Original Research

HSP70 protects rats and hippocampal neurons from central nervous system oxygen toxicity by suppression of NO production and NF-κB activation

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Impact statement

Because the pathogenesis of central nervous system oxygen toxicity (CNS-OT) remains unclear, there are few interventions available. To develop an efficient strategy against CNS-OT, it is necessary to understand its pathogenesis and in particular, the relevant key factors involved. This study examined the protective effects of heat shock protein 70 (HSP70) on CNS-OT via *in vivo* and *in vitro* experiments. Our results indicated that overexpression of HSP70 in hippocampal neurons may protect rats from CNS-OT by suppression of nNOS and iNOS-mediated NO production and the activation of NF-κB. These findings contribute to clarification of the role of HSP70 in CNS-OT and provide us a potential novel target to prevent CNS-OT. Clarification of the involvement of NO, NOS and NF-κB provides new insights into the mechanism of CNS-OT and may help us to develop new approach against it by interfering these molecules.

Abstract

During diving, central nervous system oxygen toxicity may cause drowning or barotrauma, which has dramatically limited the working benefits of hyperbaric oxygen in underwater operations and clinical applications. The aim of this study is to understand the effects and the underlying mechanism of heat shock protein 70 on central nervous system oxygen toxicity and its mechanisms *in vivo* and *in vitro*. Rats were given geranylgeranylacetone (800 mg/kg) orally to induce hippocampal expression of heat shock protein 70 and then treated with hyperbaric oxygen. The time course of hippocampal heat shock protein 70 expression after geranylgeranylacetone administration was measured. Seizure latency and first electrical discharge were recorded to evaluate the effects of HSP70 on central nervous system oxygen toxicity. Effects of inhibitors of nitric oxide synthase and nuclear factor-κB on the seizure latencies and changes in nitric oxide, nitric oxide synthase, and nuclear factor-κB levels in the hippocampus tissues were examined. In cell experiments, hippocampal neurons were transfected with a virus vector carrying the heat shock protein 70 gene (H3445) before hyperbaric oxygen treatment. Cell viability, heat shock protein 70 expression, nitric oxide, nitric oxide synthase, and NF-κB levels in neurons were measured. The results showed that heat shock protein 70 expression significantly increased and peaked at 48 h after geranylgeranylacetone was given. Geranylgeranylacetone prolonged the first electrical discharge and seizure latencies, which was reversed by neuronal nitric oxide synthase, inducible nitric oxide synthase and NF-κB inhibitors. Nitric oxide, nitric oxide synthase, and inducible nitric oxide synthase levels in the hippocampus were significantly increased after hyperbaric oxygen exposure, but reversed by geranylgeranylacetone, while heat shock protein 70 inhibitor quercetin could inhibit this effect of geranylgeranylacetone. In the *in vitro* study, heat shock protein 70-overexpression decreased the nitric oxide, nitric oxide synthase, and inducible nitric oxide synthase levels as well as the cytoplasm/nucleus ratio of nuclear factor-κB and protected neurons from hyperbaric oxygen-induced cell injury. In conclusion, overexpression of heat shock protein 70 in hippocampal neurons may protect rats from central nervous system oxygen toxicity by suppression of neuronal nitric oxide synthase and inducible nitric oxide synthase-mediated nitric oxide production and translocation of nuclear factor-κB to nucleus.

Keywords: Central nervous system oxygen toxicity, heat shock protein 70, nitric oxide synthase, nuclear factor-κB, geranylgeranylacetone, nitric oxide

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Introduction

Hyperbaric oxygen (HBO) has been widely used in commercial, recreational, and military diving, as well as in the treatment of a variety of medical conditions. However, in divers or patients, the high partial pressures of oxygen used in HBO treatment may lead to central nervous system oxygen toxicity (CNS-OT) soon after exposure begins. During diving, CNS-OT may lead to drowning or barotrauma, which has dramatically limited the working benefits of HBO in underwater operations and clinical applications.[1–3] Because the pathogenesis of CNS-OT remains unclear, there are few interventions available. To develop an efficient strategy against CNS-OT, it is necessary to understand its pathogenesis and in particular, the relevant key factors involved.

Heat shock protein 70 (HSP70) is among the most conserved and abundant non-specific cytoprotective proteins in the heat shock protein family. HSP70 has anti-oxidation and anti-apoptosis effects and regulates multiple signal transduction factors and hormones that protect the nervous system.[4,5] Recent studies showed that HSP70 could prevent seizures caused by many factors.[6] Tsuchiya *et al*.[7] found that high expression of HSP70 in rats reduced the symptoms of epileptic seizures. Geranylgeranylacetone (GGA), an acyclic polyisoprenoid drug, is mainly used in China and Japan to treat ulcer. By increasing HSP70 expression, GGA can protect the gastric mucosa against insults without affecting gastric acid secretion.[8] Recent studies showed that GGA exerted cytoprotective action in various other organs (such as spinal nerves, liver, heart, brain, and retina) by activating HSP70.[9] A single administration of GGA induced high expression of HSP70 and reduced the symptoms of kainic acid-induced seizures and nerve damage.[10] In addition, intraventricular injection of purified HSP70 significantly limited seizures,[11] while quercetin (a HSP70 inhibitor) significantly increased the severity of seizures.[12] In the study of Arieli *et al*.,[13] appropriate heat stress pretreatment prolonged the CNS-OT-induced seizure latencies and the inhibitory effect was significantly correlated with the level of HSP70 in the brain.[13]

Recent studies showed that abnormally increased cerebral blood flow is pivotal in the pathogenesis of CNS-OT.[14] Nitric oxide (NO), produced by nitric oxide synthase (NOS), is a gaseous neurotransmitter that governs cerebral blood flow.[15] Nuclear factor κB (NF-κB) is an upstream regulator of NOS.[16] Once activated, it will translocate from cytoplasm to nucleus, resulting in transcription of NOS and the following cerebrovascular disorders.[17,18] Whether NF-κB, NO, and NOS are involved in the action of HSP70 on CNS-OT raised our great curiosity. Hence, we investigated the effects of HSP70 induced by oral administration of GGA on HBO-induced CNS-OT in rats. Latency to first electrical discharge (FED) and seizure and amplitude of seizures were recorded. Effects of inhibitors of NOS and NF-κB on seizure latency and changes in NO, NOS, and NF-κB levels in the hippocampus tissues were examined. Hippocampal neurons were transfected with a virus vector carrying the HSP70 gene (H3445) to induce HSP70 expression and exposed to HBO. Cell viability, HSP70 expression, NO, NOS, and NF-κB levels in hippocampal neurons were measured to explore the roles of NO, NOS, and NF-κB.

Experimental procedures

Animals

Sprague-Dawley (SD) rats (240–250 g, male) were bought from the Experimental Animal Center of the Naval Medical University. The rats were housed under temperature $24 \pm 1^{\circ}C$ and humidity 40–75% with a regular 12-h light: 12 h dark schedule and free access to pellet diet/water. At various time points, rats were sacrificed by overdose injection of pentobarbital (100 mg/kg, i.p.). All procedures were approved by the Ethics Committee for Animal Experiments of the Naval Medical University, and were carried out following the guidelines in "Guide for the Care and Use of Laboratory Animals" (NIH Publication No. 85–23, revised 1996).

