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δ-Opioid Receptor-Nrf-2-Mediated Inhibition of Inflammatory Cytokines in Neonatal Hypoxic-Ischemic Encephalopathy

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Abstract

Neonatal hypoxic-ischemic encephalopathy (HIE) causes serious neurological disability; there are, however, currently few promising therapies for it. We have recently shown that δ-opioid receptor (DOR) is neuroprotective by downregulating TNF-α. Since hypoxia-ischemia (HI) triggers a robust inflammatory response, which exacerbates HI brain damage, we investigated, in this study, whether DOR activation could regulate inflammatory cytokine expression, thereby playing a protective effect on the neonatal brain under HI. Twenty-five neonatal rats were randomly divided into five groups: (1) control (control); (2) HI; (3) HI with saline (HI + NS); (4) DOR activation with UFP-512 (a potent and specific DOR agonist) under HI conditions (HI + U); and (5) DOR inhibition using NT treatment under HI conditions (HI + NT). The rats were sacrificed by decapitation at 24 h after HI, and their brains were rapidly removed for measurements. The protein expression of TNF-α, IL-6, ICAM-1, IL-10, IL-18, NQO-1, Nrf-2, and HO-1 was measured using Western blot. In the hemispheres exposed to HI, DOR activation significantly decreased the expressions of TNF-α, IL-6, and ICAM-1 in the cortex, while it significantly increased IL-10 and had no effect on IL-18 in the same region. In contrast, DOR had no appreciable effect on inflammatory cytokine expression in non-cortical tissues including hippocampal, subcortical, and cerebellar tissues. Moreover, HI stress triggered an upregulation of Nrf-2 nuclear protein as well as some of its downstream anti-inflammatory genes such as HO-1 and NQO-1 in the cortex, while DOR activation further augmented such a protective reaction against HI injury. DOR plays an important role in protecting against HI by regulating the expression of inflammatory and anti-inflammatory cytokines in the cortex, which is likely mediated by the Nrf-2/HO-1/NQO-1 signaling.

Keywords Hypoxic-ischemic encephalopathy · δ-Opioid receptor · Inflammatory cytokines · Nrf-2

Introduction

Hypoxic-ischemic encephalopathy (HIE) is considered the single most important cause of brain injury leading to neonatal encephalopathy and mortality in newborn individuals worldwide. However, neuroprotective therapies against hypoxic-ischemic (HI) injury remain limited, despite considerable research efforts that have been made over the past several decades [1, 2].

There is increasing evidence to show that the δ-opioid receptor (DOR) is neuroprotective against HI injury [3–7]. DOR is an oxygen-sensitive protein [8] and widely distributed in the mammalian central nervous system, especially in the cortex and the striatum [9, 10]. Our recent serial studies with molecular, transgenic, and electrophysiological techniques well support the role of DOR in neuroprotection against HI insults in different models, including cultured neurons, cortical brain slices, and in vivo brain [11–16]. However, the underlying mechanisms of DOR-induced neuroprotection are not yet well understood. Moreover, it is unknown if DOR signals have any effect on HIE. Since we have recently found that DOR activation downregulates TNF-α expression in the hypoxic neurons [17], suggesting a potential role of DOR signaling in inhibitory regulation of hypoxic/ischemic expression of

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inflammatory cytokines, we asked whether DOR activation can inhibit inflammatory cytokines in HIE.

Hypoxia/ischemia triggers a robust inflammatory response, which exacerbates brain damage [18]. Neonatal HI activates the immune system in the blood, peripheral organs, as well as in the brain. Indeed, there is an increased production of pro-inflammatory cytokines following neonatal brain injury [19, 20]. However, there is no data available as to whether DOR affects the inflammatory pathogenesis of neonatal HI brain injury. In this study, therefore, we investigated the effect of DOR on inflammatory cytokine expression and its possible mechanism in neonatal brain tissues under HI. To our knowledge, this is the first experiment of its kind in this field.

Materials and Methods

Chemicals and Reagents

UFP-512 (H-Dmt-Tic-NH-CH(CH2–COOH)-Bid), a specific and potent DOR agonist [21], was synthesized by our research team [22–25]. Naltrindole (NT) was purchased from Sigma-Aldrich (Cat: N115-10MG). Interleukin-10 (IL-10) Mouse anti-Rat Monoclonal Antibody, Interleukin-6 (IL-6) Mouse anti-Rat Monoclonal Antibodies, and Goat anti-Mouse IgG (H + L) Secondary Antibodies were purchased from Invitrogen (Cat: ARC9102, ARC0962, G-21040). Anti-tumor necrosis factor-α (TNF-α) antibodies, anti-intercellular adhesion molecules-1 (ICAM-1) antibodies, anti-interleukin-18 (IL-18) binding protein antibodies, and Goat anti-Rabbit IgG H&L (HRP), were purchased from Abcam Inc. (Cat: ab66579, ab124760, EP1088Y, ab6721). NADPH quinone oxidoreductase (NQO-1), nuclear factor erythroid 2-related factor-2 (Nrf-2), and heme oxygenase-1 (HO-1) Antibodies were purchased from Santa Cruz Biotechnology (Cat: sc-16464, sc-722, sc-1796). Rabbit monoclonal anti-β-actin and Lamin B1 were obtained from Cell Signaling Technology (Cat: 8457S, 13435S). NE-PER Nuclear and Cytoplasmic Extraction Reagents were obtained from Pierce (Cat: 78833).

