

Neuroprotection of Up-Regulated Carbon Monoxide by Electrical Acupuncture on Perinatal Hypoxic–Ischemic Brain Damage in Rats

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Abstract This study investigated the neuroprotection and potential mechanism of carbon monoxide (CO) against perinatal hypoxic–ischemic brain damage in rats by electrical acupuncture (EA). Animal behavior, morphological changes, cystathionine beta-synthase (CBS), hypoxia-inducible factor-1 α (HIF-1 α), and heme oxygenase-1 (HO-1) expression levels, and CO content in rat cortex cells were determined. Results demonstrated that EA treatment decreased the slope behavior and increased the overhang behavior of perinatal rats. The treatment also decreased the number of positive cells. The activator and inhibitor of CBS aggravated and remitted the hypoxic damage in cortex cells, respectively. EA treatment decreased CBS expression level and increased HO-1 and HIF-1 α expression levels in perinatal rat cortex cells. Compared with the control groups, the CO content of cortex cells in the EA treatment group significantly increased (** $p < 0.01$). We hypothesized that EA treatment increases cortical CO content to protect against hypoxic damage via the hydrogen sulfide/CBS–CO/HO-1–HIF-1 α system. This study

provided a significant reference for EA therapy and cued a novel protective mechanism for cerebral palsy.

Keywords Cerebral palsy · Perinatal rats · Hypoxic–ischemic brain damage · Electrical acupuncture · Carbon monoxide · Protection

Introduction

Cerebral palsy (CP) is a neurological disorder that appears in infancy or early childhood [1]. It is often complicated by mental retardation, epilepsy, perceptions of barriers, obstacles to communication behavior, and other abnormalities. CP in children has a serious effect on their quality of life and increases the burden on the society and family [2]. CP morbidity in Chinese children is 1.8–4.0 ‰ and shows an increasing trend each year [3]. It is the most common disorder among pediatric neurological diseases and disabilities [4]. However, an effective treatment for CP is unavailable to date. With the rapid development of Traditional Chinese Medicine, acupuncture and its curative effect on CP have received considerable attention worldwide [5].

Acupuncture, an original and no-drug therapy medical system, has curative effects on central and peripheral nerve injuries [6]. Electrical acupuncture (EA) can restrain the aspartate transaminase hyperplasia in the ischemic region to facilitate synapsis formation. It can also regulate electrical activity, improve brain blood circulation, increase cerebral blood oxygen supply, and enhance recession neuron metabolism [7, 8]. As our study, areas of increased and decreased signal intensities in children with CP significantly differed from those of normal children after being acupunctured at the Taichong acupoint. Areas of

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decreased signal intensity in children with CP were found to be mainly distributed in the frontal lobe (BA 10, BA 8, BA 21, and BA 46), anterior central gyrus (BA 4), and parahippocampal gyrus [9]. Meanwhile, novel gas signal molecules, such as hydrogen sulfide (H₂S) [10] and nitric oxide (NO), have significant neuroprotection against hypoxic–ischemic brain damage (HIBD) in rats subjected to EA. EA treatment can decrease NO generation via the nNOS/NO system in the cerebral cortex to protect against HIBD, and also decrease H₂S generation by down-regulating cystathionine beta-synthase (CBS) expression in the cerebral cortex to protect against HIBD [11]. Therefore, we investigated whether carbon monoxide (CO) is another mediator of EA in perinatal HIBD and explored its neuroprotective mechanism.

Materials and Methods

Experimental Animals and Groups

The 96 Sprague–Dawley pregnant rats (specific pathogen-free) were purchased and raised in the Laboratory Animal Center of the Academy of Military Medical Sciences until delivery. The rats (7 day, 13.9–22 g) were randomly divided into eight groups ($n = 12$) [Sham, Sham + EA, HIBD, HIBD + EA, HIBD + SAM (S-adenosyl-L-methionine, an activator of CBS, Sigma-Aldrich, USA), HIBD + SAM + EA, HIBD + HA (Hydroxylamine, an inhibitor of CBS, Sigma-Aldrich, USA), and HIBD + HA + EA] and subjected to HIBD. Wherein, the total of 12 animals in each group was used for the behavioural testing and further divided into two groups ($n = 6$). The total of six animals was used for HE and IHC staining, and the other 6 animals were used for CO content and Western blot assay as following. This experiment was approved by the Experiment and the Chinese people's liberation army general hospital ethics committee.

Construction of HIBD Model

The HIBD model was established according to literature [12], and the detailed procedures are as follows. The new born rats were narcotized by diethyl ether, and the four limbs were fixed to allow incision along the neck midline. The thyroid, vein, and nerve tissues were stripped to expose the left carotid artery communis, ligated with 5/0 surgical line, and then sutured. After 2 h, the rats were placed in a lower oxygen tank to maintain an appropriate environmental temperature under continuous hypoxia with 8 % oxygen and 92 % nitrogen gas in the container for 2 h. Then, the baby rats were returned to their mothers in the cage. The Sham-operated groups were subjected to surgery,

with their left carotid artery communis exposed, but they did not undergo ligation.