Experimental groups

To measure the effect of GGA on HSP70 expression, animals were randomly assigned to eight groups ($n = 8$): Control group (Con; no treatment), Vehicle group (Veh; oral administration of 2 mL 5% gum Arabic), G12 h–G72 h group (GGA-treated, six groups). The GGA group received 2 mL suspensions of GGA in 5% gum Arabic (800 mg/kg) by oral route. Rats in the G12 h–72 h group were sacrificed at 12, 24, 36, 48, 60, and 72 h after GGA administration, respectively. Rats in the Veh group and Con group were sacrificed at 12 h after gum Arabic administration. HSP70 in the hippocampus was measured by Western Blot method.

To detect the effect of GGA on CNS-OT, NOS/NO production and NF-κB translocation, rats were randomly assigned to five groups ($n = 8$): HBO group (rats received 600 kPa HBO exposure until seizure occurred), GGA group (rats were exposed to HBO for 11 min at 48 h after GGA administration), GGA + HBO (GH) group (rats were exposed to HBO at 48 h after GGA administration until seizure occurred), vehicle + HBO (VH) group (rats were exposed to HBO until seizure occurred at 48 h after oral administration of 2 mL 5% gum Arabic), GGA + quercetin + HBO (GQH) group (rats were exposed to HBO until seizure occurred after GGA administration and injected with quercetin). Normally, the HBO used by divers and/or patients does not exceed 280 kPa for 30 min. A high pressure to 600 kPa is frequently used to quickly induce CNS-OT in the rat model.[19] GGA was orally given at a dose of 800 mg/kg, as previously reported to prevent kainic acid-induced seizures.[10] The dosage is higher than the current used dosage in human, but it was reported to cause no damage in neurons.[10] Quercetin was injected as previously reported (20 mg/kg, i.p.).[20] Latency to FED and seizure and amplitude of seizures were measured by electroencephalogram (EEG).

To measure the effect of inhibitors of NOS and NF-κB on seizure latencies, rats were randomly assigned to five groups ($n = 8$): Con, 7-NI, 1400 W, L-NAME, and BAY. Rats in the 7-NI, 1400 W, L-NAME, and BAY groups

received i.p. injection of 7-NI (50 mg/kg, Cayman, Ann Arbor, MI, USA), 1400 W (1 mg/kg, Beyotime, Shanghai, China), L-NAME (5 mg/kg, Beyotime, Shanghai, China) or BAY11–7082 (10 mg/kg, InvivoGen, San Diego, CA, USA) 30 min before exposure to HBO. The dosages of 7-NI, 1400 W, L-NAME and BAY11–7082 were previously reported by others.[21–24] Rats were exposed to HBO (600 kPa, 11 min) at 48 h after GGA oral administration (800 mg/kg).

To measure the effects of HBO exposure on cell viability and NO production, neurons were assigned to three groups ($n = 6$): Con group (neurons received no treatment), HBO30 group (neurons received 600 kPa HBO exposure for 30 min), and HBO60 group (neurons received 600 kPa HBO exposure for 60 min). To measure the expression of HSP70, neurons were assigned to three groups ($n = 6$): Con, H3445 and H134. HSP70 in the neurons were examined by Western Blot method. To detect the effects of HSP70 on cell viability, NO content, nNOS and iNOS expression and NF-kB translocation, neurons were assigned to four groups ($n = 6$): Con group, HBO group, H3445 + HBO group (H3445HBO), and H134 + HBO group (H134HBO).

Hippocampal neuron cultures

Rat hippocampus cells were cultured as previously described.[25] Briefly, cells were firstly harvested from the hippocampus of neonatal 24 h old SD rats. Hippocampi were dissected and placed in Hank's balanced saline (pH 7.4). Next, these cells were separated with trituration and 0.125% trypsin. The cell suspension was centrifuged at 1500 r/min for 5 min. Afterwards, cells were re-suspended in Dulbecco's modified eagle medium without glutamine and then cultured in 6-well cell culture cluster (4×10^5 cells/mL) in a cell incubator under normal condition. For cytotoxicity assays, cells were used at seven days of culture. All reagents were purchased from Beyotime (Shanghai, China).

HBO exposure

HBO exposure of rats. Rats were put into a 120-L HBO chamber (Naval Medical University, Shanghai, China) fulfilled with 99.9% O_2 under 600 kPa. Both compression procedure and decompression procedure were done linearly in a 5-min period. To prevent over accumulation of carbon dioxide, soda lime was used to absorb carbon dioxide; during the whole exposure, the chamber was constantly ventilated with 99.9% O_2. The temperature was maintained at 23–26°C.

HBO exposure of cells. The cell HBO chamber (OxyCure 3000, OxyHeal, USA) was sterilized, the temperature was set at 37°C, and humidity was set at 100%. Cells were grown in a 6-well culture cluster (4×10^5 cells/mL) for seven days before they were placed into the chamber. The chamber was first ventilated with mixed gas of 0.83% CO_2 and 99.17% O_2 (1 L/min) for 5 min. Afterwards, it was compressed to 600 kPa (100 kPa/min) and remained at that pressure for 30 or 60 min. When HBO exposure was finished, decompression was performed linearly to ambient pressure in 5 min.

HSP70 overexpression in cells

Lentiviral vectors encoding HSP70 gene were manufactured by Obio Technology (Shanghai, China). After neurons were transfected with HSP70 gene-carrying virus vector H3445 or empty vector H134, they were cultured for another four days before HBO exposure. The overexpression efficiency was validated by Western Blot.

Western Blot

For the animal study, hippocampus tissues were harvested, washed in 0°C phosphate-buffered saline, and then homogenized in radio-immunoprecipitation assay lysis buffer with 1 mM phenylmethanesulfonyl fluoride (PMSF) on ice and centrifuged at 12,000*g* at 4°C for 15 min. For the cell study, cells were washed with sterile phosphate buffered saline. Next, 180 μL of RIPA lysate buffer with 1 mM PMSF was added into each well and mixed with neurons at 4°C for 15 min. The lysates were then collected with a clean cell scraper and transferred to a 0.5 mL Eppendorf tube using a micropipette gun. The neurons were disrupted by ultrasound (120 W, 15 s) and centrifuged at 15,000 r/min for 10 min at 4°C to get the supernatant. Equal amounts of protein were separated with 10% SDS-PAGE gels and polyvinylidene fluoride membranes (Bio-Rad, Hercules, CA, USA). After blocking with 5% milk, membranes were incubated with primary antibodies to HSP70 (1:1000), nNOS (1:1000), iNOS (1:500), NF-κB (1:1000), and β-actin (1:2000) for 24 h. All the antibodies were products of Cell Signaling Technology company (Danvers, MA, USA). Blots were washed with TBST three times, 6 min each time. After washing, blots were incubated with secondary antibodies (1:2000) for 2 h at 25°C. Finally, bands were visualized with an Electro-Chemi-Luminescence (ECL) Substrate Kit (Amersham, Rahn AG, Zurich, Switzerland) and quantified with Bio-Rad Quantity One software (CA, USA).

