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Heat shock protein 70 protects PC12 cells against ischemia-hypoxia/reoxygenation by maintaining intracellular Ca^{2+} homeostasis

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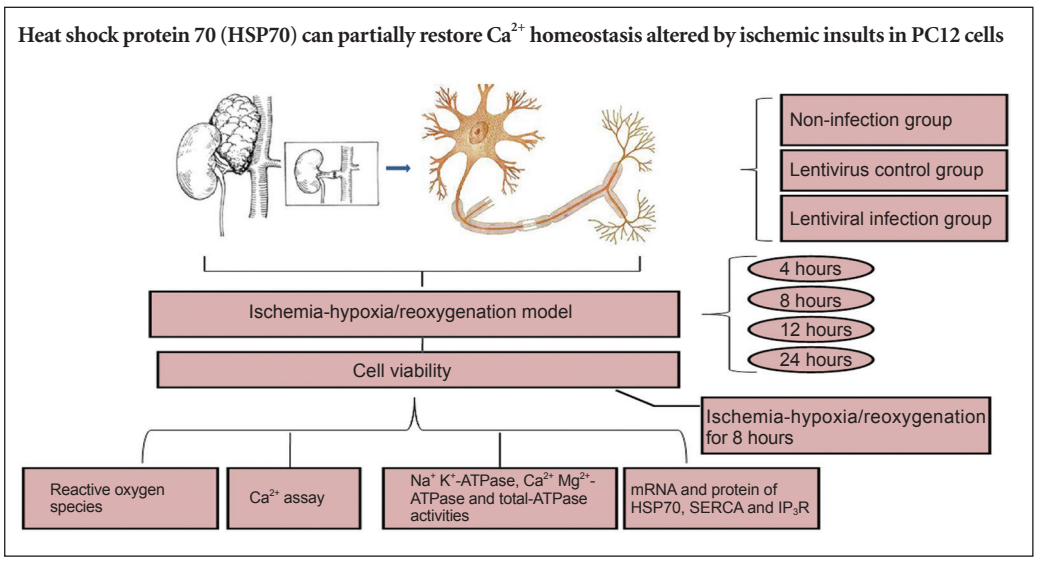
Graphical Abstract



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Abstract

Heat shock protein 70 (HSP70) maintains Ca^{2+} homeostasis in PC12 cells, which may protect against apoptosis; however, the mechanisms of neuroprotection are unclear. Therefore, in this study, we examined Ca^{2+} levels in PC12 cells transfected with an exogenous lentiviral HSP70 gene expression construct, and we subsequently subjected the cells to ischemia-hypoxia/reoxygenation injury. HSP70 overexpression increased neuronal viability and ATPase activity, and it decreased cellular reactive oxygen species levels and intracellular Ca^{2+} concentration after hypoxia/reoxygenation. HSP70 overexpression enhanced the protein and mRNA expression levels of sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA), but it decreased the protein and mRNA levels of inositol 1,4,5-trisphosphate receptor (IP_3R), thereby leading to decreased intracellular Ca^{2+} concentration after ischemia-hypoxia/reoxygenation. These results suggest that exogenous HSP70 protects against ischemia-hypoxia/reoxygenation injury, at least in part, by maintaining cellular Ca^{2+} homeostasis, by upregulating SERCA expression and by downregulating IP_3R expression.

Key Words: *nerve regeneration; exogenous heat shock protein 70; lentivirus transfection; ischemia-hypoxia/reoxygenation; PC12 cells; Ca^{2+}; endoplasmic reticulum; inositol 1,4,5-trisphosphate receptor; sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase; neural regeneration*

Introduction

Cerebrovascular ischemia is a condition in which there is insufficient blood flow to the brain to meet metabolic demand. This results in cerebral hypoxia/reoxygenation and neuronal cell death. Heat shock proteins (HSPs) are a group of conserved stress proteins found in eukaryotic and prokaryotic cells. These proteins are encoded by heat shock genes, and their expression is induced by heat stress or other adverse conditions (Pignataro et al., 2007; Fei et al., 2008). HSP70 is a 70-kDa stress protein of the inducible form, the most abundant and conserved member of the HSPs. A previous study found that HSP70 can be used as a sensitive marker