Induction of Hypoxia-Ischemia

Timed pregnant SD rats (Charles River Laboratories, Wilmington, DE, USA) at the age of 30 days old (P30) were kept under a 12-h light/12-h dark cycles and 22 °C with standard rodent chow ad libitum. Neonatal HI was induced in male rats weighing 18–22 g at postnatal day 7 (P7) according to the method described by Rice et al. [26], with some modifications. In total, 25 neonatal rats were randomly divided into five groups: (1) control (control, $n = 5$); (2) HI (HI, $n = 5$); (3) HI with saline (HI + NS, $n = 5$); (4) DOR activation using UFP-512 treatment under HI conditions (HI + U, $n = 5$); and (5) DOR inhibition using NT treatment under HI conditions (HI + NT, $n = 5$). Saline, UFP-512 (5 mg/kg, dissolved in 0.9% saline) or NT (5 mg/kg, dissolved in 0.9% saline) was administered by an intraperitoneal injection immediately before the beginning of the procedure. Then, rats were anesthetized with halothane (3.0% for induction, 1.5% for maintenance) in room air, and the left common carotid artery was isolated and ligated with 6-0 surgical silk. The procedure was completed within 10 min. The rectal temperature was maintained at 37 °C during the procedure and was controlled by a water blanket placed under the body. To expose rats to hypoxic stress, rats were placed in a plexiglass chamber (30″W × 20″D × 20″H) (Biospherix, Redfield, NY, USA). The chamber was connected with the outside environment via holes in the wall of the chamber; therefore, CO_2 levels and humidity in the chamber were kept constant at their ambient levels. O_2 levels in the chamber were strictly kept at (8 ± 0.5) % by constantly flushing with nitrogen that was automatically controlled by a ProOx P110 Oxygen Controller with an E702 Oxygen Sensor (Biospherix, Redfield, NY, USA). The rats were exposed to hypoxia for 1.5 h before being returned to their mothers. The rats was put in a temperature-controlled incubator for maintaining the rectal temperature 37 °C during the whole procedure. The control animals received a sham operation that consisted of left carotid artery exposure without ligation and exposure to hypoxia. All animal procedures were approved by the University of Texas medical school at Houston Animal Care and Use Committee and in accordance with the National Institutes of Health Guide for the Care and Use of Animals in Research.

Tissue Sampling

Rats were sacrificed by decapitation at 24 h after HI, and their brains were rapidly removed for experimental measurements. The cortical, subcortical containing striatum and thalamus, cerebellar, and hippocampal tissues were dissected on ice, frozen immediately on dry ice, and then stored at − 80 °C until use.

Western Blot Analysis

The cortical, subcortical, cerebellar, and hippocampal tissues were ultrasonically homogenized in a RIPA buffer and protease inhibitor cocktail, and the homogenates were centrifuged at 12,000×*g* for 10 min at 4 °C. To analyze the effects of DOR activation or inhibition on cortical nuclear localization of Nrf-2, NE-PER nuclear and cytoplasmic extraction reagents (Pierce Chemical Company, USA) were used to prepare cytoplasmic and nuclear extracts from cortical tissues. The protein concentration of the lysates was then determined using a BCA protein assay kit (Pierce, Rockford, IL, USA). The supernatants of tissue homogenates (40 μg protein equivalent each) of

the cortex, subcortex, cerebellum, and hippocampus from each mouse were boiled at 100 °C in a laemmli sample buffer (Abcam, Inc., Cambridge, MA, USA) for 5 min. Then, the samples were electrophoresed on 15% sodium dodecyl sulfate-polyacrylamide gel, and transferred to a 0.20-μm nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). Membranes were blocked with 5% (*m*/*v*) nonfat dry milk in 0.1% Tween 20 (TBS-T; 2 mmol/L Tris-HCl, 50 mmol/L NaCl, pH 7.4) for 1 h at room temperature and subsequently incubated overnight at 4 °C in the blocked buffer with β-actin (1:1000), TNF-α (1:500), IL-6 (1:500), ICAM-1 (1:250), IL-10 (1:250), IL-18 (1:500), NQO-1 (1:500), Nrf-2 (1:1000), HO-1 (1:250), and Lamin B1 (1:1000) antibodies, respectively. The membranes were washed with 0.1% Tween 20, and then treated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (1:10,000) for 1 h at room temperature. The blot was visualized using an enhanced chemiluminescence (ECL) Western blotting detection kit (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and exposed to X-ray film. The proteins were quantified by measuring optical densities of immunostained bands using an image analysis system (Image J; NIH, Bethesda, MD, USA). Every sample was analyzed three times, and the average value used was $n = 5$.

Statistical Analysis

All values are expressed as mean ± SEM. Student's paired *t* test was used to compare values between the left and right hemispheres of each group. One-way ANOVA, followed by post hoc Student-Newman-Keuls (SNK) test was used to compare ipsilateral hemispheres between various groups. Statistical analysis was performed using SPSS 19.0 statistical software package (SPSS, Chicago, IL, USA). Statistical significance was determined based on P values < 0.05.

Results

We focused our studies specifically on TNF-α, IL-6, ICAM-1, IL-10, and IL-18 in this work since these cytokines are differentially involved in brain responses to HI stress [27, 28].

Effects of DOR Activation/Inhibition on Cortical Inflammatory Cytokine Protein Expression Under HI

DOR is richly expressed in the cortex [9, 10, 29]. Therefore, we first assessed the role of DOR in the regulation of inflammatory cytokines in the cortex under HI (Fig. 1). Under naive control conditions, all the inflammatory cytokine proteins we were studying had equivalent expression levels between the left and right hemispheres ($P > 0.05$). On the other hand, their levels were significantly increased in the left hemisphere at 24 h after HI in comparison to the levels in the right hemisphere without HI stress ($P < 0.05$). These results suggest that HI increases the level of these cytokine proteins in our neonatal model.

In the left hemisphere, which was exposed to HI, DOR activation using UFP-512 treatment (HI + U) significantly decreased the expression of TNF-α, IL-6 and ICAM-1 ($P < 0.05$), while DOR inhibition using NT treatment (HI + NT) significantly increased their expression ($P < 0.05$). In contrast, DOR activation induced an opposite effect on the expression of IL-10 ($P < 0.05$) and had no appreciable effect on IL-18 ($P > 0.05$) (Fig. 1). In the control group (HI + NS), however, no significant difference was detected after the vehicle treatment ($P > 0.05$, HI vs. HI + NS). This data suggests that DOR differentially regulates the expression of cortical inflammatory cytokines.

Effects of DOR Activation/Inhibition on Inflammatory Cytokine Protein Expression in Hippocampal, Subcortical, and Cerebellar Tissues Under HI

The density of DOR is much lower in non-cortical regions than in the cortex [6–8]. We therefore studied the responses of non-cortical brain tissues to HI and DOR activity and compared the data to those of the cortex. We specifically chose three typical non-cortical tissues for this work. Similar to the cortical tissues, the hippocampal (Fig. 2), subcortical (Fig. 3), and cerebellar (Fig. 4) tissues had a significant increase in the expressions of TNF-α, IL-6, ICAM-1, IL-10, and IL-18 in the left hemispheres after HI ($P < 0.05$), and the vehicle treatment did not induce any appreciable change in their expression levels ($P > 0.05$). Unlike in the cortex, however, the HI-induced cytokine expression did not respond to the manipulation of DOR activity. As shown in Figs. 2, 3 and 4, neither DOR activation nor inhibition had an influence on the expression of these cytokines in the HI-treated hemisphere ($P > 0.05$). This data suggests that in spite of having similar responses to HI stress to that of the cortex, the response of non-cortical tissues to DOR activity is very different from that of the cortex, suggesting that DOR plays a small role in the regulation of inflammatory cytokine expression in these non-cortical tissues.