EEG recording

EEG recording was conducted as described in our lab previously.[25] Briefly, rats were first implanted with EEG electrodes under anesthesia. Rats were allowed to recover for four days prior to HBO treatment. When recording EEG, the electrodes on rats were connected to a PowerLab/8SP recorder (AD Instruments, Castle Hill, Australia) using wires across the chamber wall. After rats adapted for 30 min, the EEG recording began shortly prior to the HBO exposure. After the first seizure was observed, the rats were decompressed to atmosphere and sacrificed with overdose pentobarbital (100 mg/kg, i.p.). Latency to FED and seizure and amplitude of seizures were measured from the EEG graphs.

NO measurement

After the hippocampal tissues or cells were homogenized and centrifuged, the NO concentration in the supernatant was measured using an NO detection kit (Beyotime, Shanghai, China). Briefly, the supernatants, Griess Reagent I, and Griess Reagent II were added to a 96-well plate at 50 μL/well, respectively, and incubated for 10 min

under 25°C. Next, its absorbance at 540 nm was recorded with a BioTek Synergy 2 microplate reader (Winooski, VT, USA). The concentration of NO was calculated and expressed as μmol/g protein.

Cell viability measurement

Cell cytotoxicity was assessed using a lactate dehydrogenase (LDH) cytotoxicity assay kit (Beyotime, Haiman, China). Briefly, 120 μL of supernatant in each well of the 6-well culture cluster was collected immediately after HBO exposure and incubated with 60 μL of the reaction mixture provided by the kit for 30 min under 25°C. Afterwards, the absorbance of 490 nm was recorded with a BioTek Synergy2 microplate reader (Winooski, VT, USA). The release of LDH was calculated using the absorption results and used to obtain cell viability.

Data analysis

Group comparisons were performed using the Wilcoxon signed-rank test and analysis of variance with SPSS 17.0. Data were expressed as mean ± standard deviation (SD). The results were considered as "statistically significant" when P value was less than 0.05.

Results

Time course of HSP70 expression in the hippocampus of rats induced by GGA

The time course of HSP70 expression in the hippocampus of rats was measured by Western Blot (Figure 1). The results showed that the level of HSP70 increased gradually and peaked at 48 h after a single oral administration of GGA (G12 h–G72 h vs. Veh, $P < 0.01$). Based on this result, the following experiments were performed at 48 h after GGA administration.

GGA prolonged the FED and seizure latencies induced by CNS-OT

Representative EEG recordings comparing the FED and seizure latencies in rats are shown in Figure 2(a) and (b). "Latency" refers to the time between the start of HBO treatment and the occurrence of FED or seizure. When rats were treated with GGA, then exposed to HBO for 11 min, no seizure occurred in these animals (Figure 2(a), middle). When rats were treated with the same dose GGA, then exposed to HBO until seizure occurred, The FED and seizure latencies in the GH group were significantly delayed compared to the HBO group ($P < 0.05$, Figure 2(c)). There was no difference in FED and seizure latencies between the VH and the HBO group. GGA and quercetin pretreatment significantly decreased the FED and seizure latencies (GQH groups) compared with the GH group ($P < 0.05$). Figure 2 (d) shows the amplitude of seizures in each group. No significant difference was observed in the amplitude of seizures ($P > 0.05$).

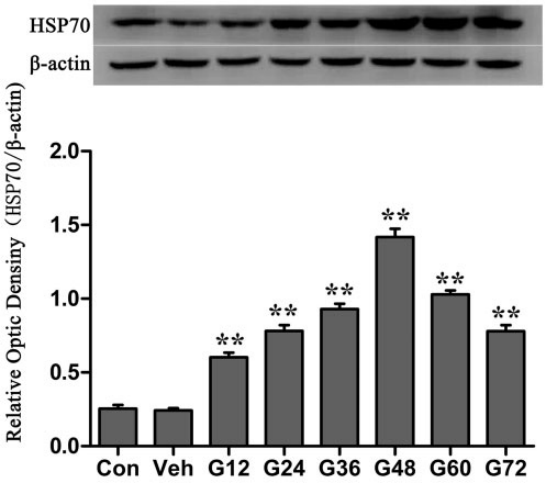

Figure 1. Time course of HSP70 expression induced by GGA in the hippocampus of rats. The expression of HSP70 in the hippocampus of GGA-treated rats was detected by Western Blot. Con: Control; Veh: Vehicle (rats received 2 mL of 5% gum Arabic); G12 h–G72 h (rats received GGA and were euthanized at 12–72 h after GGA administration). Data are expressed as mean ± SD. $^{**}P < 0.01$ compared to Veh.

Effects of inhibitors of NOS and NF-κB on seizure latencies

The roles of nNOS, iNOS, eNOS, and NF-κB in the protective effect of GGA on CNS-OT were explored by pretreatment with their respective inhibitors. The seizure latencies were significantly prolonged by nNOS inhibitor 7-NI, iNOS inhibitor 1400 W, and NF-κB inhibitor BAY11–7802 ($P < 0.01$, Figure 3). The eNOS inhibitor L-NAME had no effect on the latencies.

Changes in NO, NOS, and NF-κB levels in hippocampus tissues

Changes in NO, NOS, and NF-κB levels in the hippocampus tissues are shown in Figure 4. When rats were treated with GGA only, there was no significant change in the NO, NOS, and NF-κB levels in hippocampus tissues compared to Con group ($P > 0.05$). The NO, nNOS, and iNOS levels in the hippocampus were significantly increased in VH rats compared to Con rats, but GGA reversed these changes (Figure 4(a) and (b), $p < 0.01$). After rats in GQH group were pretreated with HSP70 inhibitor quercetin, the NO, nNOS, and iNOS levels in the hippocampus of rats were significantly increased compared with GH group (Figure 4(a) and (b), $P < 0.01$). The cytoplasm/nucleus ratio of NF-κB was significantly decreased by HBO in the VH group, but increased by GGA in the GH group (Figure 4(c), $P < 0.01$).

HBO-induced changes in viability and NO levels in hippocampal neurons

Primary hippocampal neuron cultures were treated with HBO for 30 min or 60 min before viability and NO level detection; 30-min HBO exposure had no impact on cell viability, but 60-min exposure showed remarkable inhibitory effect (Figure 5(a), $P < 0.01$). Both 30-min and 60-min HBO exposures significantly increased NO levels in