of cerebral hypoxia/reoxygenation at the early stage (Riezzo et al., 2010). Hypoxia/reoxygenation induces the expression of HSP70 in many organs, such as the liver, heart and intestine (Ramaglia and Buck, 2004; Liu et al., 2007; Sazontova et al., 2007; Orsenigo et al., 2012). Recent studies have shown that HSP70 protects the kidney, the mucosa of stress-induced gastric ulcers, and intestinal epithelial cells during hypoxia/reoxygenation (Bedirli et al., 2004; Oyake et al., 2006; Yuan et al., 2008). HSP70 mediates neuroprotection induced by ischemic preconditioning (Liu et al., 2004). Increased expression of HSP70 in the human brain has been suggested to prevent cell death in pathophysiological conditions (Radons and Multhoff, 2005). HSP70 tightly regulates Ca^{2+} homeostasis in PC12 cells and appears to have a strong anti-apoptotic function (Hu et al., 2015). However, the effects of HSP70 in the human brain against hypoxic damage and Ca^{2+} overload are unclear.

PC12 cells are a clonal cell line derived from rat adrenal medulla pheochromocytoma. PC12 cells have similar characteristics to neurons *in vitro*, and consequently, they have been widely used to study neuronal biology and pharmacology (Dijkmans et al., 2008). In the present study, we used PC12 cells to study neuronal Ca^{2+} homeostasis, as in a previous study (Smaili et al., 2001).

Ca^{2+} overload is involved in the pathology of cerebral hypoxia/reoxygenation. In the brain, ryanodine receptor (RyR) and inositol 1,4,5-trisphosphate receptor (IP_3R) are Ca^{2+} release channels located on the endoplasmic and/or sarcoplasmic reticulum. Sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) resides in the sarcoplasmic reticulum and transfers Ca^{2+} from the cytosol to the lumen of the sarcoplasmic reticulum at the expense of ATP hydrolysis. Na^+/Ca^{2+} exchanger (NCX) is a critically important membrane antiporter that removes Ca^{2+} from cells. NCX removes a single Ca^{2+} ion in exchange for three Na^+ ions. NCX is present in many different cell types and species (Brustovetsky et al., 2010).

Ca^{2+} accumulation in ischemia-hypoxia/reoxygenation is either abolished or significantly attenuated by overexpression of HSP70 (Hu et al., 2015). However, the mechanisms by which HSP70 maintains Ca^{2+} homeostasis have not been elucidated. Therefore, in the present study, we investigated the changes in Ca^{2+} levels in PC12 cells transfected with an exogenous lentiviral HSP70 gene expression construct and subjected to hypoxia/reoxygenation injury.

Materials and Methods

Cell culture

Differentiated PC12 cells, provided by the Department of Physiology, Qingdao University, Qingdao, Shandong Province, China, were cultured in Dulbecco's modified Eagle's medium supplemented with 5% horse serum, 10% fetal bovine serum, penicillin (100 U/mL) and streptomycin (100 μg/mL), under 5% CO_2 and 95% air (20% O_2) at 37°C (Yuan et al., 2005). An optical microscope (Olympus BX51, Tokyo, Japan) was used to observe cellular morphology. The final concentration of cells in each group was 5×10^5 cells/mL. The cells were divided into three groups: lentiviral infection group (lentiviral HSP70 gene delivery vector; Shanghai R&S Biotechnology Co., Ltd., Shanghai, China), lentivirus control group (empty vector, only containing lentivirus without the HSP70 gene; Shanghai R&S Biotechnology Co., Ltd.) and non-infection group. Technology for lentiviral infection was provided by Gene Chemical Company, Shanghai, China. Virus was screened with puromycin.

Cell culture model of ischemia-hypoxia/reoxygenation

The three different groups of cells were incubated with serum-free medium under sterile conditions. The cells were covered with culture solution and placed in a sealed container under 95% N_2 and 5% CO_2 at a flow rate of 10 L/min for 4–5 minutes. Samples were incubated in a hypoxic environment (approximately 1% O_2) in a 37°C incubator for 8 hours. Afterwards, normal medium was added, and the cells were cultured for 24 hours to simulate reoxygenation (Galán-Cobo et al., 2013).

In vitro cell viability assay

Neuronal cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. PC12 cells were seeded in 96-well plates at 5×10^3 cells/well for 24 hours, and incubated with serum-free medium under sterile conditions for 8 hours. At the end of the incubation period, 20 μL of MTT solution, 5 mg/mL, was added to each well and incubated for 4 hours. After removal of the medium, 150 μL dimethyl sulfoxide was added to each well to dissolve the formazan crystals. The optical density of each well was measured at 490 nm with a microplate reader (Biotek Synergy H1, Winooski, VT, USA).

