with a fluorescence microscope at 488 nm excitation and 525 nm emission wavelengths. To assess the mitochondrial ROS generation (mROS), the sections were stained MitoSOX Red (5 μmol/L) in dark at 37°C for 30 minutes and then were visualized under a fluorescence microscope at 510 nm excitation and 580 nm emission wavelengths. The fluorescence intensity was quantified with ImageJ software.

ATP Determination

ATP concentration in perihematoma basal ganglia was examined by ATP Colorimetric Assay Kit (Biovision CA) according to the manufacturer's instructions. The optical density value was read at 570 nm using a microplate reader (Bio-Tek, VT). The concentration of ATP was calculated based on the standard curve.

Western Blotting

The perihematoma basal ganglia were washed with PBS and then lysed in radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology) containing .1% protease and phosphatase inhibitors (Pierce Biotechnology, IL). The protein concentration of each sample was determined by a bicinchoninic acid kit (Bio-Rad, CA). Samples were electrophoresed on 8%-10% SDS-PAGE gels and then transferred onto nitrocellulose membranes (Millipore, MA). The membranes were blocked in 5% skim milk and probed with the primary antibodies as follows: peroxisome proliferator-activated receptor gamma coactivator 1-α (PGC-1α) (1:500) (Abcam, MA), uncoupling protein 2 (UCP2), manganese-dependent superoxide dismutase (MnSOD), and GAPDH (1:1000) (Santa Cruz Biotechnology, CA). Afterward, the blots were visualized by HRP conjugated secondary antibodies (Beyotime Institute of Biotechnology) using enhanced chemiluminescence kit according to the manufacturer's instructions (Amersham Pharmacia, NJ). The intensity of each band was quantified by ImageJ software. GAPDH was used as an internal loading control.

Statistical Analysis

All data were expressed as mean ± SEM. n value represented the number of animals. One-way or 2-way ANOVA followed by Bonferroni multiple comparison test was used to compare differences among the groups. Kruskal-Wallis test was used to analyze the behavioral data. $P < .05$ was considered to be statistically significant.

Results

Dex Ameliorates ICH-Induced the Neurological Deficits

To investigate the effects of Dex on neurobehavioral outcomes in ICH mice, several kinds of behavior tests were performed. Morriz water maze test showed that Dex