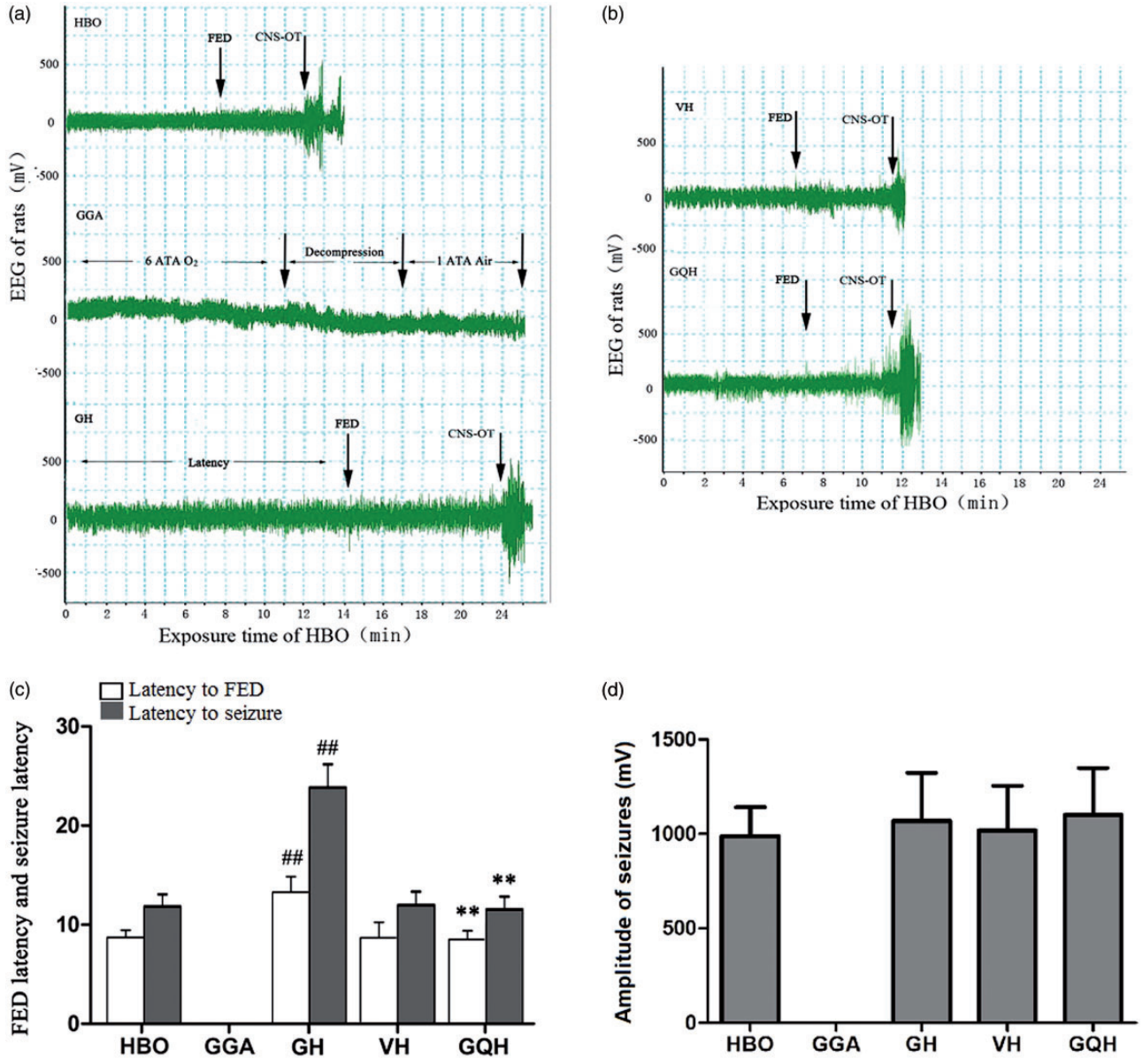

Figure 2. Latency to first electrical discharge (FED) and seizure and amplitude of seizures. Representative EEG recordings of seizures in rats: (a) (top), HBO group; a (middle), GGA group; a (bottom), GH group; (b) (top), VH group; and b (bottom), GQH group. (c) histogram comparing the FED and seizure latencies between the five groups. (d) amplitude of seizures. HBO group: rats received only HBO exposure until seizure occurs; GGA group: rats received GGA before 11-min HBO exposure; GH group: rats received GGA before HBO exposure until seizure occurs; VH group: rats received vehicle before HBO exposure until seizure occurs; GQH groups: rats received GGA and a HSP70 inhibitor quercetin before HBO exposure until seizure occurs. Data in c and d are expressed as mean ± SD. $^{\#\#}P < 0.01$ compared to VH; $^{**}P < 0.01$ compared to GH. (A color version of this figure is available in the online journal.)

hippocampal neuron cultures; the increase in 60-min HBO group was much higher than that in the 30-min HBO group (Figure 5(b), $P < 0.01$).

Effects of HSP70 overexpression on cell viability

Neurons were transfected with a virus vector carrying the HSP70 gene (H3445) or an empty vector (H134), and the overexpression efficiency was validated by Western Blot. HSP70 expression was significantly enhanced by H3445 transfection (Figure 6(a), $P < 0.01$), but was unaffected by H134. To further confirm the effects of HSP70 on CNS-OT, after transfection, neuron cultures were exposed to HBO and cell viability was examined. Cell viability in the H3445-tranfected cells had a significant increase compared to uninfected HBO group (Figure 6(b), $P < 0.01$); transfection with H134 caused no change in cell viability.

Effects of HSP70 overexpression on NO, NOS, and NF-κB levels in primary hippocampal neurons

As shown in Figure 7, H3445 significantly decreased NO, nNOS, and iNOS levels in the hippocampal neurons, while H134 had no effect on these parameters. Furthermore, the cytoplasm/nucleus ratio of NF-κB was significantly increased by H3445, but not changed by H134 (Figure 7(c)).

Discussion

Since Paul Bert first identified CNS-OT in the 19th century, many scholars have investigated its mechanisms and suggested various theories, including membrane permeability changes, lipid peroxidation, free radicals, vascular dysfunction, and CNS metabolic disorders.[14,26–28] The complicated mechanism makes it difficult to prevent CNS-OT effectively. Although a variety of prevention methods have been proposed, few measures have been applied to practice except controlling the pressure and duration of HBO exposure.[29–32]

In earlier studies, GGA, an anti-ulcer drug, was found to increase HSP70 expression in the gastrointestinal mucosa, liver, heart, and other tissues.[33] It was reported that GGA could also induce HSP70 expression in the CNS.[34] GGA can phosphorylate heat shock protein transcription factor I (HSFI) by increasing protein kinase C, which in turn increases HSP70 expression.[35] In present study, the expression of HSP70 in the hippocampus of rats was significantly increased and peaked at 48 h after GGA administration. The HSP70 expression gradually decreased after 48 h, but still remained significantly higher than Control at 72 h. These results confirmed the effectiveness of GGA to induce HSP70 in the CNS. The FED and seizure latencies were significantly prolonged from 8.66 ± 1.79 min and 11.83 ± 1.45 min to 17.25 ± 3.82 min and 23.83 ± 3.72 min by GGA, respectively. These effects were abolished by HSP70 inhibitor quercetin, which indicated that GGA delayed the occurrence of CNS-OT by inducing HSP70. When we treated a group of animals with the same dose GGA, then exposed them with HBO for 11 min and continued to measure the EEG measurement, the results showed that no seizure occurred in this group, suggesting that GGA treatment could actually prevent the seizures under the same HBO exposure. The amplitude of seizures in each group, however, was not significantly changed between groups. This result indicates that although rats in the GH group were exposed to longer HBO exposure, the amplitude of seizures was not increased, possibly due to the protection exerted by GGA.