Effects of DOR Activation/Inhibition on Cortical Nuclear Localization of Nrf-2, HO-1, and NQO-1 Protein Expression Under HI

Since recent evidence suggests that DOR promotes the relocation of Nrf-2 from the cytoplasm to the nucleus and, thus, induces cytoprotection [3], we investigated whether the Nrf-2 pathway helps to cause the DOR effects to explore the mechanism underlying the DOR regulation of HI-induced cytokine expression. We examined the nuclear localization of Nrf-2 and the expression of HO-1 and NQO-1, which are the

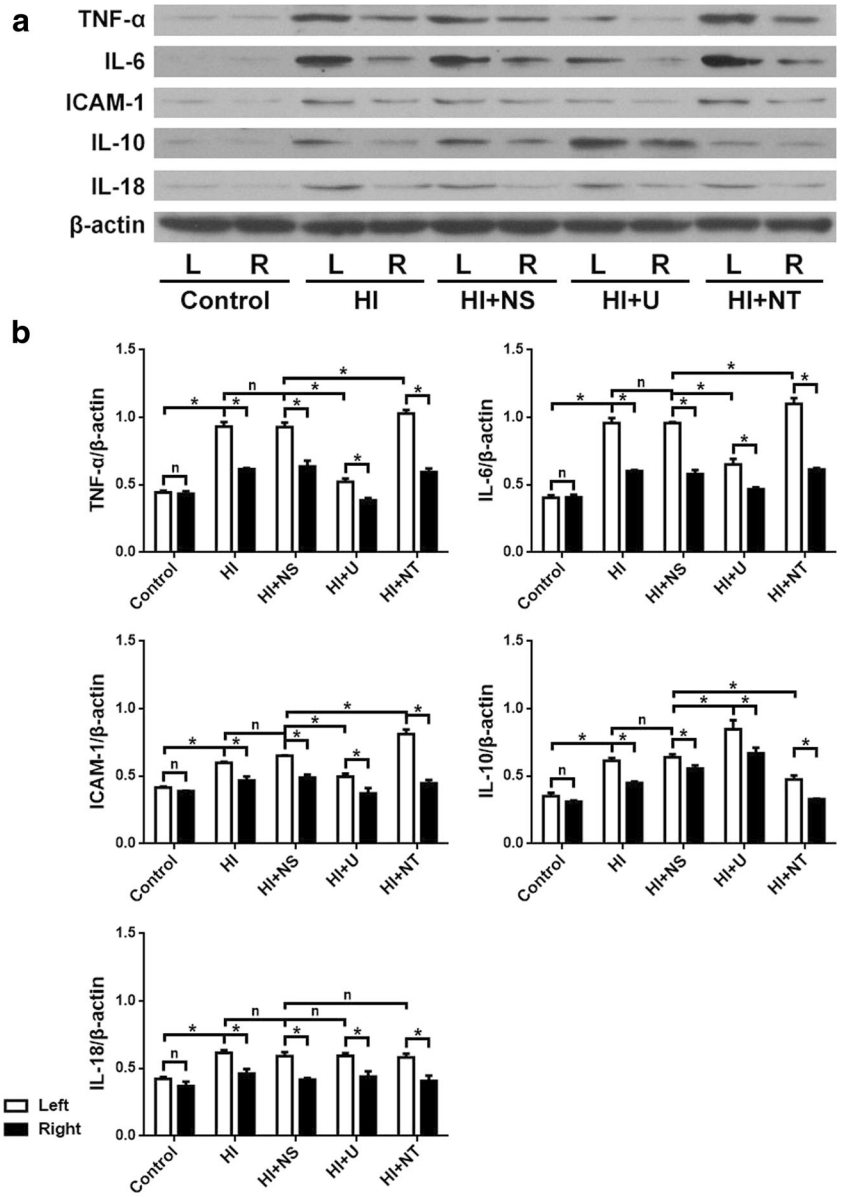

Fig. 1 Effects of DOR activation/inhibition on cortical inflammatory cytokine protein expression under HI. Protein expression levels of TNF-α, IL-6, ICAM-1, IL-10, and IL-18 in the cortex did not show any significant difference between the left and right hemispheres in control groups ($^nP > 0.05$), while they increased significantly in the left hemispheres in all other groups ($^*P < 0.05$). The levels of expression of these inflammatory cytokines in the left hemispheres were significantly increased in both the HI and the HI + NS groups in comparison to the control group ($^*P < 0.05$). Their levels of expression in the left hemispheres were not significantly different between the HI and the HI + NS group ($^nP > 0.05$). When compared with the HI + NS group, activating DOR using UFP-512 treatment under HI conditions (HI + U) significantly decreased the levels of expression of TNF-α, IL-6, and ICAM-1 ($^nP > 0.05$), while inhibiting DOR using naltrindole treatment (HI + NT) significantly increased their levels of expression ($^nP > 0.05$) in left hemispheres. Activating and inhibiting DOR had opposing effects on the levels of expression of IL-10 ($^*P < 0.05$) and no effect on the levels of expression of IL-18 ($^nP > 0.05$). Note that HI increased these cytokine proteins in the neonatal model, and that DOR differentially regulates their levels of expression in cortical tissues

downstream target genes of Nrf-2 for the regulation of inflammatory reactions to HI stress, and assessed their responses to DOR activation or inhibition.

Interestingly, HI significantly increased Nrf-2 nuclear protein in both HI (left) and non-HI (right) cortices (Fig. 5), which is very different from the changes between cytokines in both cortical and non-cortical tissues. DOR activation by pretreatment using UFP-512 before HI further elevated the level of Nrf-2 nuclear protein by 70% in the HI-exposed cortex ($P < 0.05$). On the other hand, DOR inhibition using the pretreatment with NT completely abolished the HI- and DOR-induced increases in Nrf-2 nuclear protein. In the control, the vehicle treatment did not have any significant effect on Nrf-2 nuclear protein levels.