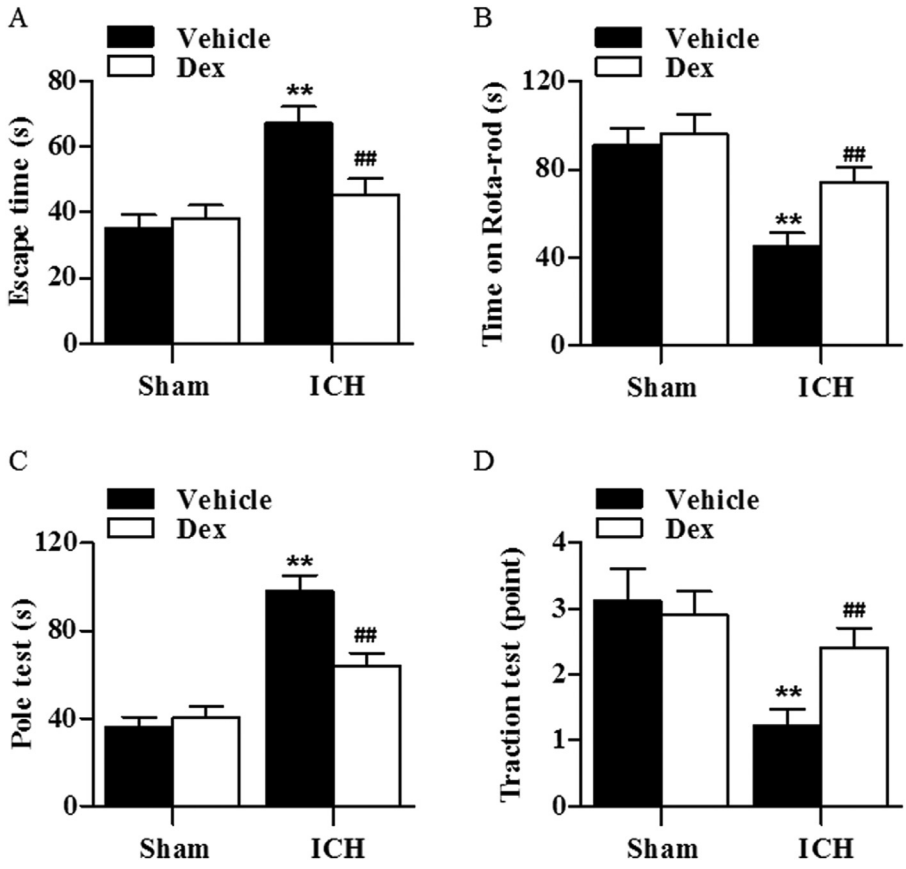

Figure 1. ***Dexmedetomidine (Dex) improves ICH-induced dyskinesia.*** *(A) Sham or ICH mice were intraperitoneally injected with Dex (50 μg/kg) or vehicle for 48 hours. The learning ability and spatial memory were examined by Morris water maze test. (B and C) The motor function of mice was assessed by rota-rod test (B) and string test (C). (D) The limb movements were determined and scored by traction test. All data were present as mean ± SEM.* $^{**}P < .01$ *versus sham + vehicle;* $^{\#\#}P < .01$ *versus ICH + vehicle, n = 10 per group.*

administration had no effect on the ability of mice to find the platform under sham conditions. After ICH surgery, mice took significantly longer time to reach the platform in vehicle-treated group, but not in Dex-treated group (Fig 1, A). The rota-rod test revealed that ICH significantly impaired the performance, which was attenuated in Dex-treated mice (Fig 1, B). Moreover, ICH-induced the increase in total locomotor activity was also ameliorated by Dex treatment, as evidenced by pole test (Fig 1, C). In addition, a significant decrease in the scores of traction test was observed in ICH mice compared with the sham-operated mice. This decrease was markedly inhibited after Dex treatment (Fig 1, D).

Dex Inhibits ICH-Induced Brain Edema and Neuronal Loss

Brain edema is considered an important index for assessing ICH severity.[14] Here, we determined brain water content 48 hours after ICH to observe the effects of Dex on ICH-induced brain edema. The results showed that ICH evoked a significant increase in brain water content. However, Dex treatment was associated with reduced brain water content (Fig 2, A). We also examined the effects of Dex on neuronal activity. Intrastriatal injection of autologous whole blood resulted in a marked decrease in nissl substance, and treatment with Dex attenuated this neuronal loss (Fig 2, B and C). Similarly, FJB staining revealed that the number of degenerating neurons was increased after ICH surgery. The increase in FJB-positive neurons was remarkably attenuated after Dex treatment (Fig 2, D and E).

Dex Limits ICH-Induced the Mitochondrial Dysfunction-Derived Oxidative Stress

Given that oxidative stress plays a critical role in the pathogenesis of ICH,[3] we next investigated the effects of Dex on oxidative stress. There were no significant differences in the levels of GPx, SOD, MDA, and NO between sham vehicle-treated and Dex-treated mice. Two days after ICH surgery, GPx and SOD levels were significantly decreased, while MDA and NO levels were increased compared with sham mice. However, treatment with Dex markedly inhibited the decrease in GPx and SOD levels and increase in MDA and NO levels (Fig 3, A-D). Similarly, Dex treatment significantly inhibited ICH-induced the increase in intracellular ROS production (Fig 3, E and F).