Electric Acupuncture Intervention Experiment and Intraperitoneal Injection

The rats in the EA group were acupunctured at the BaiHui (crossing point before and after the skull and the linkline of the two ears) and DaZhui (below the detail of the cervical spine) acupoints with an electric acupuncture (frequency: 2/100 Hz, intensity: 3 mA, 30 min/day/time) for 14 day. The limbs of the rats in the control group were only fixed, and was not performed EA.

The young rats in the HIBD + HA and HIBD + HA + EA groups were injected with 12.5 mg/kg/day HA (dissolved in saline) via the peritoneal cavity 20 min prior to the acupuncture procedure or fixation. Similarly, the young rats in the HIBD + SAM and HIBD + SAM + EA groups were injected with 50 mg/kg/d SAM (dissolved in saline) via the peritoneal cavity 20 min prior to the EA procedure or fixation. The control group was injected with an equal volume of normal saline.

Behavioral Testing

For overhang experiment, the front legs of the rats were grasped in a horizontal glass rod (diameter 0.6 cm). These rats were suspended on the table with a distance of 45 mm, and the overhanging time was recorded. For slope experiment, the rats were placed headdown on a plane to an angle of 45° inclination. The time was measured when the rat was in the up-headed position.

Hematoxylin and Eosin (HE) Staining

The total of six baby rats were after behavior testing was executed and further prepared wax block. The wax block was sectioned and the slides (3–4 μ m) were deparaffinized and rehydrated, and over-stained with hematoxylin for 3 min followed with differentiation and destaining using acidic alcohol. Bicarbonate was applied (2 min) until the nuclei stand out sharply in blue. The sections were placed in 70 % ethanol (3 min) and then in eosin (2 min). The images of the cortex were captured using a microscope connected to a CCD camera at 200 \times magnification at the same image acquisition settings.

Immunohistochemistry (IHC) Assay

The total of six baby rats were after behavior testing was executed and further prepared wax block. The wax block was sectioned and the slides (3–4 μ m) were deparaffinized and rehydrated, and post-fixed with 4 % paraformaldehyde for 10 min, and then washed with phosphate-buffered

saline (PBS). Endogenous peroxidase was inactivated by 3 % H₂O₂ (30 min), and incubated with 10 % normal goat serum in PBS for 30 min at room temperature. Then, the sections were incubated with rabbit anti-CBS (SC-67154, 1:200; Santa Cruz, the United States), anti-HIF-1 α (AH-337-1, 1:100; Biyuntian Company, China), and anti-HO-1 (ab13243, 1:300; Abcam, England) monoclonal in PBS containing 0.3 % Triton X-100 overnight at 4 °C and washed with PBS, and then incubated in peroxidase-conjugated goat anti-rabbit IgG (1:200; ZYMED, the United States) for 1 h at room temperature. Finally, the sections were developed with diaminobenzidine (Sigma, the United States) in PBS containing 0.001 % H₂O₂ for 30 min. The images were captured at the same image acquisition settings, and the amount of immuno-positive cells and total positive area in the assigned subregions was measured by Image-Pro Plus 7.0 software (http://www.mediacy.com/index.aspx?page=IP_Premier) and histogram analysis using Origin 9.0 software (<http://www.originlab.com/>).

CO Content Assay

The other six baby rats were after behavior testing was executed and the rat brain cortex tissues (about 50 mg) were ground and added to nine volumes of normal saline. The homogenate was centrifuged at 2,500 rpm for 10 min, and 200 μ L of supernatant was obtained to measure A₅₆₈ and A₅₈₁. Then, the absolute absorbance value (A₅₆₈ – A₅₈₁) was determined. Subsequently, HbCO % and Hb contents (g/L) were calculated according to the functions HbCO % = 0.822x + 0.001 (x: A₅₆₈ – A₅₈₁) and Hb = A_{540nm} \times 367.7, respectively. Then, CO content was calculated according to the function: CO = HbCO % \times Hb \times 10⁶ \times 4/(64456x) (x: protein concentration, g/L). Histogram analysis was carried out by Origin 9.0 software (<http://www.originlab.com/>).