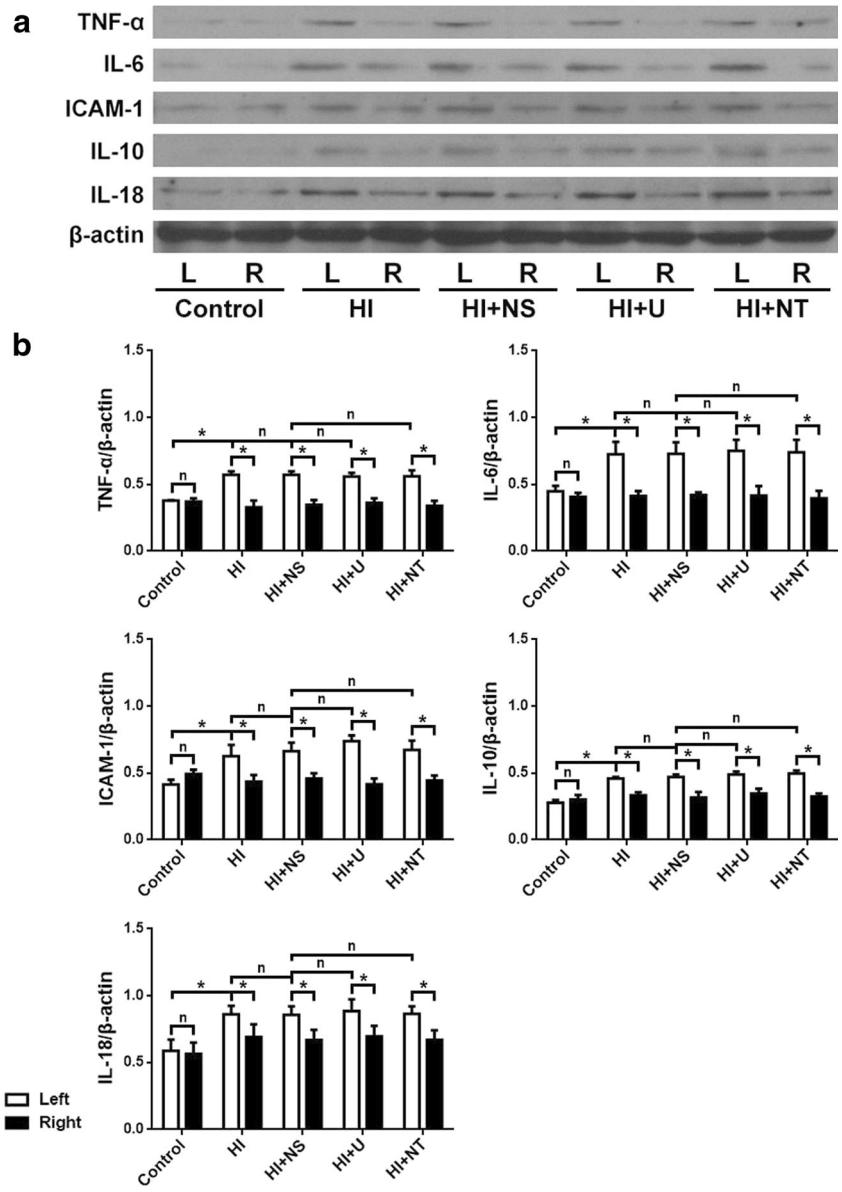

Fig. 2 Effects of DOR activation/inhibition on inflammatory cytokine protein expressions in hippocampal tissues under HI. The levels of expression of TNF-α, IL-6, ICAM-1, IL-10, and IL-18 in hippocampal tissues significantly increased in the left hemispheres in the HI, HI + NS, HI + U, and HI + NT groups (*$P <$ 0.05). The levels of expression of these inflammatory cytokines in left hemispheres were significantly increased in both the HI and the HI + NS groups in comparison to the control group (*$P <$ 0.05). Their levels of expression were not significantly different in left hemispheres between the HI and the HI + NS groups ($^{n}P > 0.05$). Neither DOR activation nor DOR inhibition had an effect on the levels of expression of these inflammatory cytokines in left hippocampal tissues in comparison to the HI + NS group ($^{n}P > 0.05$). Note that DOR had no effect on the regulation of inflammatory cytokine expression in hippocampal tissues

Similarly, the levels of HO-1 and NQO-1 proteins also increased by 28% and 65% respectively, in response to HI stress (Fig. 6), while DOR activation further increased the levels of HO-1 and NQO-1 proteins by 36% and 42%, respectively (Fig. 6). In sharp contrast, DOR inhibition by pretreatment with naltrindole largely decreased the levels of HO-1 and NQO-1 proteins ($P < 0.05$). In fact, DOR inhibition completely eliminated the upregulation of HO-1 and NQO-1 proteins under HI even with DOR activation (Fig. 6). The vehicle treatment did not induce any appreciable effect on the expression of HO-1 and NQO-1 proteins.

These results suggest that HI stress triggers an upregulation of Nrf-2 nuclear protein as well as its downstream anti-inflammatory genes such as HO-1 and NQO-1, while DOR activation further augments such a protective reaction against HI injury.

Discussion

We have made several novel and interesting findings in this work: (1) in the cortex of a HI-exposed hemisphere, DOR activation significantly decreased the expression of TNF-α, IL-6, and ICAM-1, while it also significantly increased the expression of IL-10 with no appreciable effect on IL-18; (2) DOR inhibition induced an opposite effect on these cytokines in the exposed cortex; (3) DOR had no effect on inflammatory cytokine expression in the non-cortical tissues; and (4) HI stress triggered an upregulation of nuclear Nrf-2 protein as well as its downstream anti-inflammatory genes such as HO-1 and NQO-1, while DOR activation further augmented such a protective reaction against HI injury in the cortex.