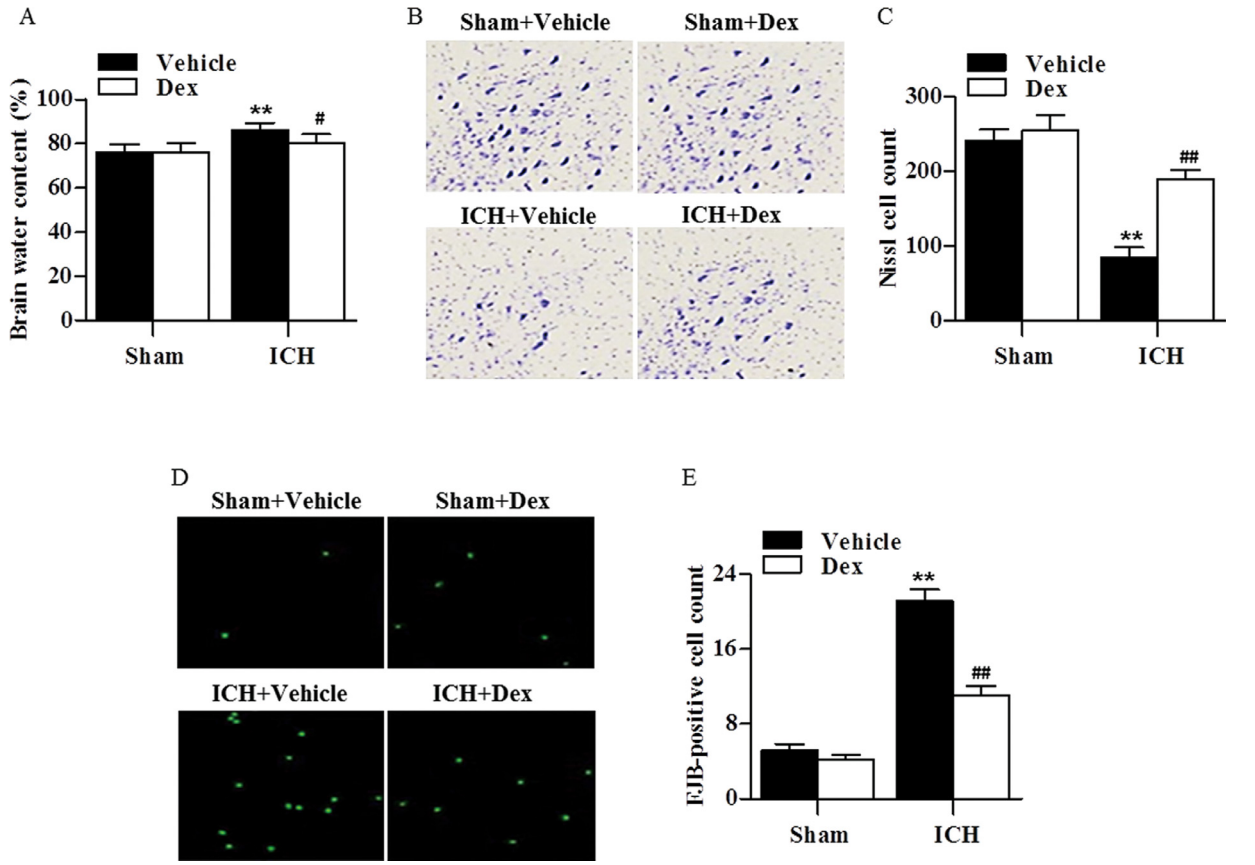

Figure 2. ***Dex prevents ICH-induced brain edema and neuronal loss.*** *(A) Brain water content was measured. n = 6 per group. (B) The nissl staining in brain sections. (C) Representative images were shown. (D) The degenerating neurons were stained by Fluoro-Jade B (FJB) and the representative images were shown. (E) Quantitative analysis of FJB-positive cells. n = 8 per group.* $^{**}P < .01$ *versus sham + vehicle;* $^{\#}P < .01$, $^{\#\#}P < .01$ *versus ICH + vehicle.*

Notably, Dex had no effects on the increased NADPH oxidase activity induced by ICH (Fig 3, G). Moreover, MitoSOX Red staining revealed that ICH-induced the increase in mROS production was dramatically inhibited by Dex (Fig 3, H and I), indicating Dex limits oxidative stress mainly via inhibiting mitochondria-derived ROS production. Further, ICH led a decrease in ATP concentration, and this decrease was attenuated by Dex (Fig 3, J). Taken together, these results suggest that Dex prevents ICH-induced mitochondrial dysfunction and subsequently decreases oxidative stress.

Induction of mROS Abolishes Dex-Mediated the Inhibition of Neurological Deficits and Oxidative Stress

To confirm the importance of mROS in the effects of Dex on neurological function, Dex-treated mice were administrated with mROS stimulator rotenone. The results showed that rotenone administration had no effects on spatial memory, locomotor activity, and limb movements in sham Dex-treated mice. After ICH surgery, rotenone markedly abrogated the improvement of performance in Morriz water maze test, rota-rod test, pole test, and traction test in Dex-treated mice (Fig 4, A-D). Moreover, the inhibitory effects of Dex on oxidative stress were completely abolished by rotenone administration (Fig 4, E-H). These data indicate that inhibition of mROS may underlie the protective action of Dex in neurological function.