Western Blot Assay

The other six baby rats were after behavior testing was executed and the rat brain cortex tissues were ground, and added 50 μ l cell lysate buffer (50 mM Tris-Cl pH 6.8, 15 mM NaCl, 5 mM EDTA, 0.5 % NP-40, 1 mM PMSF). The homogenate was centrifuged at 10,000 rpm for 10 min and added 50 μ l 2 \times loading buffer to boil at 95 °C for 10 min. Approximately 35 mg of protein samples was fractionated by 12 % polyacrylamide gels and transferred to a polyvinylidene difluoride membrane following the manufacturers' instructions. The membrane was probed with the first antibody, rabbit-derived anti-HO-1 antibody (1:2,000 in TBST; Abcam, England), rabbit-derived anti-HIF-1 α antibody (1:1,000 in TBST; Biyuntian Company, China), rabbit-derived anti-CBS antibody (1:500 in TBST;

Table 1 Animal slope and overhang experiment assay

Groups	Data	
	Animal slope experiment (Mean \pm SD, n = 6, s)	Animal overhang experiment (Mean \pm SD, n = 6, s)
Sham	4.8058 \pm 0.9104	31.5280 \pm 2.5603
Sham + EA	4.3720 \pm 0.5263	32.3611 \pm 11.7491
HIBD	8.5850 \pm 1.3375	10.6621 \pm 2.1383
HIBD + EA	6.2718 \pm 1.2698**	16.5948 \pm 2.8045**
HIBD + SAM	7.5076 \pm 1.2875	11.7773 \pm 3.4761
HIBD + SAM + EA	6.2854 \pm 0.6759**	17.2709 \pm 2.8763**
HIBD + HA	6.6476 \pm 1.0800	13.0923 \pm 2.7124
HIBD + HA + EA	5.6669 \pm 0.6710*	19.8972 \pm 3.3812**

* $p < 0.05$, ** $p < 0.01$

Santa Cruz, the United States), and mouse-derived anti- β -actin antibody (1:1,500 in TBST; Beijing Zhongshan Biotechnology, China) for 1.5 h at room temperature. Then, the membrane was incubated with horseradish peroxidase-conjugated goat anti-mouse secondary antibody (1:5,000 in TBST; Beijing Zhongshan Biotechnology, China) at room temperature for 1 h. Chemiluminescence substrate luminol reagent (GE Healthcare, the United States) and exposure to X-ray film were used to examine the immunolabeled bands. The optical density of the bands was scanned and quantified using the ImageJ 1.46 software (<http://rsb.info.nih.gov/ij/download.html>).

Statistical Analysis

All data were expressed as mean \pm SD. Statistical analysis was performed using SPSS software (version 21.0, SPSS; <http://spss.en.softonic.com/>). In Student's t test, $p < 0.05$ and $p < 0.01$ were considered to indicate significant difference and highly significant difference, respectively.

Results

EA Improves Animal Slope and Overhang Behavior

The results of behavioral assessment demonstrated that EA decreased the slope time in the Sham + EA (4.3720 \pm 0.5263 s), HIBD + EA (6.2718 \pm 1.2698 s), HIBD + SAM + EA (6.2854 \pm 0.6759 s), and HIBD + HA + EA (5.6669 \pm 0.6710 s) groups compared with the control groups, including Sham (4.8058 \pm 0.9104 s), HIBD (8.5850 \pm 1.3375 s), HIBD + SAM (7.5076 \pm 1.2875 s), and HIBD + HA (6.6476 \pm 1.0800 s). This result indicates that hypoxia can increase the slope time in HIBD in contrast to the Sham-operated group (Table 1).