Measurements of Na^+/K^+-ATPase, Ca^{2+}/Mg^{2+}-ATPase and total-ATPase activities

Cells were seeded in 24-well plates after hypoxia/reoxygenation treatment for 8 hours. A 3-mL aliquot of 1×10^6 cells/mL cell suspension was disrupted with an ultrasonic disrupter (Solarbio, Shanghai, China) (parameter settings: 160 Hz; pulse duration, 6 seconds; interval, 10 seconds; total of 20 pulses). Cells were centrifuged at $45 \times g$ and 4°C for 10 minutes. The supernatant was collected for protein quantification. Na^+/K^+-ATPase, Ca^{2+}/Mg^{2+}-ATPase and total-ATPase activities were measured in accordance with the instructions in the assay kit (Beyotime, Haimen, Jiangsu Province, China). Optical density was measured at 636 nm in a spectrophotometer (NanoDrop 2000, Thermo Scientific, Waltham, MA, USA).

Quantitative real time-polymerase chain reaction (qRT-PCR)

Total cellular RNA was extracted using the TRIzol one-step method after hypoxia/reoxygenation treatment for 8 hours. A 2-μg sample of RNA was reverse-transcribed using oligo(dT) primers and the Roche Reverse Transcriptase Kit (Roche, Basel, Switzerland) according to the manufacturer's protocol. The mRNA expression levels of the various genes were normalized to the expression level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the same cDNA sample. Quantitative real-time PCR was

performed with a LightCycler96 instrument (Roche) (Yan et al., 2014). The PCR reaction contained 2× FastStart Essential DNA Green Master Mix (10 μL), upstream and downstream primers (0.4 μM each), cDNA template (2 μL; ≤ 0.1 μg), and RNase-free water to a final volume of 20 μL. The reaction conditions were as follows: pre-denaturation at 95°C for 5 minutes; 35 cycles of 94°C for 15 seconds, annealing at 60°C for 30 seconds, extension at 70°C for 30 seconds. Specificity of the PCR products was verified by melting curve analysis. Differential expression of mRNA was calculated using the $2^{-\Delta\Delta Ct}$ method. The primers are listed in **Table 1**.

Western blot assay

HSP70, IP3R and SERCA protein levels were determined using a previously reported method (Honisch et al., 2015). Briefly, cells were washed three times with ice-cold phosphate buffered saline and suspended in 400 μL ice-cold radioimmune precipitation assay lysis buffer and 4 μL ice-cold phenylmethyl sulfonylfluoride lysis buffer (Thermo Fisher). Protein concentration was determined using the Bradford assay (BioRad, München, Germany). Samples containing 50 μg of total protein were solubilized in sample buffer at 100 °C for 5 minutes. The samples were subjected to 10% sodium dodecyl sulfate polyacrylamide gel electropheresis analysis and then electro-transferred onto polyvinylidene fluoride membranes and blocked with 5% non-fat milk in Tris-buffered saline/0.10% Tween 20 at room temperature for 2 hours. The membranes were incubated with primary antibodies; rabbit anti-rat HSP70 (1:1,000; Abcam, Cambridge, UK), rabbit anti-rat SERCA (1:1,000; Cell Signaling Technology, Danvers, MA, USA), rabbit anti-rat IP_3R (1:1,000; Cell Signaling Technology) or rabbit anti-rat β-actin (1:2,000; Cell Signaling Technology) at 4°C overnight. After washing with Tris-buffered saline/0.10% Tween 20, the blots were incubated with secondary goat anti-rabbit and anti-mouse antibodies (1:4,000; Boster, Wuhan, Hubei Province, China) for 2 hours at room temperature. Signals were visualized with enhanced chemiluminescence (Beyotime).

Measurements of reactive oxygen species (ROS)

To analyze the kinetics of ROS generation (Xu et al., 2009), PC12 cells were exposed to ischemia-hypoxia/reoxygenation for 8 hours, and then incubated in normal medium at 37°C for 24 hours. ROS were detected using the fluorescent probe dihydroethidium (Beyotime), dihydrorhodamine 123 (Molecular Probes) and 3-amino-4-aminomethyl-29,79-difluorescein diacetate (Molecular Probes). Cells were incubated with 2 mM dihydroethidium, 5 mM dihydrorhodamine 123 and 5 mM 3-amino-4-aminomethyl-29,79-difluorescein diacetate for 30 minutes at 37°C in the dark. The fluorescence intensity of ROS probes was analyzed by flow cytometric analysis (CyFlow® Counter, PARTEC, Munster, Germany).