Dex Reverses ICH-Induced the Decrease in PGC-1α, UCP2, and MnSOD Expression

PGC-1α signaling plays a key role in mitochondrial biogenesis and oxidative stress. We next investigated whether Dex inhibits ICH-induced mitochondrial dysfunction via regulating PGC-1α signaling. Western blotting results showed that intrastriatal injection of autologous blood significantly decreased PGC-1α and its downstream UCP2 and MnSOD expression. However, Dex treatment was associated with restoration of these genes expression (Fig 5, A-C).

Discussion

The protective role of Dex has been well characterized in preclinical studies in a wide range of organ system.[10-12,15] In this study, we utilized autologous blood injection model to investigate the effects of Dex on ICH in mouse. Our results showed that Dex

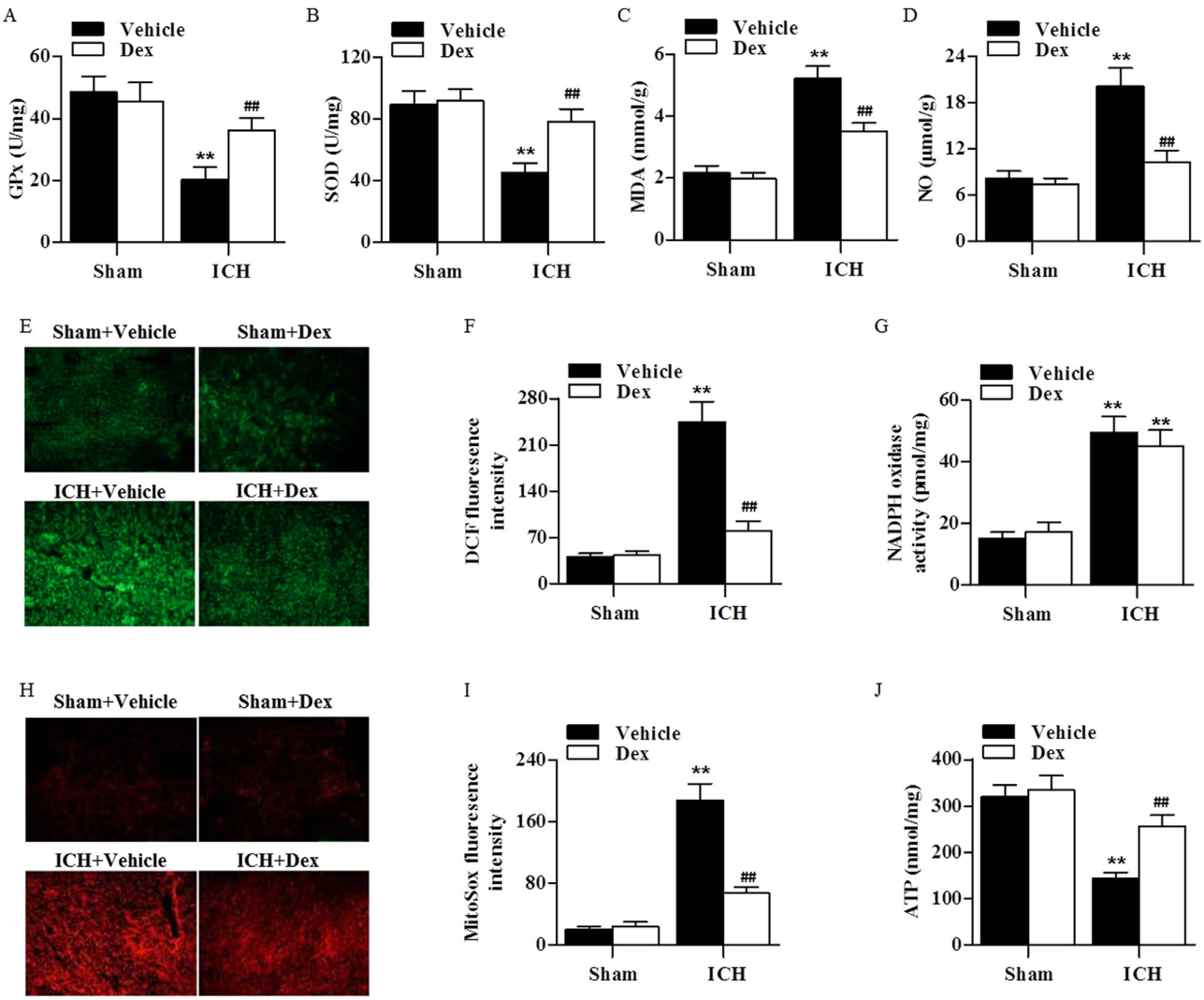

Figure 3. ***Dex inhibits ICH-induced the mitochondrial dysfunction-assocaited oxidative stress.*** *(A-D) The oxidative stress indicators glutathione peroxidase (GPx) (A), superoxide dismutase (SOD) (B), malondialdehyde (MDA) (C), and nitric oxide (NO) (D) concentrations in brain tissues were determined by commercial kits as described in method section. (E) Intracellular ROS production in brain sections was assessed by H_2DCF-DA staining. (F) Quantitative analysis of ROS fluorescence intensity. (G) The activity of NADPH oxidase was measured. (H) Mitochondrial ROS generation was determined using MitoSOX Red staining. (I) Quantitative evaluation of MitoSOX fluorescence intensity. (J) ATP synthesis was determined by ATP colorimetric assay kit.* $^{**}P < .01$ *versus sham + vehicle;* $^{\#}P < .01$, $^{\#\#}P < .01$ *versus ICH + vehicle. n = 6 per group.*