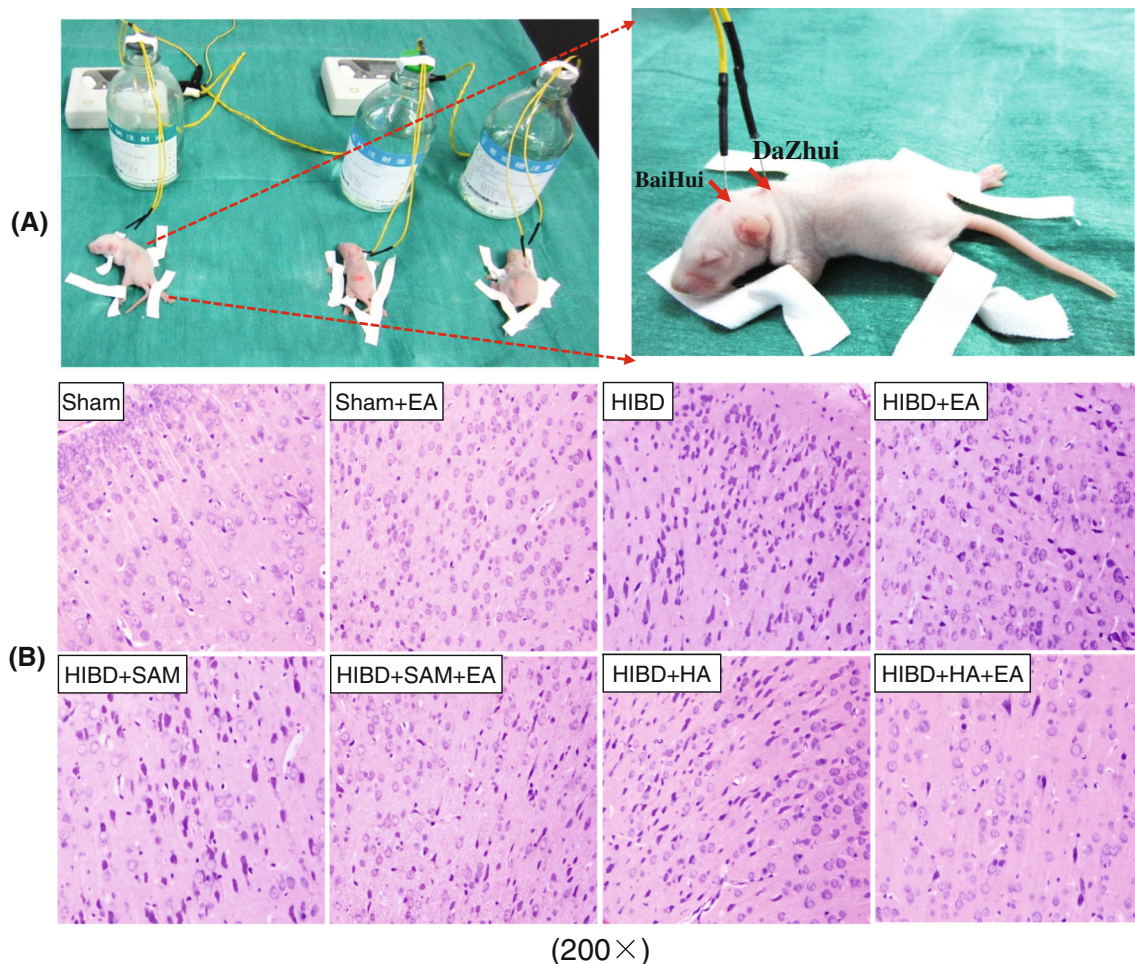


Fig. 1 The acupuncture image of new born rats and HE staining of cortex. **a** The acupuncture image of new born rats; **b** HE staining of cortex. EA was beneficial to alleviate the cortex damage in perinatal HIBD rats. The red arrow indicated the acupuncture points (Color figure online)

In addition, EA increased the overhang time in the Sham + EA (32.3611 ± 11.7491 s), HIBD + EA (16.5948 ± 2.8045 s), HIBD + SAM + EA (17.2709 ± 2.8763 s), and HIBD + HA + EA (19.8972 ± 3.3812 s) groups compared with the control groups, including Sham (31.5280 ± 2.5603 s), HIBD (10.6621 ± 2.1383 s, $**p < 0.01$), HIBD + SAM (11.7773 ± 3.4761 s, $**p < 0.01$), and HIBD + HA (13.0923 ± 2.7124 s, $**p < 0.01$). This finding indicates that hypoxia can significantly decrease the overhang time in HIBD in contrast to the Sham-operated group (Table 1, $**p < 0.01$).

EA Alleviates Cortex Damage in Perinatal HIBD in Rats

For acupuncture images exhibiting (Fig. 1a), the new born rats was fixed on the table to performed acupuncture.

EA alleviated the cortex damage in perinatal HIBD in rats and decreased the number of positive cells in the Sham + EA, HIBD + EA, HIBD + SAM + EA, and

HIBD + HA + EA groups compared with the control groups, including Sham, HIBD, HIBD + SAM, and HIBD + HA (Fig. 1b). Hypoxia can also trigger severe damage of the cortex cells. The CBS activator SAM aggravated this damage, whereas the CBS inhibitor HA alleviated this damage (Fig. 1b).

EA Decreases CBS Expression Level in Rat Cortex

EA decreased CBS expression level in the cortex cells compared with the control groups (Fig. 2a). A significant difference in CBS expression level was found between the Sham + EA and Sham groups (Fig. 2b, $**p < 0.01$). Similarly, significant difference in CBS expression level was also found between the other EA groups and control groups, including HIBD (Fig. 2b, $**p < 0.01$), HIBD + SAM (Fig. 2b, $**p < 0.01$), and HIBD + HA (Fig. 2b, $**p < 0.01$). As expected, the CBS activator SAM and the CBS inhibitor HA significantly increased its expression level in the cortex cells, respectively.