Ca^{2+} assay

The levels of free cytosolic Ca^{2+} were measured using the cell-permeable Ca^{2+}-sensitive fluorescent dye Fluo-3/AM. PC12 cells were exposed to hypoxia/reoxygenation for 8 hours, and then incubated in normal meduim at 37°C for 24 hours. PC12 cells were incubated with 5 mM Fluo-3/AM (Beyotime) for 30 minutes at 37°C. The fluorescence intensity of Fluo-3/AM probes was analyzed by flow cytometric analysis (CyFlow® Counter, PARTEC).

Statistical analysis

Data were analyzed with SPSS 17.0 software (SPSS, Chicago, IL, USA) and were expressed as the mean ± SEM. One-way analysis of variance followed by the least significant difference test was used to compare differences between groups. Intergroup differences in cell viability measurements were compared using two-way analysis of variance followed by the Student-Newman-Keuls test. $P < 0.05$ was considered statistically significant.

Results

Effects of ischemia-hypoxia/reoxygenation on PC12 cell morphology

Under an optical microscope, PC12 cells were small and translucent immediately after passage in suspension. At 24 hours, most of the adherent cells showed the emergence of processes. At 48 hours, the cells were plump, and formed a network (**Figure 1A**). After an 8-hour period of ischemia-hypoxia/reoxygenation, PC12 cells exhibited no obvious morphological changes (**Figure 1B**).

HSP70 overexpression increased neuronal viability after ischemia-hypoxia/reoxygenation

MTT assay showed that PC12 cell viability was significantly higher in the lentiviral infection group than in the lentivirus control and non-infection groups after ischemia-hypoxia/reoxygenation for 8 hours ($P < 0.05$). There was no difference in cell viability between the lentivirus control group and non-infection group ($P > 0.05$; **Figure 2A**). After ischemia-hypoxia/reoxygenation for 8 hours, the three groups underwent reoxygenation for 7 days. Cell viability was significantly higher in the lentiviral infection group than in the lentivirus control group or the non-infection group ($P < 0.05$; **Figure 2B**).

HSP70 overexpression increased ATPase activities in PC12 cells after ischemia-hypoxia/reoxygenation

The activities of Na^+/K^+-ATPase, Ca^{2+}/Mg^{2+}-ATPase and total-ATPase were significantly higher in the lentiviral infection group than in the lentivirus control group or the non-infection group after ischemia-hypoxia/reoxygenation for 8 hours in PC12 cells ($P < 0.01$). There was no difference between the lentivirus control group and the non-infection group ($P > 0.05$; **Figure 3**).

HSP70 overexpression upregulated SERCA2a and SERCA2b mRNA expression and downregulated IP_3R mRNA expression in PC12 cells after ischemia-hypoxia/reoxygenation

HSP70, SERCA2a, SERCA2b and IP_3R mRNA expression

levels in PC12 cells were assessed by qRT-PCR after ischemia-hypoxia/reoxygenation for 8 hours. mRNA expression levels of HSP70, SERCA2a and SERCA2b were higher in the lentiviral infection group compared with the lentivirus control group or the non-infection group ($P < 0.01$). IP_3R mRNA expression was lower in the lentiviral infection group compared with the lentivirus control group or the non-infection group ($P < 0.01$; **Table 2**).

HSP70 overexpression upregulated SERCA protein levels and downregulated IP_3R protein levels in PC12 cells after ischemia-hypoxia/reoxygenation

HSP70, SERCA and IP_3R protein levels were detected by western blot assay after PC12 cells were exposed to ischemia-hypoxia/reoxygenation for 8 hours. HSP70 and SERCA protein levels were upregulated in the lentiviral infection group, while IP3R protein expression was downregulated, compared with the lentivirus control group or the non-infection group (**Figure 4**).

HSP70 overexpression decreased intracellular ROS production in PC12 cells after ischemia-hypoxia/reoxygenation

ROS production was measured in PC12 cells for 8 hours. ROS levels were significantly lower in the lentiviral infection group than in the lentivirus control group or the non-infection group ($P < 0.01$). There was no difference between the lentivirus control and non-infection groups ($P > 0.05$; **Table 3**).