improves the neurological outcome after ICH, and also ameliorates brain injury. This is the first report to demonstrate that the Dex-induced neuroprotection may be associated with inhibition of mitochondrial dysfunction-mediated oxidative stress.

It is widely accepted that neurological deficit is one of main classic characteristics in the early stage of ICH.[1,14] In the current study, ICH mice produced memory impairment and behavior disorder, as evidenced that Morriz water maze test, rota-rod test, pole test, and traction test. However, administration with Dex decreased the time to find the platform, prolonged the duration of mice on the rota-rod, and increased the grasping force and the rate of climbing pole. Furthermore, we investigated whether Dex improves dyskinesia through protective against ICH-induced brain injury. Brain edema is a remarkable phenotype during ICH and the leading fatal cause of death.[16] We found that Dex significantly inhibited ICH-induced the increase in brain water content, indicating that Dex prevents against blood-brain barrier breakdown. Moreover, ICH is also known to decrease neuronal activity and thereby induce neuron degeneration.[2,17,18] Both nissl and FJB staining showed that Dex treatment in ICH mice could increase neuronal activity and inhibit neuron loss. The results demonstrate that Dex can protect against brain injury in ICH mice. This concurs with neuroprotection of Dex in hypertensive cerebral hemorrhage,[9] and subarachnoid haemorrhage.[12,19] In clinic, for the procedural sedation of Precedex, a loading dose of 1 μg/kg in 10 minutes followed by a maintenance infusion of .6 μg/kg/h is titrated to the desired clinical effect with doses ranging from .2 to 1 μg/kg/h. The time