Infants and children possess an incredible resilience from injury in comparison to adults owing to the powerful ability of

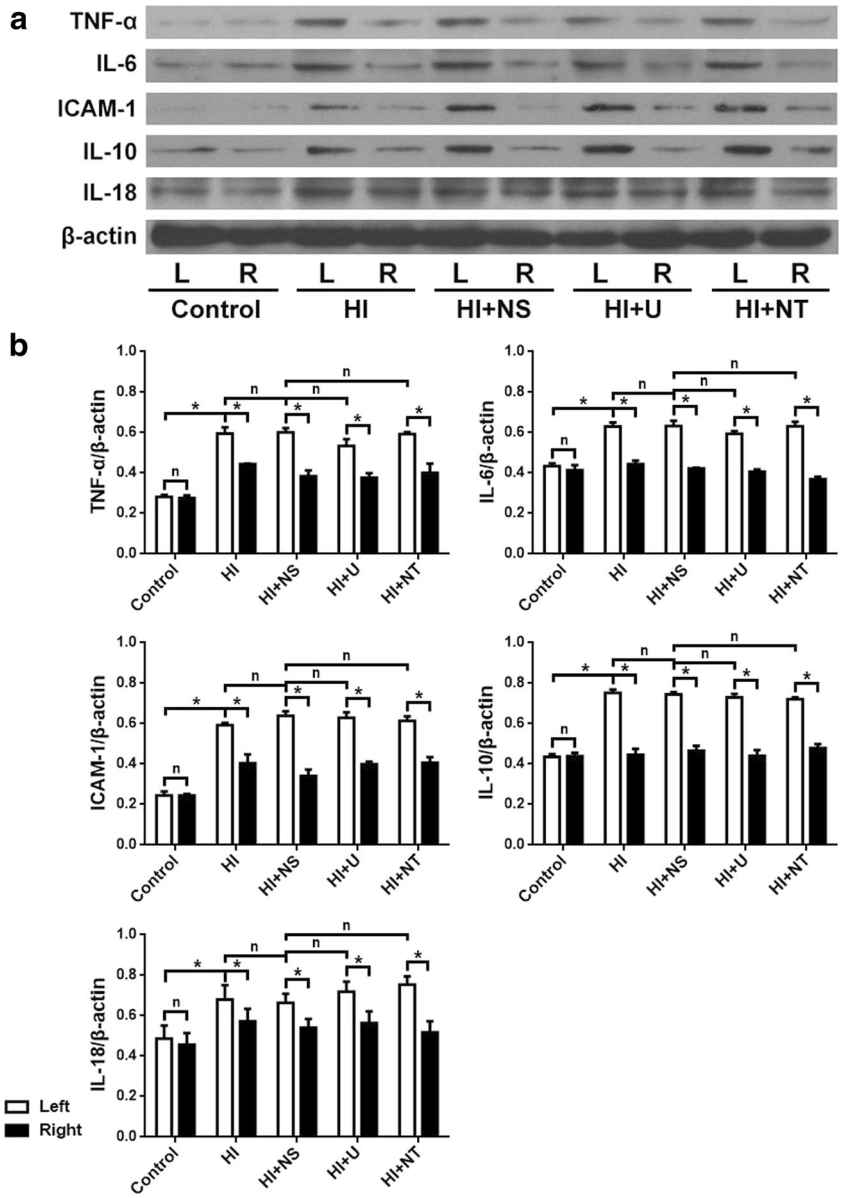

Fig. 3 Effects of DOR activation/inhibition on inflammatory cytokine protein expression in subcortical tissues under HI. Expression levels of TNF-α, IL-6, ICAM-1, IL-10, and IL-18 in subcortical tissues were significantly increased in left hemispheres in the HI, HI + NS, HI + U, and HI + NT groups (*$P <$ 0.05). The levels of expression of these inflammatory cytokines in left hemispheres were significantly increased in both the HI and the HI + NS groups in comparison to the control group (*$P <$ 0.05). Their expression levels in left hemispheres were not significantly different between the HI and the HI + NS groups ($^{n}P > 0.05$). Neither DOR activation nor DOR inhibition had an effect on the expression levels of these inflammatory cytokines in left subcortical tissues in comparison to the HI + NS group ($^{n}P > 0.05$). Note that DOR had no effect on the regulation of inflammatory cytokine expression in subcortical tissues

the developing brain to modify its own structure and function in efforts to compensate for loss of function due to injury [30, 31]. This ability, known as neuroplasticity, is particularly active during childhood as the synaptic organization and white matter pathways are still undergoing development in the developing brain [32]. Perinatal hypoxia/ischemia causes a major brain injury in the newborns leading to encephalopathy which may culminate in cognitive, motor, and behavioral deficits later in life [33, 34]. Plasticity seems to play a major role for spontaneous recovery of the HI-injured newborn brain. However, the exact mechanism for recovery in relations to brain function and reorganization of the neural networks has not yet been well elucidated. Consequently, there remains a lack of effective interventional methods available for the treatment of HI brain injury. Therefore, it is imperative to find new method to treat HIE. Our present studies suggest, for the first time, that DOR, an endogenous protector in the cortex [7, 8, 15, 22–24, 35], is neuroprotective against HI injury by upregulating pro-inflammatory cytokines and downregulating pro-inflammatory cytokines in the cortex. The reason we did not see the same outcomes in the non-cortical regions is very likely related to the low density of DOR in these regions at this age. As shown in our previous studies, the density of DOR is much higher in the cortex than in non-cortical regions with a postnatal development from the low density in the immature to the high density in the adult [7, 10, 36]. The amount of DOR might not be enough to induce sufficient DOR signals for the regulation of pro- and/or anti-inflammatory cytokines in non-cortical regions at this age. Moreover, although a short/mild stress may upregulate DOR expression [35, 37], a prolonged/severe insult may reduce its expression [7, 8, 15, 35, 37–39]. HI is a severe insult and may