HSP70 overexpression decreased intracellular Ca^{2+} concentration in PC12 cells after ischemia-hypoxia/reoxygenation

Ca^{2+} concentration was measured in PC12 cells after ischemia-hypoxia/reoxygenation for 8 hours. Intracellular Ca^{2+} concentration was significantly lower in the lentiviral infection group than in the lentivirus control and non-infection groups ($P < 0.01$). There was no difference between the lentivirus control and non-infection groups ($P > 0.05$; **Table 3**).

Discussion

Our findings demonstrate that ischemia-hypoxia/reoxygenation for 8 hours increases the expression of HSP70. Compared to normal cells, cell morphology was altered. We infer that HSP70 may not rescue cells acutely. However, cells gradually recovered their normal morphology over time. Seven days after reoxygenation, cell viability gradually reached the level of normal cells. We found that HSP70 overexpression decreased ROS production and Ca^{2+} concentration in PC12 cells exposed to ischemia-hypoxia/reoxygenation for 8 hours. PC12 cells are significantly damaged by ischemia-hypoxia/reoxygenation for 8 hours (Hu et al., 2015). Moreover, the decreased Ca^{2+} concentration was related to the upregulation of SERCA and the downregulation of IP_3R. Our results are consistent with a previous study (Amin et al., 1996), showing that overexpression of HSP70 protects cultured sensory neurons from nerve injury or ischemia. HSP70, in particular, has been demonstrated to play important roles in cerebrovascular disease (Zhang et al., 2009). It has been shown that HSP expression is correlated with ischemic vulnerability and neuronal survival (Nakka et al., 2010). The results of our study suggest that 8 hours of hypoxia/reoxygenation produces the most significant increase in HSP70 expression. Recent research has shown that Ca^{2+} plays a key role in cerebral ischemia (Kumar et al., 2014). Several proteins are responsible for cellular Ca^{2+} homeostasis. IP_3R and RyR are Ca^{2+} release channels located on the sarcoplasmic reticulum in all cell types. SERCA is located in the sarcoplasmic reticulum within nerve cells. It is a Ca^{2+}-ATPase that transfers Ca^{2+} from the cytosol to the lumen of the sarcoplasmic reticulum at the expense of ATP hydrolysis. NCX is an antiporter membrane protein that removes Ca^{2+} from cells. It has been reported that HSP70 may decrease Ca^{2+} overload in myocardial cells during myocardial ischemia (Chen et al., 2003). HSP70 may increase myocardial SERCA and RyR expression, enhance Ca^{2+} release from the endoplasmic reticulum into the cytosol by RyR, and increase cytosolic Ca^{2+} reuptake into the endoplasmic reticulum by SERCA. Furthermore, HSP70 increases NCX activity, suggesting that it may regulate Ca^{2+} homeostasis by affecting NCX function as well (Xu et al., 2009).

Ischemia-hypoxia/reoxygenation impacts Ca^{2+} flux and reduces reoxygenation injury. HSP70 decreases the tethering of the endoplasmic reticulum to mitochondria and prevents mitochondrial Ca^{2+} overload and reduces cell death after ischemia-hypoxia/reoxygenation (Dremina et al., 2012). Smaili et al. (2001) suggested that, in intact hepatocytes, cyclophilins play a role in Ca^{2+} cycling between the endoplasmic reticulum and mitochondria by showing that cyclosporine modifies IP_3-dependent Ca^{2+} signals. The present data expand on this notion because both genetic and pharmacological inhibition of cyclophilin D leads to decreased Ca^{2+} transfer from the endoplasmic reticulum to mitochondria through IP_3R, even in the *in vivo* cardiomyocyte model. Studies suggest that several isoforms of IP_3R are enriched in the mitochondria-associated endoplasmic reticulum membrane (Mendes et al., 2005; Szabadkai et al., 2006; Hayashi et al., 2009). In the brain, the type-2 isoform of IP_3R (IP_3R_2) is highly expressed in neurons (Vermassen et al., 2004). Most of the functions of IP_3R are attributed to IP_3R_2, and our results show that HSP70 preferentially interacts with this isoform. Our results also demonstrate that HSP70 regulates endoplasmic reticulum Ca^{2+} in PC cells and that its absence from these cells can alter Ca^{2+} homeostasis. Our results also demonstrate that perturbed Ca^{2+} homeostasis plays an important pathophysiological role in neurons.