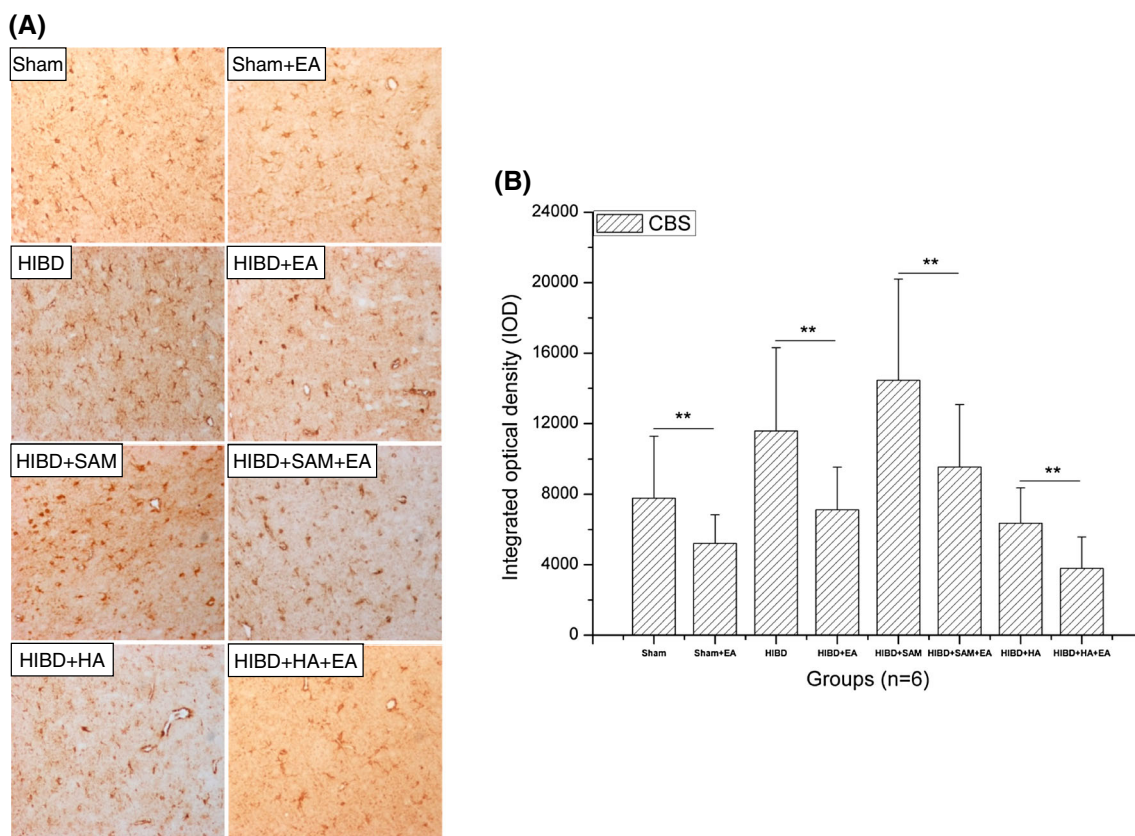


Fig. 2 The cortex CBS expression level assay by IHC and histogram analysis. **a** IHC assay. **b** Histogram analysis. EA could decrease CBS in the cortex, and had a significant different compare with each control. (** $p < 0.01$)

EA Increases HIF-1 α Expression Level in Rat Cortex

EA increased HIF-1 α expression level in the cortex cells compared with the control groups (Fig. 3a). A significant difference in HIF-1 α expression level was found between the Sham + EA and Sham groups (Fig. 3b, ** $p < 0.01$). Similarly, a significant difference in HIF-1 α expression level was found between the other EA groups and control groups, including HIBD (Fig. 3b, ** $p < 0.01$), HIBD + SAM (Fig. 3b, * $p < 0.05$), and HIBD + HA (Fig. 3b, ** $p < 0.01$). Unexpectedly, HIF-1 α expression level slightly decreased in the HIBD group compared with that in the Sham group.

EA Increases HO-1 Expression Level to Up-Regulate CO Content in Rat Cortex

EA increased HO-1 expression level in the cortex cells compared with the control groups (Fig. 4a). A significant difference in HO-1 expression level was found between the Sham + EA and Sham groups (Fig. 4b, ** $p < 0.01$). Similarly, a significant difference in HO-1 expression level was also found between the other EA groups and the control groups, including HIBD (Fig. 4b, ** $p < 0.01$),

HIBD + SAM (Fig. 4b, ** $p < 0.01$), and HIBD + HA (Fig. 4b, ** $p < 0.01$). Subsequently, the CO content in the cortex cells significantly increased with EA compared with the control groups (Fig. 5). A significant difference in CO content in the cortex cells was found between the Sham + EA and Sham groups (** $p < 0.01$). Similarly, a significant difference in the CO content in the cortex cells was also found between the other EA groups and the control groups, including HIBD (** $p < 0.01$), HIBD + SAM (** $p < 0.01$), and HIBD + HA (** $p < 0.01$). Additionally, hypoxia can reduce more CO in the HIBD group compared with the Sham group.

EA Down-Regulates CBS Expression Level to Up-Regulate HIF-1 α and HO-1 in the Cortex of HIBD in Rats

EA down-regulated CBS expression level (** $p < 0.01$, compared with each control group) and up-regulated HIF-1 α (** $p < 0.01$, compared with each control group) and HO-1 expression levels (* $p < 0.05$, ** $p < 0.01$, compared with each control group). Hypoxia decreased CBS expression level and increased HIF-1 α and HO-1 expression levels. Meanwhile, the CBS activator SAM increased