In addition to inhibiting neuronal apoptosis and oxidative stress, studies have shown that HSP70 also regulated neural excitability and improved neuronal tolerance to

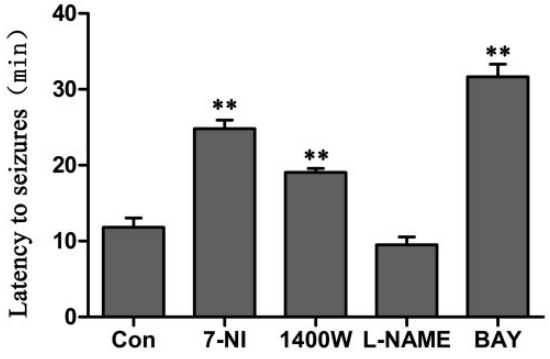

Figure 3. Effects of inhibitors of NOS and NF-κB on seizure latency. Rats were given different inhibitors 30 min before HBO exposure. The effects of inhibitors on seizure latencies were measured by EEG. Control (Con); 7-NI, nNOS inhibitor; 1400 W, iNOS inhibitor; L-NAME, eNOS inhibitor; BAY11–7802 (BAY), NF-κB inhibitor. Data are expressed as mean ± SD. $^{**}P < 0.01$ compared to Con.

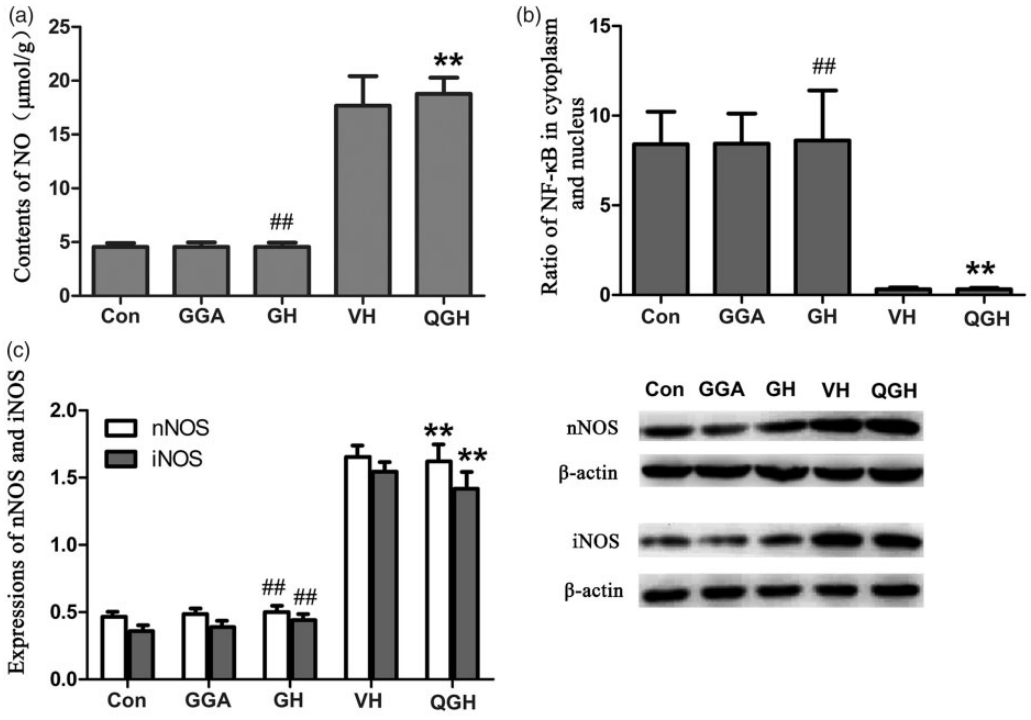

Figure 4. Changes of NO, NOS, and NF-κB levels in rat hippocampus. (a) changes of NO levels; (b) translocation of NF-κB from the cytoplasm to the nucleus; (c) changes of iNOS and nNOS protein expression and examples of Western Blots. Con group: rats received no treatment; GGA group: rats received GGA only; GH group: rats received GGA before HBO exposure; GQH groups: rats received GGA and the HSP70 inhibitor quercetin before HBO exposure; VH group: rats received 5% gum Arabic before HBO exposure. Data are expressed as mean ± SD. $^{\#\#}P < 0.01$ compared to VH; $^{**}P < 0.01$ compared to GH.

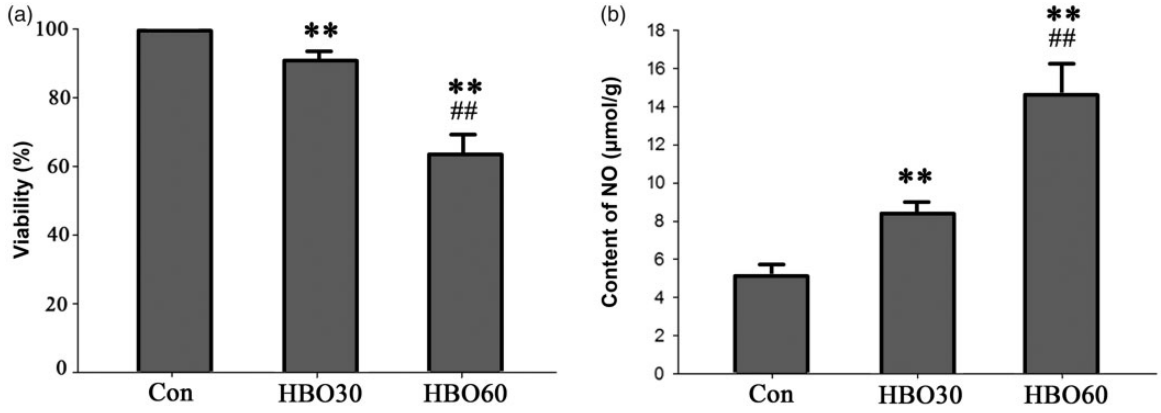

Figure 5. Changes in cell viability and NO levels in primary hippocampal neurons. Neurons were exposed to HBO for 30 min (HBO30) or 60 min (HBO60). (a) Changes of cell viability after HBO exposure. (b) Effects of HBO on NO levels. Con: cells received no treatment. Data are expressed as mean ± SD. $^{**}P < 0.01$ compared to Con; $^{\#\#}P < 0.01$ compared to HBO30.

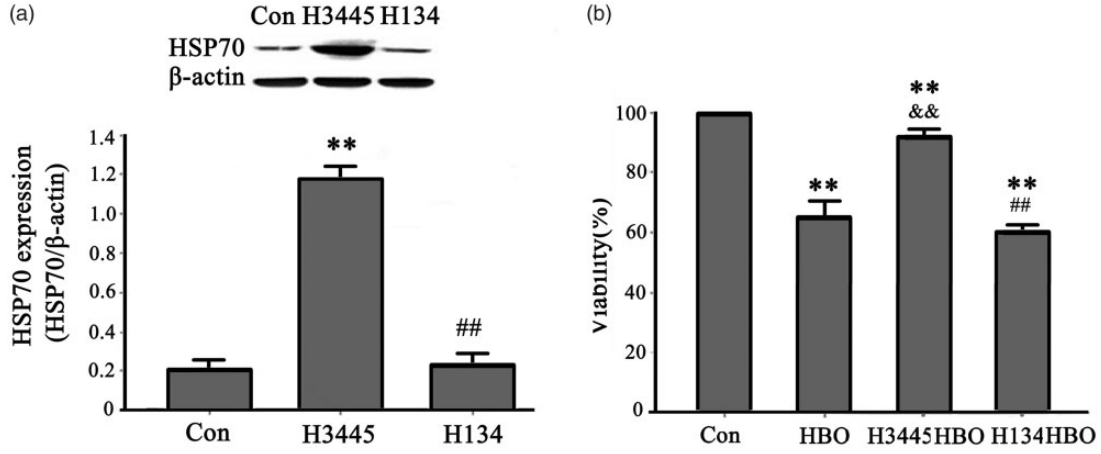

Figure 6. Effects of HSP70 transfection on HSP70 expression and cell viability. Primary hippocampal neurons were transfected with a virus vector carrying the HSP70 gene (H3445) or an empty vector (H134). The transfection efficiency was validated by Western Blot. (a) The expression of HSP70 after transfection. (b) Changes of cell viability after HBO exposure. Data are expressed as mean ± SD. $^{**}P < 0.01$ compared to Con; $^{\#\#}P < 0.01$ compared to H3445 (a) or H3445HBO (b); $^{\&\&}P < 0.01$ compared to HBO.