The essential role of HSP70 in Ca^{2+} homeostasis is unexpected given the other cell types studied thus far (Guo et al., 2004; Shahlaie et al., 2013). It is conceivable that synaptic transmission produces a moderate but persistent deficit in endoplasmic reticulum calcium concentration because of release through IP_3R and RyR channels. Whereas primary Ca^{2+} signals in hematopoietic and other nonexcitable cells involve brief but massive Ca^{2+} release and require rapid refilling of depleted intracellular stores, higher HSP70 levels may be

Table 1 Primers for quantitative real time-polymerase chain reaction

Gene	Sequence (5′–3′)
HSP70	Forward: GCA AGG CCA ACA AGA TCA CCA TCA Reverse: TCC TCT TTC TCA GCC AGC GTG TTA
SERCA2a	Forward: ACT TCT TGA TCC TCT ACG TG Reverse: AAA TGG TTT AGG AAG CGG TT
SERCA2b	Forward: ACT TCT TGA TCC TCT ACG TG Reverse: AGA CCA GAA CAT ATC GCT AA
IP_3R	Forward: GTG GAG GTT TCA TCT GCA AGC Reverse: GCT TTC GTG GAA TAC TCG GTC
GAPDH	Forward: TGC ACC ACC AAC TGC TAG C Reverse: GGC ATG GAC TGT GGT CAT GAG

HSP70: Heat shock protein 70; SERCA: sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase; IP_3R: inositol 1,4,5-trisphosphate; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

Table 2 Effect of HSP70 overexpression on SERCA2a, SERCA2b and IP_3R mRNA ((copies/mL)^2) expression in PC12 cells after ischemia-hypoxia/reoxygenation

	Non-infection	Lentivirus control	Lentiviral infection
HSP70	0.428±0.019	0.423±0.023	0.785±0.018$^{**\#\#}$
SERCA2a	0.367±0.014	0.347±0.012	0.971±0.018$^{**\#\#}$
SERCA2b	3.015±0.091	2.941±0.091	8.869±0.162$^{**\#\#}$
IP_3R	0.439±0.020	0.433±0.040	0.183±0.020$^{**\#\#}$

Data are presented as the mean ± SEM and were analyzed by analysis of variance followed by the least significant difference test. $^{**}P < 0.01$, *vs.* lentivirus control group; $\#\#P < 0.01$, *vs.* non-infection group. The experiment was performed in triplicate. Lentiviral infection group: Lentivirus-mediated HSP70 gene transfected PC12 cells; lentivirus control group: empty vector transfected PC12 cells; non-infection group: untransfected PC12 cells. Cells were exposed to ischemia-hypoxia/reoxygenation for 8 hours. HSP70: Heat shock protein 70; SERCA: sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase; IP_3R: inositol 1,4,5-trisphosphate.

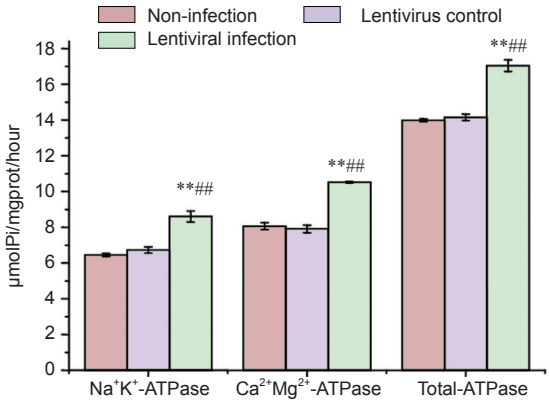

Figure 3 Effects of exogenous HSP70 on Na^+/K^+-ATPase, Ca^{2+}/Mg^{2+}-ATPase and total-ATPase activities in PC12 cells after ischemia-hypoxia/reoxygenation for 8 hours.
Data are presented as the mean ± SEM and were analyzed by analysis of variance followed by the least significant difference test. $^{**}P < 0.01$, *vs.* lentivirus control group, $\#\#P < 0.01$, *vs.* non-infection group. The experiment was performed in triplicate. Lentiviral infection group: Lentivirus-mediated HSP70 gene transfected PC12 cells; lentivirus control group: empty vector transfected PC12 cells; non-infection group: untransfected PC12 cells. Cells were exposed to ischemia/hypoxia/reoxygenation for 8 hours. HSP70: Heat shock protein 70.