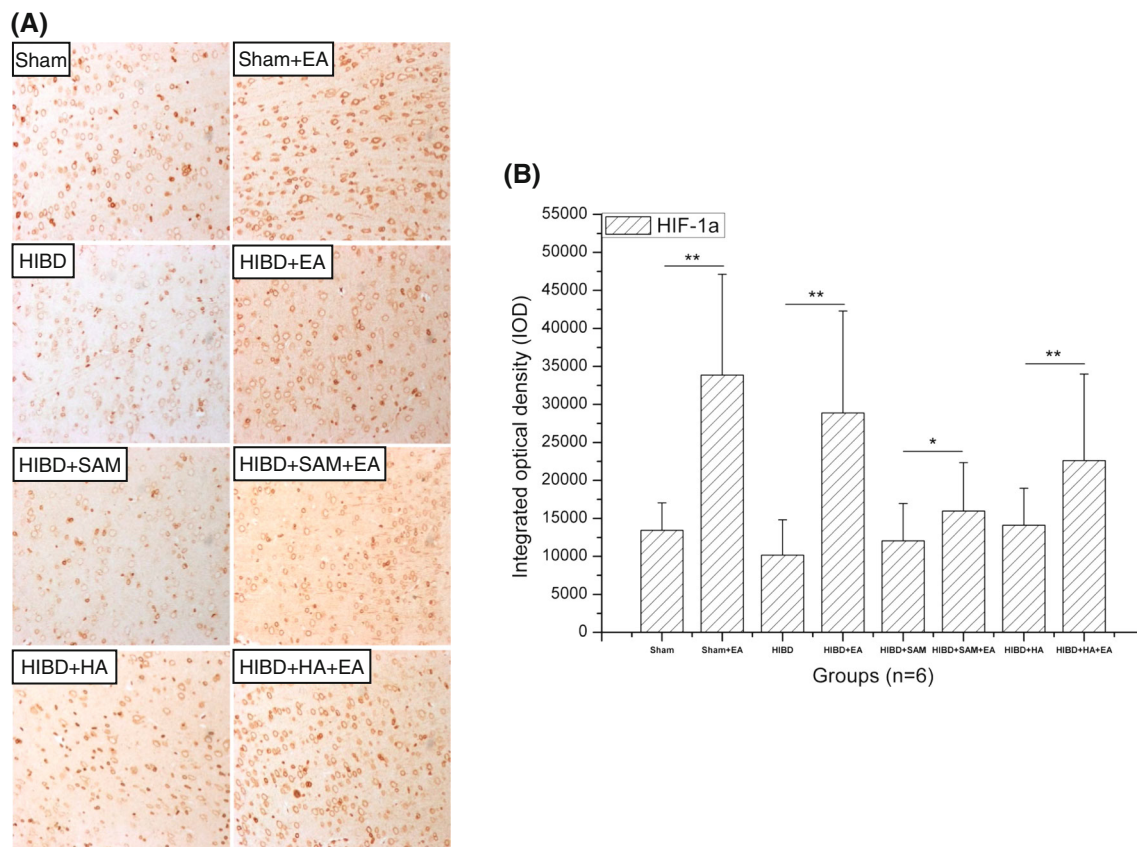


Fig. 3 The cortex HIF-1 α expression level assay by IHC and histogram analysis. **a** IHC assay. **b** Histogram analysis. EA could increase HIF-1 α in the cortex, and had a significant different compare with each control. (* $p < 0.05$; ** $p < 0.01$)

CBS and HO-1 expression levels and significantly decreased HIF-1 α expression level. The CBS inhibitor HA decreased CBS and HIF-1 α expression levels and increased HO-1 expression level (Fig. 6).

Discussion

We demonstrated that EA can alleviate hypoxia-induced cortex cell damage in perinatal HIBD in rats. The number of positive cells significantly decreased with EA compared with each control. Additionally, EA can increase HIF-1 α expression level, decrease CBS expression level, and increase HO-1 expression level in the perinatal rat cortex. The CO content in the rat cortex also increased with EA.

Gas biology is an emerging discipline that is receiving considerable attention [13–16]. Currently, several novel gas signal molecules (H₂S, NO, and CO) involved in the regulation of the structure and function of the nervous system under physiological and pathological conditions have been explored [17–20]. These gas signal molecules are characterized by low molecular weight, continued

generation, rapid diffusion, and extensive effect; these molecules can also freely transfer a variety of biomembranes to several organs.

In Traditional Chinese Medicine, acupuncture can regulate the gas signal molecules to protect an organism. For instance, acupuncture can increase NO local generation [21]; Acupuncture can increase NO generation on the skin surface of acupoints [22]; Acupuncture can reduce NO content in rat corpus striatum of transient middle cerebral artery occlusion and further alleviate brain damage [23]. Acupuncture can decrease the nNOS/NO system to recover the neuronal function in HIBD in rats [11]. All these results indicated that NO gas signal molecule had a significant neuroprotection against HIBD. However, no study has reported on the neuroprotection of the gas signal molecule CO in HIBD, especially in children with CP. Therefore, we constructed a perinatal HIBD model to determine it. This study validated that the over-expressed CO had a significant protection against perinatal HIBD in rats and that CBS, HO-1, and HIF-1 α were involved in this process. On the one hand, acupuncture decreased CBS expression level in the cortex to reduce H₂S content in our previous study