ischemia and other types of CNS damage.[36,37] Recent studies reported that HSP70 prevented a variety of injury induced by seizures and reduced the onset of symptoms.[7,10–11] In this study, when rats in the GGA-HBO group received quercetin, the FED and seizure latencies were significantly decreased. These results indicated the role of HSP70 in the protection of GGA against CNS-OT. To further confirm the effects of HSP70 on HBO-induced cell injury, we treated primary cultured hippocampal neurons with HBO, and then measured the changes of cell viability. The 60-min HBO exposure significantly decreased cell viability. After transfecting neurons with a virus vector carrying the HSP70 gene (H3445) or an empty vector (H134), HSP70 expression and cell viability were significantly enhanced in the H3445-transfected cells, but were not affected in H134-tranfected cells, indicating the possible role of HSP70 in the protection against HBO-induced neuronal injury.

During HBO exposure, NO plays an important role by dilating blood vessels, elevating cerebral blood flow, and ultimately triggering CNS-OT.[38,39] HBO treatment produces an excessive amount of oxygen molecules as a reaction substrate and promotes the reaction of NOS to catalyze NO production, which in turn dilates cerebral blood vessels and increases the excitability of nerve cells, leading to CNS-OT onset.[40] NO may also react with hydrogen peroxide, hydroxyl radicals, or superoxide anion, which facilitates CNS excitability and increases CNS-OT. During prolonged HBO exposure, over loaded superoxide anion leads to peroxynitrite production by reacting with NO, which has strong oxidizing and nitrification abilities, and finally causes tissue damage. Some stimuli can induce the release of arginine into nNOS-positive or iNOS-positive neurons and accelerate the formation of NO.[41] To explore the participation of NOS and NO in CNS-OT, rats were treated with HBO, GGA, and nNOS inhibitor 7-NI, iNOS inhibitor 1400 W, or eNOS inhibitor L-NAME before seizure latency measurement. The results showed that 7-NI and 1400 W, but not L-NAME, significantly abolished the effect of GGA and prolonged the seizure latencies,

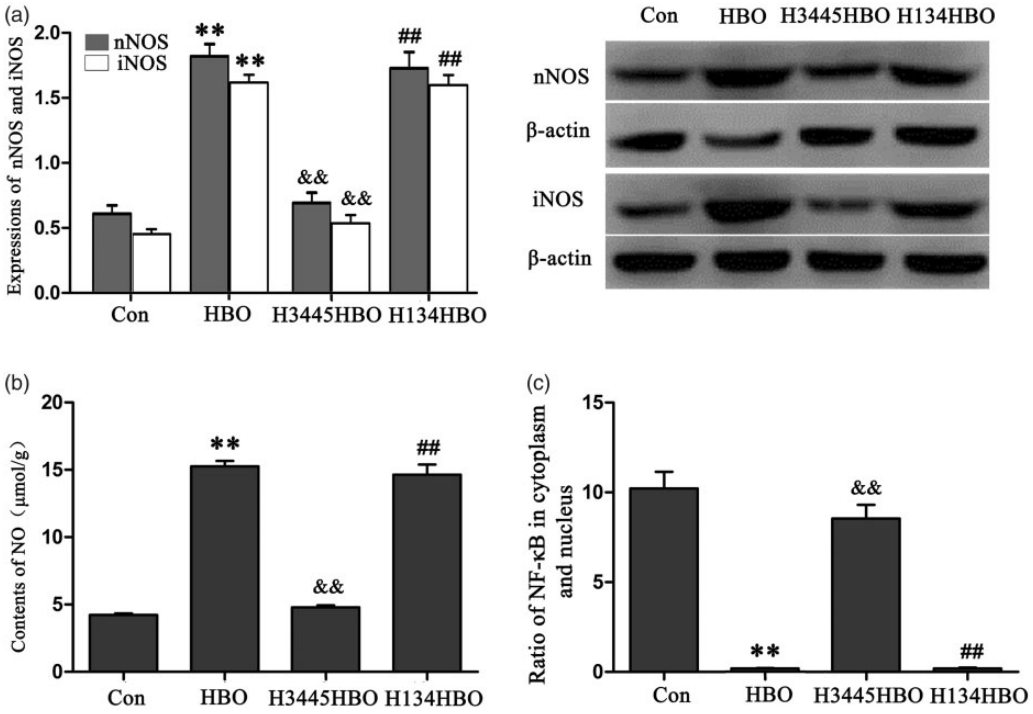

Figure 7. Effects of HSP70 transfection on NO, NOS, and NF-κB levels in primary hippocampal neurons. (a) Expression of nNOS and iNOS detected by Western Blot. (b) NO levels. (c) Translocation of NF-κB from the cytoplasm to the nucleus. Data are expressed as mean ± SD. $^{**}P < 0.01$ compared to Con; $^{\&\&}P < 0.01$ compared to HBO; $^{\#\#}P < 0.01$ compared to H3445.

suggesting the involvement of nNOS and iNOS in the mechanism of GGA protection. Furthermore, NO, nNOS, and iNOS levels in the hippocampus were significantly increased by HBO, but this effect of HBO was reversed by GGA. When rats were treated with GGA only, there was no significant change in the NO and NOS levels in hippocampus tissues compared to Con group, suggesting that GGA did not affect the normal expression of NOS and NO production. Quercetin, an inhibitor of HSP70, reversed the action of GGA on production of NO, nNOS, and iNOS. In cell experiments, HBO exposure increased NO levels in primary hippocampal neurons in a duration-dependent manner. Transfection of neurons with a virus vector carrying the HSP70 gene (H3445) significantly decreased the NO, nNOS, and iNOS levels. These results indicated that nNOS, iNOS, and NO might participate in the action of GGA-induced HSP70. HBO may stimulate the expression of nNOS and iNOS and production of NO and trigger CNS-OT. HSP70 can be induced by GGA treatment and may inhibit the expression of nNOS and iNOS and limit the production of NO, and thus reduce the severity of CNS-OT.