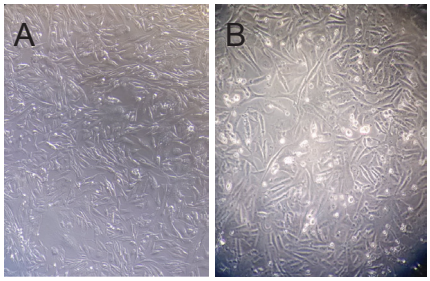

Figure 1 Morphology of differentiated PC12 cells after ischemia-hypoxia/reoxygenation for 8 hours (× 200).
(A) After plating for 48 hours, normal PC12 cells were plump and interconnected, forming a network. (B) After ischemia-hypoxia/reoxygenation for 8 hours, PC12 cells displayed no obvious morphological changes.

Table 3 Effect of HSP70 overexpression on cellular ROS levels (U) and intracellular Ca^{2+} concentration (U) in PC12 cells after ischemia-hypoxia/reoxygenation

Group	ROS	Ca^{2+}
Non-infection	58.03±1.97	48.20±3.02
Lentivirus control	57.72±2.35	46.80±2.75
Lentiviral infection	30.54±1.23$^{**\#\#}$	34.50±2.05$^{**\#\#}$
F	34.67	26.03
P	< 0.01	< 0.01

Data are presented as the mean ± SEM and were analyzed by analysis of variance followed by the least significant difference test. $^{**}P < 0.01$, *vs.* lentivirus control group; $\#\#P < 0.01$, *vs.* non-infection group. The experiment was performed in triplicate. Lentiviral infection group: Lentivirus-mediated HSP70 gene transfected PC12 cells; lentivirus control group: empty vector transfected PC12 cells; non-infection group: untransfected PC12 cells. Cells were exposed to ischemia-hypoxia/reoxygenation for 8 hours. HSP70: Heat shock protein 70; ROS: reactive oxygen species.

needed in neurons.

PC12 cells overexpressing HSP70 exhibited a significant increase in viability and ATPase activity, as well as decreased cellular ROS and intracellular Ca^{2+} concentration, after hypoxia/reoxygenation. HSP70 overexpression increased the mRNA and protein expression levels of SERCA, but it decreased the mRNA and protein levels of IP_3R, thereby decreasing intracellular Ca^{2+} concentration after hypoxia/reoxygenation. These results suggest that HSP70 overexpression improves the ischemia-induced perturbation in Ca^{2+} homeostasis in neuronal cells.

In summary, lentivirus-mediated HSP70 overexpression protects PC12 cells against ischemic/hypoxic injury by maintaining cellular Ca^{2+} homeostasis. Our findings suggest that lentivirus-mediated exogenous HSP70 overexpression may have clinical potential for the prevention and treatment of cerebral ischemia-hypoxia/reoxygenation.

Author contributions: *YQ and DH conceived and designed the study. YL and QSL performed the experiments. YL wrote the paper. XCW, SRH and ZL reviewed and edited the paper. All authors approved the final version of the paper.*
Conflicts of interest: *None declared.*