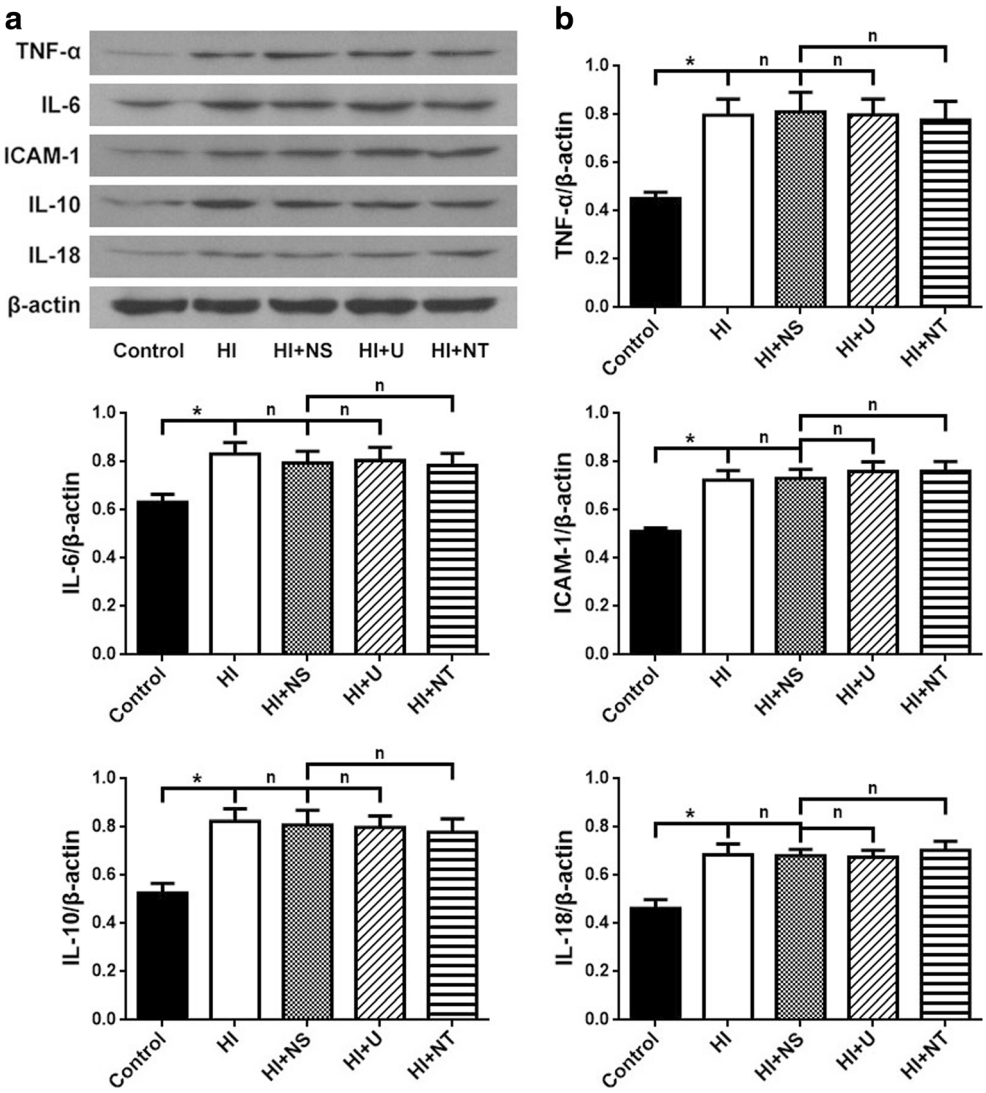

Fig. 4 Effects of DOR activation/inhibition on inflammatory cytokine protein expressions in cerebellar tissues under HI. Expression levels of TNF-α, IL-6, ICAM-1, IL-10, and IL-18 in cerebellar tissues were significantly increased in both the HI and the HI + NS groups in comparison to the control group (*$P < 0.05$), but were not significantly different between the HI and the HI + NS groups ($^{n}P > 0.05$). Neither DOR activation nor DOR inhibition had an effect on the levels of expression of these inflammatory cytokines in cerebellar tissues in comparison to the HI + NS group ($^{n}P > 0.05$). Note that DOR had no effect on the regulation of inflammatory cytokine expression in cerebellar tissues

reduce neural density of DOR, especially in the HI-vulnerable tissues like the hippocampus, which may further reduce DOR action in these brain regions of the immature.

In the pathogenesis of HIE, inflammation plays a critical role in the condition of neonatal HI brain injury. Inflammatory signals are generated within minutes after the HI insult and can expand for weeks and even months [40–43] with differential contributions to different cell types. Emerging evidence has demonstrated the association between cytokine profiles with HI severity in animals and humans [44–46]. Depending on the experimental protocol, cytokines peak within 12–24 h after HI insult [46, 47]. We therefore chose 24 h post HI as the time point for measuring the inflammatory cytokine expression in this study. On the other hand, several studies have shown that hypothermia suppresses inflammation [48–52]. Since DOR activation may produce a hypothermic response in rats [53] and ischemia or the use of an anesthetic could induce hypothermia [54], we assessed animal temperature and maintained it at 37 °C during and after the operation until the rats were sacrificed in order to minimize the effect of the body temperature on the inflammatory cytokines. Therefore, we are confident that the present results reflect the effects of DOR signaling, but not due to other factors, on these cytokines at 24 h post HI.

It is interesting to note that although the whole brain was exposed to hypoxia, the increase in inflammatory cytokine protein expression occurred only in the left hemisphere exposed to HI, but not in the right hemisphere without ischemic insult. Vannucci RC et al. [55] used the similar HI model, e.g., 7-day-old rats subjected to right common carotid artery ligation and then hypoxia with 8% O_2 at 37 °C and showed that blood flow to the subcortical white matter was reduced to the greatest extent of all the brain regions examined (15% of the control), while blood flows to contralateral cerebral hemispheric structures were relatively unchanged from pre-hypoxic values. Their results showed the vulnerability of the immature brain to HI with little effect of ischemia in one hemispheres on contralateral cerebral hemispheric structures.