Normally, NF-κB is maintained in an inactive state in the cytoplasm. Under stress conditions, NF-κB can be activated by rapid phosphorylation and translocate into the nucleus. Holtman's study found that the onset of epilepsy was related to NF-κB, and inhibition of NF-κB activation reduced seizures and mortality in rats.[42] NF-κB activation may be a common cause of various epilepsies. A possible mechanism is that NF-κB regulates intracellular NOS expression after its translocation into the nucleus.[17,18] In the case of CNS-OT, HBO may activate NF-κB in the neurons and increase the expression of NOS and production of NO. In turn, NO dilates cerebral blood vessels and increases brain levels of acetylcholine, glutamate, and other neurotransmitters, leading to CNS-OT.[16,43] In present study, it was shown that administration of a NF-κB inhibitor BAY11–7802 significantly prolonged the seizure latency by nearly 2-fold, confirming the importance of NF-κB in CNS-OT. The results indicated that HBO exposure significantly activated NF-κB, while GGA inhibited it. When rats were treated with GGA only, there was no significant change in the NF-κB levels in hippocampus tissues compared to Con group, suggesting that GGA did not affect the normal activity of NF-κB. In cell experiments, the cytoplasm/nucleus ratio of NF-κB was significantly increased by H3445 transfection, suggesting the role of HSP70 in the activation of NF-κB. Taken together, these results suggested that NF-κB participated in the protective effects of HSP70 on CNS-OT. There have been some studies exploring the interaction between HSP70, NF-κB and NOS, but the conclusion appears controversial. Zhou *et al.*[44] reported that induction of HSP70 by heat treatment could suppress the activation of NF-κB and activated NOS/NO in a mice model of post-infection irritable bowel syndrome. In another study, HSP70 was found to suppress iNOS expression in brain glial cells by decreasing NF-κB p65 subunit nuclear accumulation.[17] In rat C6 glioma cells, HSP70 cold also inhibit iNOS induction by interfering NF-κB activation.[18] Furthermore, in an animal model of seizure, HSP70 could suppress IκB kinase (IKK) activity and phosphorylation of IκBα, thus protecting against apoptotic cell death induced by iNOS in the hippocampal and glial cells.[45] In our animal

model of CNS-OT, it appears that HSP70 can inhibit the activation of NF-κB and suppress nNOS and iNOS-mediated NO production, similarly to previous findings in CNS. The different action of HSP70 on NOS and NF-κB pathway in different organ and cells needs further exploration.

In conclusion, this study examined the protective effects of HSP70 on CNS-OT via *in vivo* and *in vitro* experiments. It was indicated that overexpression of HSP70 in hippocampal neurons may protect rats from CNS-OT by suppression of nNOS and iNOS-mediated NO production and the activation of NF-κB.

Authors' contributions: Hongjie Yi and Guoyang Huang contributed equally to this article. Hongjie Yi and Guoyang Huang carried out the experiments and wrote the paper; Kun Zhang helped to perform the cell experiments; Shulin Liu designed the study and revised the manuscript; Weigang Xu supervised the whole study and revised the manuscript.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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REFERENCES

1. Boison D, Chen JF, Fredholm BB. Adenosine signaling and function in glial cells. *Cell Death Differ* 2010;**17**:1071–82
2. Mahon RT, Dainer HM, Gibellato MG, Soutiere SE. Short oxygen prebreathe periods reduce or prevent severe decompression sickness in a 70-kg swine saturation model. *J Appl Physiol* 1985;**106**:1459–63
3. Petersen K, Soutiere SE, Tucker KE, Dainer HM, Mahon RT. Oxygen breathing accelerates decompression from saturation at 40 msw in 70-kg swine. *Aviat Space Environ Med* 2010;**81**:639–45
4. Franklin TB, Krueger-Naug AM, Clarke DB, Arrigo AP, Currie RW. The role of heat shock proteins Hsp70 and Hsp27 in cellular protection of the central nervous system. *Int J Hyperthermia* 2005;**21**:379–92
5. Fu ES, Tummala RP. Neuroprotection in brain and spinal cord trauma. *Curr Opin Anaesthesiol* 2005;**18**:181–7
6. Ammon-Treiber S, Grecksch G, Angelidis C, Vezyraki P, Hollt V, Becker A. Pentylenetetrazol-kindling in mice overexpressing heat shock protein 70. *Naunyn Schmiedebergs Arch Pharmacol* 2007;**375**:115–21
7. Tsuchiya D, Hong S, Matsumori Y, Kayama T, Swanson RA, Dillman WH, Liu J, Panter SS, Weinstein PR. Overexpression of rat heat shock protein 70 reduces neuronal injury after transient focal ischemia, transient global ischemia, or kainic acid-induced seizures. *Neurosurgery* 2003;**53**:1179–87
8. Fujimoto M, Yamanaka T, Bessho M, Igarashi T. Effects of geranygeranylacetone on gastrointestinal secretion in rats. *Eur J Pharmacol* 1982;**77**:113–8
9. Kikuchi S, Shinpo K, Takeuchi M, Tsuji S, Yabe I, Niino M, Tashiro K. Effect of geranylgeranylaceton on cellular damage induced by proteasome inhibition in cultured spinal neurons. *J Neurosci Res* 2002;**69**:373–81
10. Fujiki M, Kobayashi H, Inoue R, Ishii K. A single oral dose of GGA attenuates kainic acid-induced seizures and neuronal cell death in rat hippocampus. *Brain Res* 2004;**24**:281–5
11. Ekimova IV, Nitsinskaya LE, Romanova IV, Pastukhov YF, Margulis BA, Guzhova IV. Exogenous protein Hsp70/Hsc70 can penetrate into brain structures and attenuate the severity of chemically-induced seizures. *J Neurochem* 2010;**115**:1035–44
12. Nitsinskaia LE, Ekimova IV, Guzhova IV, Feizulaev BA, Pastukhov Iu F. [EFfect of quercetin on the severity of chemically induced seizures and the content of heat shock protein 70 in the rat brain structures]. *Ross Fiziol Zh Im I M Sechenova* 2010;**96**:283–92
13. Arieli Y, Eynan M, Gancz H, Arieli R, Kashi Y. Heat acclimation prolongs the time to central nervous system oxygen toxicity in the rat. Possible involvement of HSP72. *Brain Res* 2003;**962**:15–20
14. Demchenko IT, Boso AE, Bennett PB, Whorton AR, Piantadosi CA. Hyperbaric oxygen reduces cerebral blood flow by inactivating nitric oxide. *Nitric Oxid* 2000;**4**:597–608
15. Liu W, Li J, Sun X, Liu K, Zhang JH, Xu W, Tao H. Repetitive hyperbaric oxygen exposures enhance sensitivity to convulsion by upregulation of eNOS and nNOS. *Brain Res* 2008;**27**:128–34
16. Allen BW, Demchenko IT, Piantadosi CA. Two faces of nitric oxide: implications for cellular mechanisms of oxygen toxicity. *J Appl Physiol* 1985;**106**:662–7
17. Feinstein DL, Galea E, Aquino DA, Li GC, Xu H, Reis DJ. Heat shock protein 70 suppresses astroglial-inducible nitric-oxide synthase expression by decreasing NFkappaB activation. *J Biol Chem* 1996;**271**:17724–32
18. Feinstein DL, Galea E, Reis DJ. Suppression of glial nitric oxide synthase induction by heat shock: effects on proteolytic degradation of IkappaB-alpha. *Nitric Oxide* 1997;**1**:167–76
19. Liu S, Li R, Ni X, Cai Z, Zhang R, Sun X, Quock RM, Xu W. Perfluorocarbon-facilitated CNS oxygen toxicity in rats: reversal by edaravone. *Brain Res* 2012;**30**:56–65
20. Jo SK, Ko GJ, Boo CS, Cho WY, Kim HK. Heat preconditioning attenuates renal injury in ischemic ARF in rats: role of heat-shock protein 70 on NF-kappaB-mediated inflammation and on tubular cell injury. *J Am Soc Nephrol* 2006;**17**:3082–92
21. Coelho CH, Martins TF, Oliveira-Pelegrin GR, da Rocha MJA. Inhibition of neuronal nitric oxide synthase activity does not alter vasopressin secretion in septic rats. *Pituitary* 2017;**20**:333–9
22. Haddad Y, Couture R. Interplay between the kinin B1 receptor and inducible nitric oxide synthase in insulin resistance. *Br J Pharmacol* 2016;**173**:1988–2000
23. Luetic K, Sucic M, Vlainic J, Halle ZB, Strinic D, Vidovic T, Luetic F, Marusic M, Gulic S, Pavelic TT, Kokot A, Seiwerth RS, Drmic D, Batelja L, Seiwerth S, Sikiric P. Cyclophosphamide induced stomach and duodenal lesions as a NO-system disturbance in rats: L-NAME, L-arginine, stable gastric pentadecapeptide BPC 157. *Inammopharmacol* 2017;**25**:255–64
24. Wang ZR, Li YX, Lei HY, Yang DQ, Wang LQ, Luo MY. Regulating effect of activated NF-κB on edema induced by traumatic brain injury of rats. *Asian Pac J Trop Med* 2016;**9**:274–7
25. Kim HJ, Kim TH, Choi SJ, Hong YJ, Yang JS, Sung KW, Rhie DJ, Hahn SJ, Yoon SH. Fluoxetine suppresses synaptically induced [Ca(2)(+)]i spikes and excitotoxicity in cultured rat hippocampal neurons. *Brain Res* 2013;**15**:23–34
26. Arai M, Takata K, Takeda Y, Mizobuchi S, Morita K. The excitement of multiple noradrenergic cell groups in the rat brain related to hyperbaric oxygen seizure. *Acta Med Okayama* 2011;**65**:163–8
27. Tunnicliff G, Urton M, Wood JD. Susceptibility of chick brain L-glutamic acid decarboxylase and other neurotransmitter enzymes to hyperbaric oxygen in vitro. *Biochem Pharmacol* 1973;**22**:501–5
28. Yildiz S, Aktas S, Cimsit M, Ay H, Togrol E. Seizure incidence in 80,000 patient treatments with hyperbaric oxygen. *Aviat Space Environ Med* 2004;**75**:992–4
29. Block ER. Effect of superoxide dismutase and succinate on the development of hyperbaric oxygen toxicity. *Aviat Space Environ Med* 1977;**48**:645–8
30. Oury TD, Ho YS, Piantadosi CA, Crapo JD. Extracellular superoxide dismutase, nitric oxide, and central nervous system O2 toxicity. *Proc Natl Acad Sci U S A* 1992;**89**:9715–9