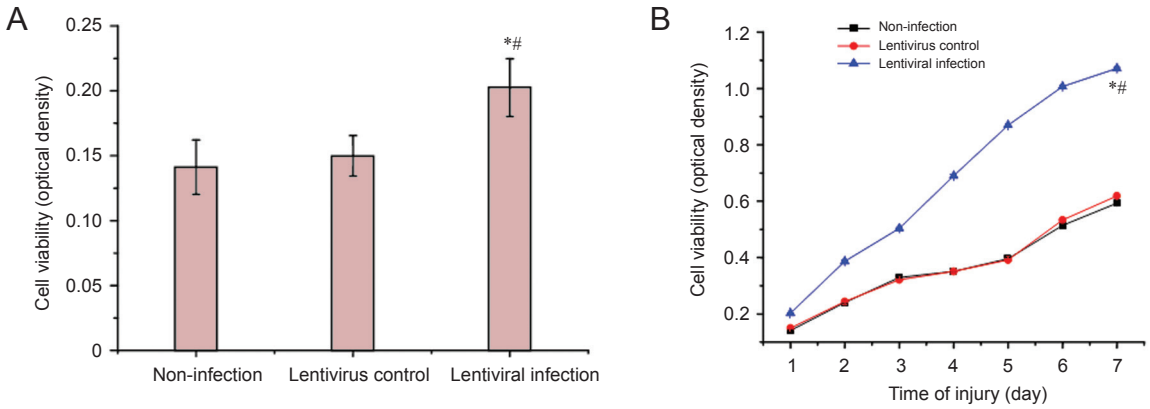

Figure 2 Effect of HSP70 overexpression on neuronal viability after ischemia-hypoxia/reoxygenation.
(A) Effects of exogenous HSP70 on cell viability of PC12 cells after 8 hours of ischemia-hypoxia/reoxygenation. (B) PC12 cells were then incubated for 1–7 days to simulate reoxygenation. Percent cell viability was assessed by MTT assay. Data are expressed as the mean ± SEM. *$P < 0.05$, *vs.* lentivirus control group; #$P < 0.05$, *vs.* non-infection group (two-way analysis of variance followed by Student-Newman-Keuls test). The experiment was performed in triplicate. Lentiviral infection group: Lentivirus-mediated HSP70 gene transfected PC12 cells; lentivirus control group: empty vector transfected PC12 cells; non-infection group: untransfected PC12 cells. Cells were exposed to ischemia-hypoxia/reoxygenation for 8 hours. HSP70: Heat shock protein 70; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

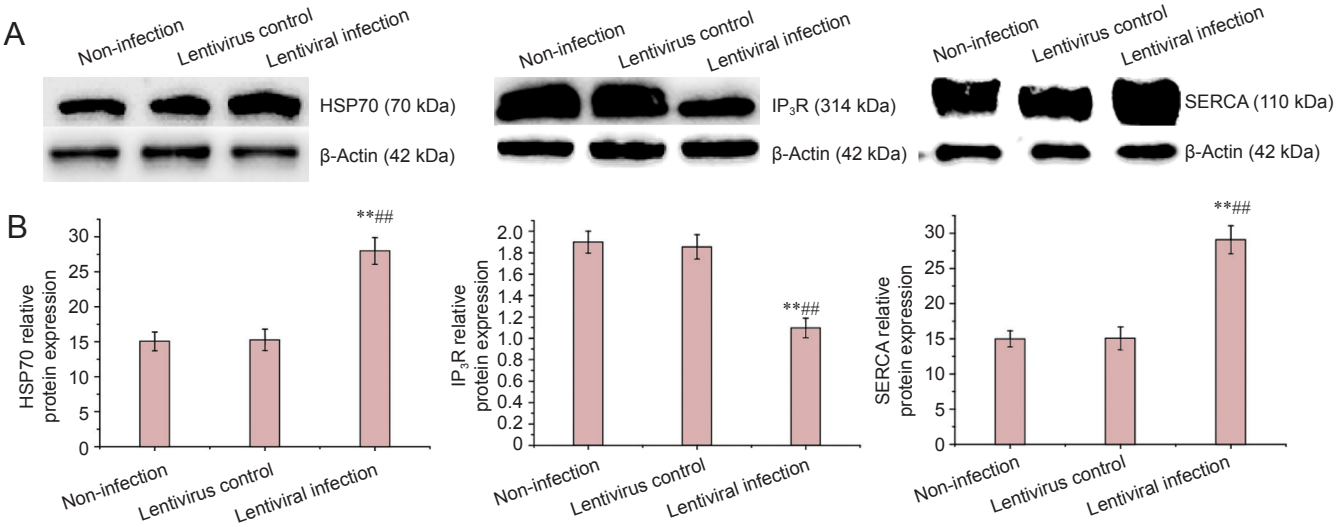

Figure 4 Effects of exogenous HSP70 on HSP70, SERCA and IP_3R protein expression levels in PC12 cells exposed to ischemia-hypoxia/reoxygenation for 8 hours.
(A) Western blot assay was used to assess HSP70, SERCA and IP_3R protein levels. Relative protein expression was calculated as the optical density ratio to that of β-actin. β-Actin was used as a loading control. (B) Statistical analysis. Data are presented as the ratio of HSP70, SERCA and IP_3R to β-actin. Each bar represents the mean ± SEM, and data were analyzed by analysis of variance followed by the least significant difference test. **$P < 0.01$, *vs.* lentivirus control group; ##$P < 0.01$, *vs.* non-infection group. The experiment was performed in triplicate. Lentiviral infection group: Lentivirus-mediated HSP70 gene transfected PC12 cells; lentivirus control group: empty vector transfected PC12 cells; non-infection group: untransfected PC12 cells. Cells were exposed to ischemia-hypoxia/reoxygenation for 8 hours. HSP70: Heat shock protein 70; SERCA: sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase; IP_3R: inositol 1,4,5-trisphosphate.

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Peer review: *This paper was double-blinded and stringently reviewed by international expert reviewers.*

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