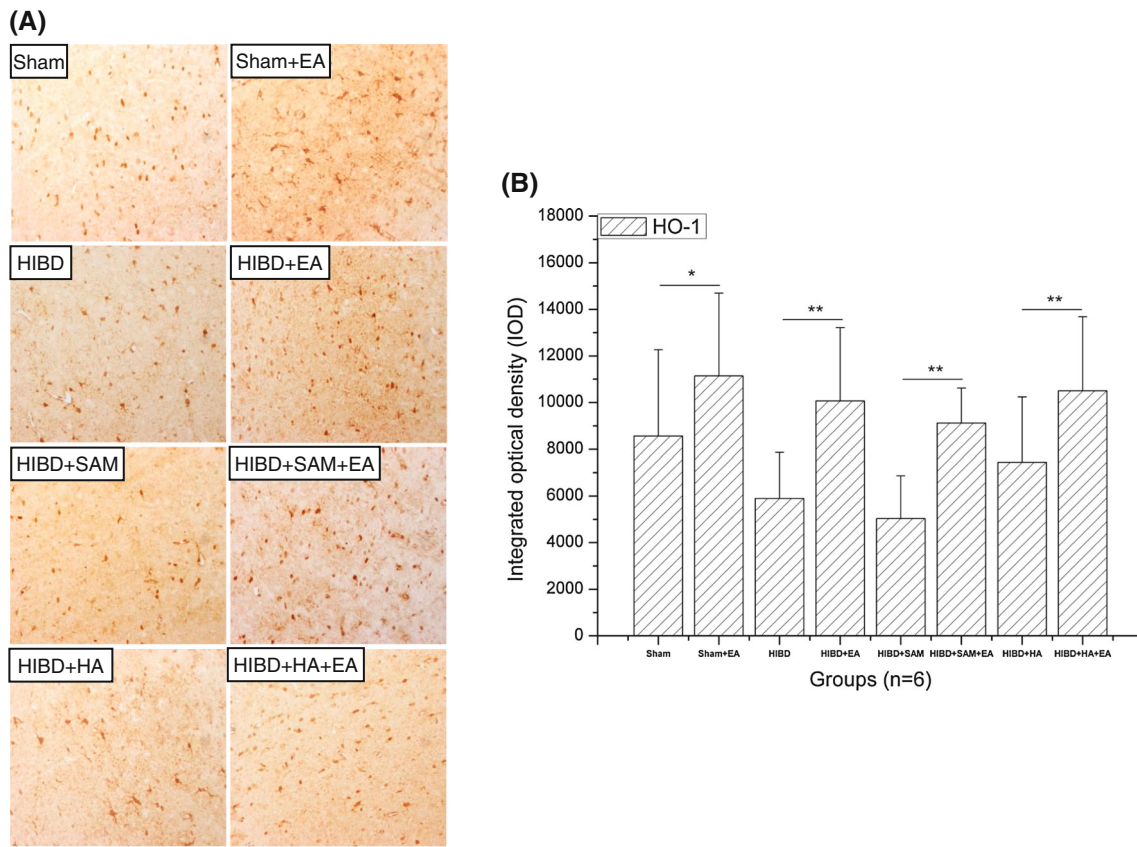
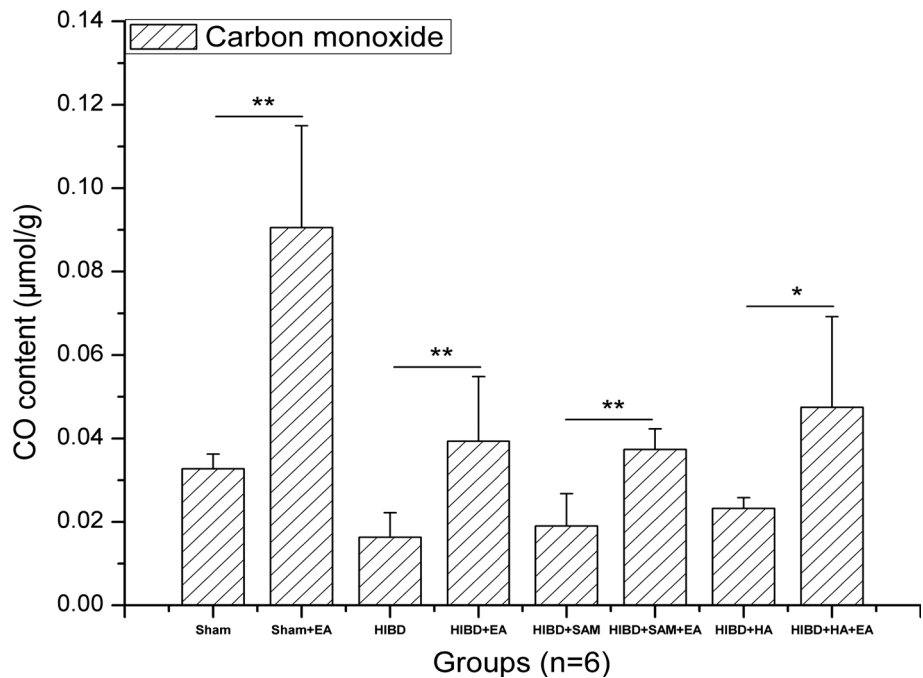