31. Shupak A, Tal D, Pratt H, Sharoni Z, Hochman A. Attenuation of cerebral oxygen toxicity by sound conditioning. *Otol Neurotol* 2004;**25**:186–92
32. Tsan MF, White JE, Caska B, Epstein CJ, Lee CY. Susceptibility of heterozygous MnSOD gene-knockout mice to oxygen toxicity. *Am J Respir Cell Mol Biol* 1998;**19**:114–20
33. Ohta Y, Kobayashi T, Imai Y, Inui K, Yoshino J, Nakazawa S. Effect of gefarnate on acute gastric mucosal lesion progression in rats treated with compound 48/80, a mast cell degranulator, in comparison with that of teprenone. *Biol Pharm Bull* 2005;**28**:1424–30
34. Fujiki M, Kobayashi H, Abe T, Ishii K. Astroglial activation accompanies heat shock protein upregulation in rat brain following single oral dose of GGA. *Brain Res* 2003;**991**:254–7
35. Uchida S, Fujiki M, Nagai Y, Abe T, Kobayashi H. Geranylgeranylacetone, a noninvasive heat shock protein inducer, induces protein kinase C and leads to neuroprotection against cerebral infarction in rats. *Neurosci Lett* 2006;**396**:220–4
36. Malyshev IY, Wiegant FA, Mashina SY, Torshin VI, Goryacheva AV, Khomenko IP, Kruglov SV, Pokidyshev DA, Popkova EV, Pshennikova MG, Vlasova MA, Zelenina OM, Manukhina EB. Possible use of adaptation to hypoxia in Alzheimer's disease: a hypothesis. *Med Sci Monit* 2005;**11**:25
37. Takeuchi H, Kobayashi Y, Yoshihara T, Niwa J, Doyu M, Ohtsuka K, Sobue G. Hsp70 and Hsp40 improve neurite outgrowth and suppress intracytoplasmic aggregate formation in cultured neuronal cells expressing mutant SOD1. *Brain Res* 2002;**949**:11–22
38. Atochin DN, Demchenko IT, Astern J, Boso AE, Piantadosi CA, Huang PL. Contributions of endothelial and neuronal nitric oxide synthases to cerebrovascular responses to hyperoxia. *J Cereb Blood Flow Metab* 2003;**23**:1219–26
39. Hagioka S, Takeda Y, Zhang S, Sato T, Morita K. Effects of 7-nitroindazole and N-nitro-l-arginine methyl ester on changes in cerebral blood flow and nitric oxide production preceding development of hyperbaric oxygen-induced seizures in rats. *Neurosci Lett* 2005;**382**:206–10
40. Jones K, Evans AW, Bristow RG, Levin W. Treatment of radiation proctitis with hyperbaric oxygen. *Radiother Oncol* 2006;**78**:91–4
41. Grima G, Benz B, Do KQ. Glutamate-induced release of the nitric oxide precursor, arginine, from glial cells. *Eur J Neurosci* 1997;**9**:2248–58
42. Holtman L, van Vliet EA, Edelbroek PM, Aronica E, Gorter JA. Cox-2 inhibition can lead to adverse effects in a rat model for temporal lobe epilepsy. *Epilepsy Res* 2010;**91**:49–56
43. Iadecola C, Xu X, Zhang F, el-Fakahany EE, Ross ME. Marked induction of calcium-independent nitric oxide synthase activity after focal cerebral ischemia. *J Cereb Blood Flow Metab* 1995;**15**:52–9
44. Zhou X, Dong L, Yang B, He Z, Chen Y, Deng T, Huang B, Lan C. Preinduction of heat shock protein 70 protects mice against post-infection irritable bowel syndrome via NF-kappaB and NOS/NO signaling pathways. *Amino Acids* 2015;**47**:2635–45
45. Chang CC, Chen SD, Lin TK, Chang WN, Liou CW, Chang AY, Chan SH, Chuang YC. Heat shock protein 70 protects against seizure-induced neuronal cell death in the hippocampus following experimental status epilepticus via inhibition of nuclear factor-kappaB activation-induced nitric oxide synthase II expression. *Neurobiol Dis* 2014;**62**:241–9

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