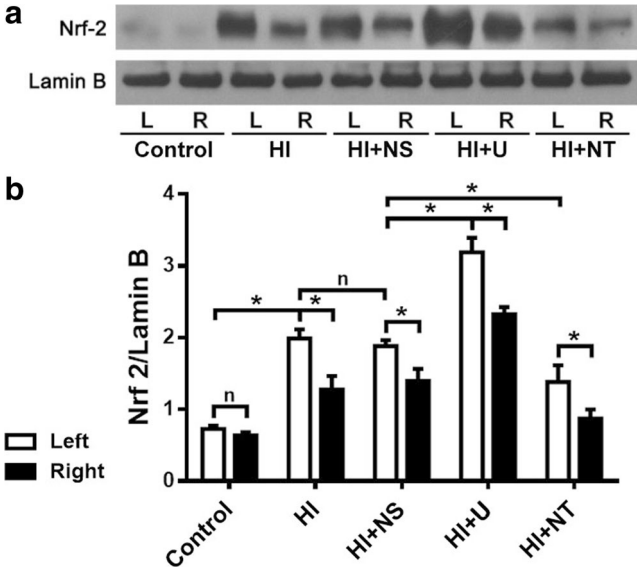

Fig. 5 Effects of DOR activation/inhibition on cortical nuclear localization of Nrf-2 under HI. HI significantly increased Nrf-2 nuclear protein levels in left hemispheres in both the HI and the HI + NS groups in comparison to the control group ($^{*}P < 0.05$) and were not significantly different between the HI and the HI + NS groups ($^{n}P > 0.05$). DOR activation using UFP-512 treatment under HI conditions (HI + U) caused a significant increase in Nrf-2 nuclear protein levels ($^{*}P < 0.05$), while the expression of Nrf-2 nuclear protein was significantly decreased in the NT pretreatment group (HI + NT) compared to the HI + NS group ($^{*}P < 0.05$). Note that HI stress triggered an upregulation of Nrf-2 nuclear protein while DOR activation further augments such a protective reaction against HI injury

Moreover, based on our long-term experiences in hypoxic research, a short-term hypoxia at 8% of oxygen might not induce any measurable change in most proteins in the brain. Therefore, it is not surprising for us to see major changes in the inflammatory cytokines of the left hemispheres exposed to both ischemia and hypoxia, but not in the right one exposed to hypoxia alone.

The present study suggests that pro- and anti-inflammatory cytokines differentially respond to DOR signaling. As documented in Fig. 1, in the cortical tissue of the left hemisphere that was exposed to HI, DOR activation significantly decreased the expression of pro-inflammatory cytokines such as TNF-α, IL-6, and ICAM-1, but induced an opposite effect on the expression of IL-10, a neuroprotective cytokine in HI [56] with no appreciable effect on IL-18. Clearly, some cytokines are sensitive to DOR signaling, while others are insensitive to it. DOR activation induced a "beneficial" effect on the HI-exposed brain by selectively regulating some cytokines, except for IL-18.

Our previous study [57] suggest that hypoxia causes a downregulation of brain-derived neurotrophic factor (BDNF)-TrkB signaling, leading to an increase in TNF-α in the cortex, while DOR activation upregulates BDNF-TrkB signaling, thereby decreasing the level of TNF-α in the hypoxic cortex. This mechanism may be involved in the DOR reaction against the HI-induced upregulation of TNF-α. On the other hand, BDNF can upregulate IL-10 expression [58].

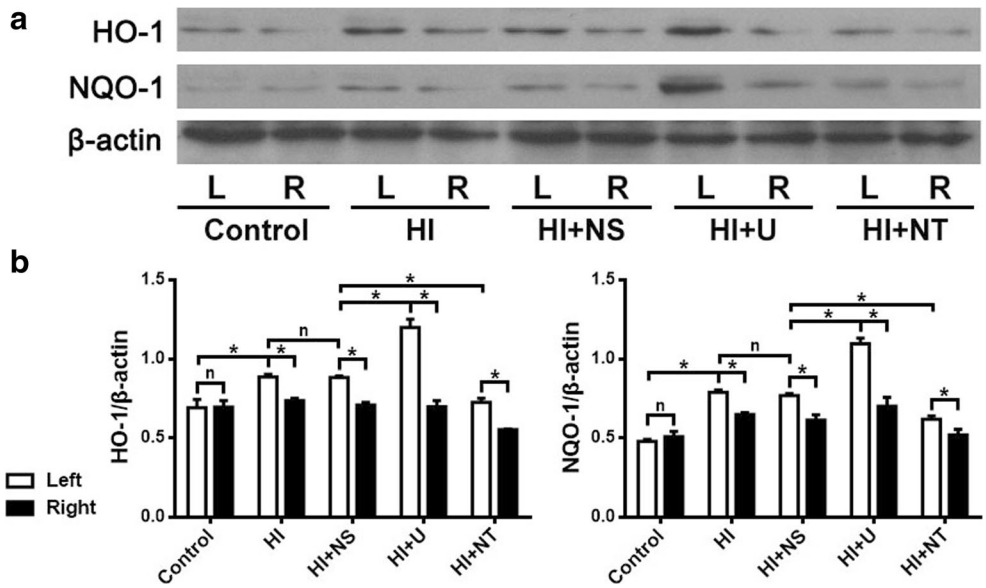

Fig. 6 Effects of DOR activation/inhibition on cortical HO-1 and NQO-1 protein expressions under HI. HI significantly increased the expression levels of HO-1 and NQO-1 in left hemispheres in both the HI and the HI + NS groups in comparison with the control group ($^{*}P < 0.05$) and were not significantly different between the HI and the HI + NS groups ($^{n}P > 0.05$). Cortical protein levels of HO-1 and NQO-1 were significantly increased in the HI + U group ($^{*}P < 0.05$), while the levels of expression of HO-1 and NQO-1 were significantly decreased in the HI + NT group in comparison to the HI + NS group ($^{*}P < 0.05$). Note that HI stress triggered an upregulation of HO-1 and NQO-1, while DOR activation further augmented such a protective reaction against HI injury

In the present study, we observed that IL-10 protein expression tended to increase after DOR activation. This can also be, at least partially, attributed to the BDNF-TrkB signaling. There is no clue yet concerning the association between DOR and IL-6 regulation. However, IL-10, as an anti-inflammatory cytokine, may have an inhibitory effect on pro-inflammatory cytokines, especially TNF, IL-1, and IL-6 [59, 60]. As such, the DOR-induced upregulation of IL-10 can further suppress the expression of TNF-α and IL-6. The expression of ICAM-1 is particularly responsive to TNF-α [61]. Therefore, the DOR suppression on the level of TNF-α may more or less influence the level of ICAM-1.

IL-18 is a member of the IL-1 family and a major pro-inflammatory cytokine in mediating neuroinflammation in brain injury [62]. An upregulation of IL-18 expression can exacerbate HI injury in the immature brain [63, 64]. Surely enough, our data showed that HI significantly increased the level of IL-18 in our neonatal model. However, DOR activation/inhibition had no appreciable effect on IL-18. It is reported that IL-18 and IL-1 act through their related receptor complexes to trigger common signaling pathways and that IL-18 is more related to IL-1β than any other cytokine [65]. Since our previous study showed that DOR activation had no appreciable effect on the expression of IL-1β [17] in both astrocytes and PC-12 cells, we believe that DOR activity has a limited influence on the IL-18 and IL-1β complex, at least at the level of expression, unlike its effect on TNF-α, IL-6, ICAM-1, and IL-10. Nevertheless, a decrease in TNF-α, IL-6, and ICAM-1 with an increase in IL-10 are sufficient enough to provide significant protection to the neonatal cortex under HI stress.