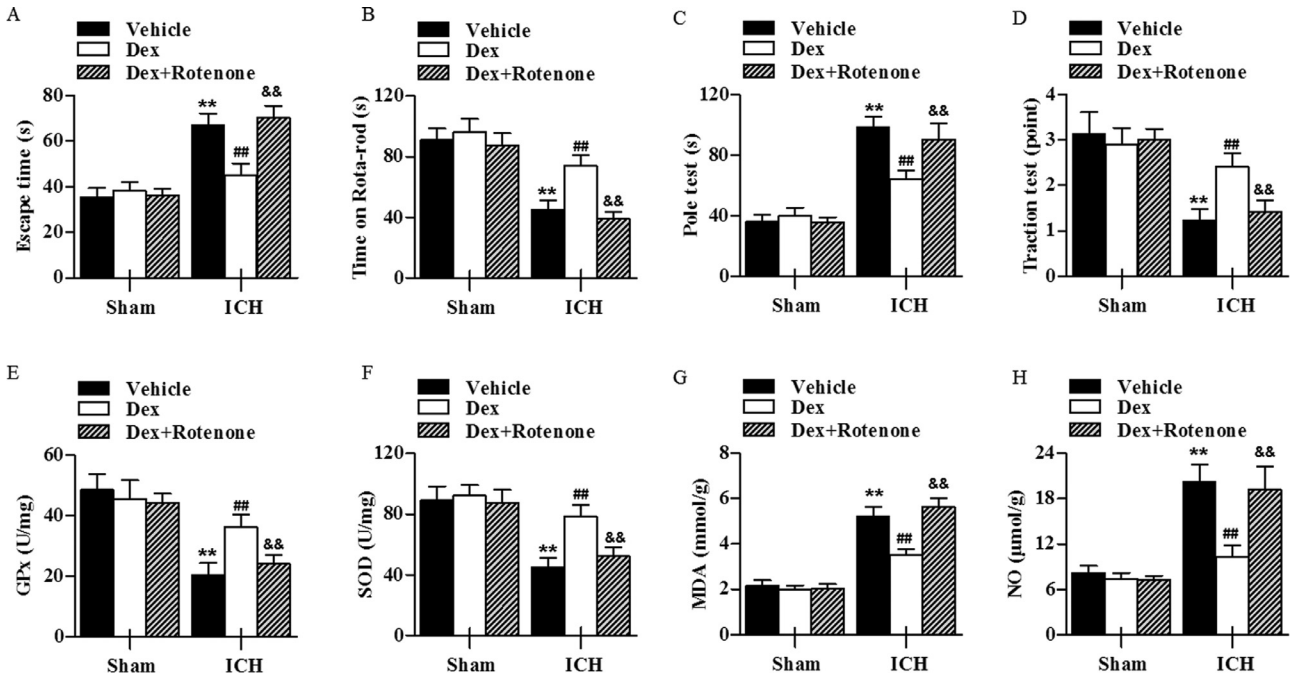

Figure 4. ***The effects of Dex on ICH-induced neurological dysfunction are abolished by mitochondrial ROS induction.*** *(A-D) Dex-treated sham or ICH mice were injected intraperitoneally with rotenone at a dose of 500 μg/kg. Neurological deficits were assessed by Morris water maze test (A), rota-rod test (B), string test (C), and traction test (D). n = 8 per group. (E-H) The oxidative stress indicators glutathione peroxidase (GPx) (E), superoxide dismutase (SOD) (F), malondialdehyde (MDA) (G), and nitric oxide (NO) (H) concentrations in brain tissues were determined by commercial kits as described in method section. n = 6 per group.* $^{**}P < .01$ *versus sham + vehicle;* $^{\#\#}P < .01$ *versus ICH + vehicle;* $^{\&\&}P < .01$ *versus ICH + Dex.*

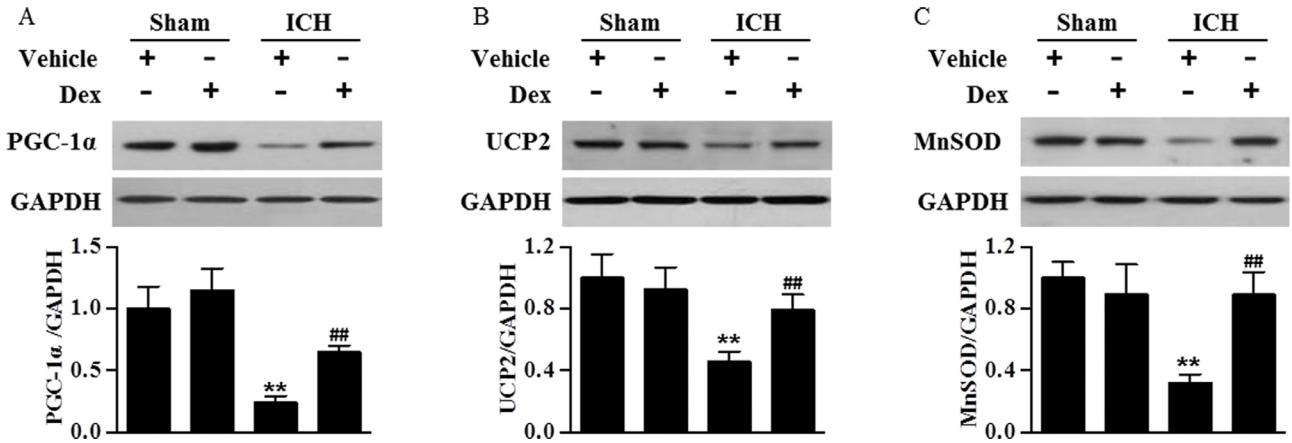

Figure 5. ***Dex attenuates ICH-induced the decrease in PGC-1α, UCP2 and MnSOD expressions.*** *(A-C) Brain tissues from sham and ICH mice with vehicle or Dex treatment were analyzed for PGC-1α (A), UCP2 (B), and MnSOD (C) protein expressions using western blotting.* $^{**}P < .01$ *versus sham + vehicle;* $^{\#\#}P < .01$ *versus ICH + vehicle, n = 6 per group.*