Fig. 4 The cortex HO-1 expression level assay by IHC and histogram analysis. **a** IHC assay. **b** Histogram analysis. EA could increase HO-1 in the cortex, and had a significant different compare with each control. (* $p < 0.05$; ** $p < 0.01$)

Fig. 5 The histogram analysis of the CO content in the cortex. EA could increase the CO content in the cortex, and had a significant different compare with each control. (** $p < 0.01$)



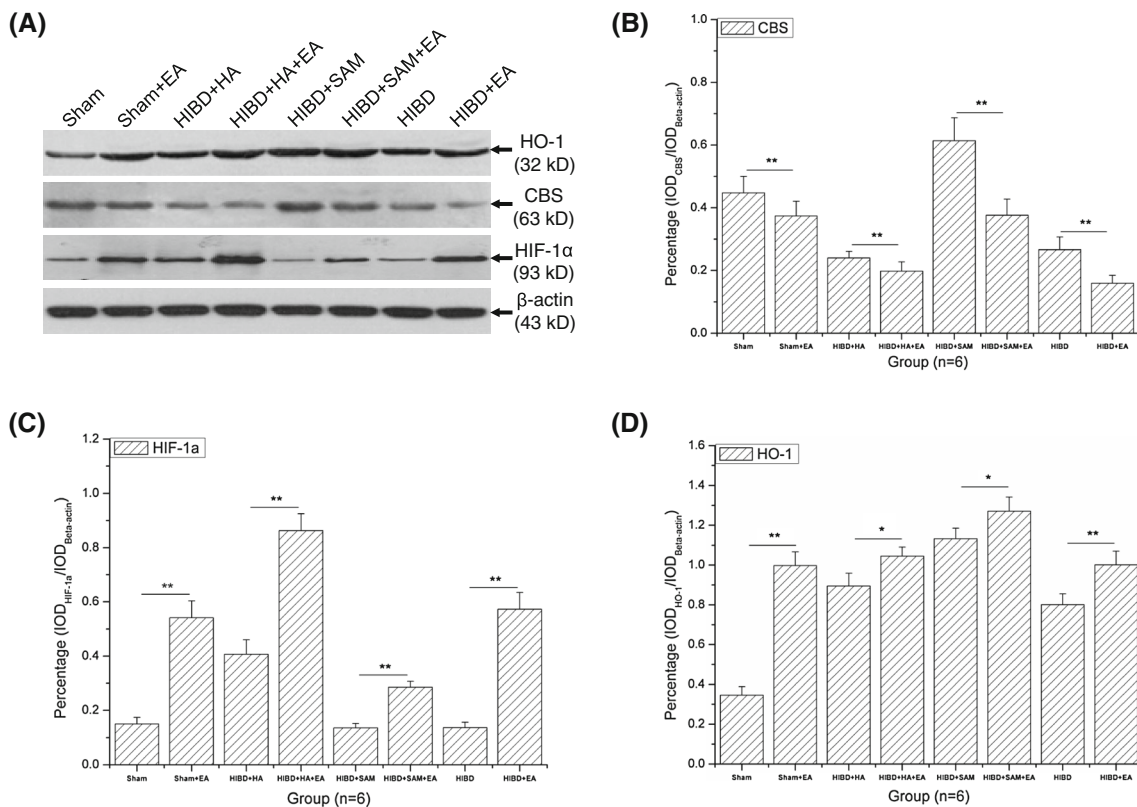


Fig. 6 The cortex CBS, HIF-1α and HO-1 expression level assay by Western blot and histogram analysis. **a** Western blot assay. **b–d** Histogram analysis. EA could decrease the CBS expression level and

up-regulated the HIF-1α and HO-1 expression level, it had a significant different (**p* < 0.05; ***p* < 0.01)

[10]. On the other hand, the down-regulated CBS increased HIF-1α and HO-1 expression levels, and the over-expressed HO-1 can generate excessive CO in the cortex to protect against HIBD.

For behavior testing, the overhang experiment was used to measure the myodynamia, and further assessed the movement of new born rats. The slope experiment was used to measure the balance function of new born rats. Both of these experiments were used to estimate the curative effect of acupuncture after HIBD. By comparing, the EA treatment could decrease the slope time and increase the overhang time, indicating the EA is favor of improving the balance and the movement, especially after HIBD.

Taken together, the recent study revealed that the acupuncture could alleviate the HIBD of rats via up-regulating of the CO content in the cortex cells. It provided a significant reference for the acupuncture therapy in CP and cued a novel protective mechanism for CP.

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