While exploring the signal transduction between DOR activation and the regulation of the cytokines, the first thing that came to our mind was Nrf-2, the inducible transcription factor, because our recent work shows that DOR activation promotes the translocation of Nrf-2 from the cytoplasm to the nucleus and upregulates the expression of anti-inflammatory genes in a cell model [29]. There is no information in the literature regarding the DOR regulation of Nrf-2 in neither neonatal nor adult brains. Our data provides strong evidence for the DOR-mediated upregulation of nuclear Nrf-2 and anti-inflammatory genes such as HO-1 and NQO-1 in the neonatal cortex exposed to HI, which is well consistent with our findings made in the cell model [29]. A variety of studies have reported that Nrf-2 can increase the expression of a variety of phase II detoxifying

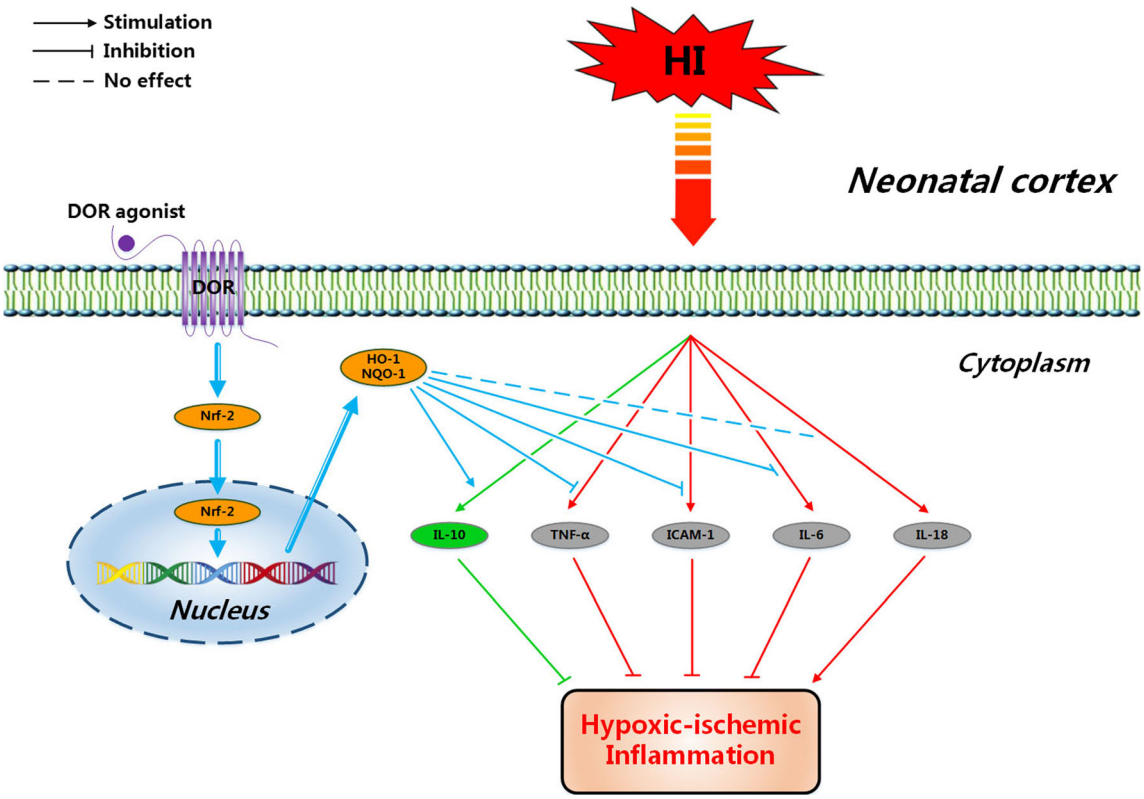

Fig. 7 Schematic demonstration of the mechanisms underlying DOR neuroprotection against HI-induced inflammatory cytokines. HI increases the expression of inflammatory cytokine protein such as IL-10, TNF-α, ICAM-1, IL-6, and IL-18. DOR activation significantly inhibits the HI-induced TNF-α, ICAM-1, and IL-6 production and increases the level of IL-10 production with no appreciable effect on the IL-18 expression in the neonatal rat cortex, which is mediated, at least in part, by the Nrf-2/HO-1/NQO-1 signaling pathway. Since IL-10 is an anti-inflammatory cytokine and TNF-α, ICAM-1, IL-6, IL-18 are pro-inflammatory cytokines, DOR activation induces a "beneficial" regulation of these cytokines except in the case of IL-18. Blue lines indicate the effect of DOR activation. Green lines indicate the effect of anti-inflammatory cytokine IL-10. Red lines indicate the effect of pro-inflammatory cytokines TNF-α, ICAM-1, IL-6, and IL-18

enzymes including HO-1 and NQO-1 and can subsequently limit oxidative stress, which can further suppress pro-inflammatory cytokines [66–68]. More recent studies have indicated that the activation of Nrf-2 inhibits the production of pro-inflammatory mediators in many animal and cell models [69, 70], while the inhibition of Nrf-2 (−/−) in mice causes an increase in the expression of inflammatory cytokines [71, 72]. Therefore, our data strongly suggests that Nrf-2 is an essential mediator for DOR's anti-inflammatory function.

Conclusions

In conclusion, we present the first data to demonstrate that DOR activation significantly inhibits HI-induced TNF-α, ICAM-1, and IL-6 production and increases the level of IL-10 production with no appreciable effect on the IL-18 expression in the neonatal rat cortex, which is mediated, at least in part, by the Nrf-2/HO-1/NQO-1 signaling pathway. The mechanisms underlying DOR neuroprotection against HI-induced inflammatory cytokines are schematically illustrated in Fig. 7. To our knowledge, these are the first findings indicating a new mechanism by which DOR protects the neonatal brain against HI, which suggests a novel therapeutic target for the treatment of HIE.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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