of infusion ranges from .1 to 6.2 h, and the total dosages are from .5 to 6.7 μg/kg approved by the Food and Drug Administration, which for a mouse will be less than 60 μg/kg. Additionally, the concentration of Dex was selected according to a previous publication, which has been demonstrated to effectively improve brain injury-induced acute lung injury.[20]

The most significant finding of this study was that Dex was able to decrease the level of oxidative stress. Accumulating evidences have indicated that oxidative stress plays an important role in the pathogenesis of neurological diseases.[3-6] Excessive production of ROS causes caused oxidative damage in brain, accompanied by decreased GPx and SOD, and increased MDA and NO levels.[21] In this study, along with ICH-induced injury, MDA and NO levels and ROS production was expectedly increased in the brain, whereas GPx and SOD levels were reduced. The above alterations were inhibited by Dex treatment. These results were consistent with the discoveries that Dex could reduce oxidative stress and subsequently exerts

protective effects against neurotoxicity, myocardial ischemia/reperfusion injury, and kidney injury.[22-24] A recent study showed that Dex ameliorated traumatic brain injury in rats via the PGC-1α signaling pathway.[22] In addition, Chen et al reported that the reduced oxidative stress by Dex was associated with inhibition of the JNK signaling pathway.[24] Here, given that NADPH oxidase and mROS are the 2 major sources of intracellular ROS production,[25] we thus examined the effect of Dex on NADPH oxidase activity and mROS production. Our data showed that inhibition of mitochondria-derived ROS may contribute to the effects of Dex on total ROS production and neurological deficits, because Dex treatment largely abolished ICH-induced the increase in mROS production. However, induction of mROS using rotenone abrogated the effects of Dex on neurological dysfunction. Although several studies have reported that rotenone could induce neurotoxicity for the establishment of Parkinson's disease mouse model at the concentration ranging from 5 to 30 mg/kg,[26-28] our pilot experiment showed that rotenone ranging from 100 to 1000 ug/kg did not cause any neurological deficits (such as Parkinson's symptoms) under basal condition (data were not shown). Moreover, in this study, we also evidenced that rotenone had no effects on spatial memory, locomotor activity, limb movements, and oxidative stress in sham mice. Thus, the dosage of rotenone at 500 ug/kg was selected.

Another significant finding of this study was that Dex inhibited mitochondrial dysfunction and subsequent oxidative stress by inducing PGC-1α activation. It is well documented that mitochondrial dysfunction plays a critical role in sustained oxidative stress.[5,25] Moreover, dysregulation of mitochondria biogenesis is the major contributor of mitochondrial dysfunction.[3,25] This is also supported by a previous study reporting that restriction of calorie intake can increase mitochondrial biogenesis and lifespan, concomitantly with high ATP production and yet lower oxidative stress.[29] In agreement with this study, we found that Dex also restored ICH-induced the decrease in ATP synthesis. PGC-1α is a well-characterized regulator of mitochondrial biogenesis and cell metabolism.[30-32] Deficiency of PGC-1α can induce mitochondrial dysfunction and oxidative stress.[33,34] In this study, we found that Dex treatment reversed ICH-induced the decrease in PGC-1α expression and downstream oxidative stress-protective molecules UCP2 and MnSOD expressions, suggesting that the restoration of PGC-1α pathway activation may be the underlying mechanism by which Dex improves mitochondrial dysfunction.

In conclusion, our results demonstrate that Dex attenuates PGC-1α pathway inactivation and mitochondrial dysfunction, which in turn inhibits oxidative stress and ameliorates neurological deficits and brain injury in ICH mice. These findings provide a novel understanding of the neuroprotective effect of Dex.

Supplementary Material

Supplementary data to this article can be found online at doi:10.1016/j.jstrokecerebrovasdis.2019.01.016.

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