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Tat-HSP70 protects neurons from oxidative damage in the NSC34 cells and ischemic damage in the ventral horn of rabbit spinal cord

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ABSTRACT

Heat shock protein 70 (HSP70) is an ATP-dependent molecular chaperone, and it has been shown that its levels increase after exposure to various types of stress, including ischemia. In the present study, we investigated the effects of HSP70 against H_2O_2-induced neuronal stress in NSC34 cells and against spinal cord ischemia in rabbits. Tat-HSP70 proteins facilitated the intracellular delivery of HSP70 into the NSC34 cells and enabled them to cross the blood-brain barrier in the rabbit spinal cord. Tat-HSP70 was effectively transduced into NSC34 cells in a concentration- and time-dependent manner, while control-HSP70 protein could not be delivered intracellularly at any concentration or time after treatment. Treatment with Tat-HSP70 reduced the generation of reactive oxygen species and cell death induced by H_2O_2, while the control-HSP70 did not show any significant effect on the NSC34 cells exposed to H_2O_2. In rabbit spinal cord, the administration of Tat-HSP70 showed significant amelioration of neurological defects and neuronal death in the ventral horn of spinal cord. In addition, Tat-HSP70 treatment significantly reduced lipid peroxidation and increased Cu, Zn-superoxide dismutase activities in the spinal cord, but glutathione peroxidase and Mn-superoxide dismutase activities remained unchanged. These results suggest that Tat-HSP70, not control-HSP70, decreases cell damage by reducing oxidative stress in NSC34 cells and rabbit spinal cord, and it can be employed for the reduction of neuronal damage caused after spinal cord ischemia.

1. Introduction

In clinics, paraplegia develops as an unpredictable complication after reconstruction surgery of the descending and thoracoabdominal aorta between 1 and 21 days following surgery depending on the duration and degree of ischemia (Crawford and Coselli, 1991; Etz et al., 2006; Kouchoukos, 1991; Panthee and Ono, 2015). Rabbit is the most suitable animal for the study of spinal cord ischemia, as rabbits have no collateral blood vessels in the thoracic spinal cord (Mazensky et al., 2011). Occlusion of the abdominal aorta at subrenal levels causes massive, but delayed, neuronal damage in the ventral horn of spinal cord in rabbits (Zivin and DeGirolami, 1980). One of most accepted hypotheses regarding delayed neuronal death is the increased generation of reactive oxygen species (ROS) and subsequent DNA and cell membrane damage due to the high levels of unsaturated fatty acids and high consumption of oxygen by neurons (Lin et al., 2003; Song et al., 2013). Free radicals generated during ischemia/reperfusion cause protein breakdown, lipid peroxidation, and DNA damage in neurons (Olmez and Ozyurt, 2012). Endogenous defense mechanisms, such as antioxidants, are activated to quench the excess free radicals and attenuate cell damage. Several studies have demonstrated that antioxidant treatment significantly reduces the neuronal damage induced

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by spinal cord ischemia (Hwang et al., 2015; Kim et al., 2012; Zhu et al., 2012).

Heat shock proteins (HSPs) are stress response proteins that are ubiquitously expressed in physiological conditions at very low levels. However, in the pathological conditions, HSPs are induced, and they facilitate protein folding to prevent protein aggregation (Kovacs, 2017). HSPs are divided into various subfamilies depending on their molecular weight, such as HSP27, HSP40, HSP60, HSP70, HSP90, and HSP110. Several reports have demonstrated that HSPs play an important role in cellular processes following oxidative stress including ischemia (Hecker and McGarvey, 2011; Kim et al., 2018). In particular, HSP70, an ATP-dependent molecular chaperone, is induced in the motor neurons in degenerative disease models (Brown, 2007) and after spinal cord ischemia in rats (Carmel et al., 2004; Cizkova et al., 2004). This protein facilitates the folding of unfolded or denatured proteins (Giffard et al., 2004; Mayer, 2013). Overexpression of HSP70β in mice induces neuroprotection against ischemic and oxidative damage in the hypothalamus (Chen et al., 2009). In contrast, it was seen that the administration of an HSP70 inhibitor induces cell damage in HEK and SH-SY5Y cells (Shabbir et al., 2015). Ischemic preconditioning significantly induces HSP70 expression in the CA1 neurons and exhibits neuroprotective effects against ischemic damage in the rat brain (Ge et al., 2008). However, there is conflicting evidence regarding whether early ischemic preconditioning changes the HSP70 expression level in the pig spinal cord after thoracoabdominal aortic occlusion or not (Kyrou et al., 2013).

Many studies have demonstrated the neuroprotective effects of HSP70 against ischemic damage (Doeppner et al., 2009; Kim et al., 2018; Zhan et al., 2010), but there are limitations to the therapeutic approaches of HSP70 in the central nervous system, as it cannot efficiently penetrate the blood-brain barrier. In our previous study, we demonstrated that certain cell-penetrating peptides, such as trans-activating transcriptional activator (TAT) and PEP-1, efficiently transduced into the brain (Eum et al., 2004) and the spinal cord (Jung et al., 2016). Administration of Fv-Hsp70, which is an Hsp70 protein attached to a modified antibody, or TAT-Hsp70, an Hsp70 attached to a TAT motif, improves the penetration of the protein across the blood-brain barrier and shows neuroprotective effects against ischemic damage in rats (Zhan et al., 2010) and mice (Doeppner et al., 2009). However, there have been no reports on the effects of HSP70 on the ventral horn of the spinal cord after ischemia-reperfusion. Therefore, in the present study, we decided to investigate the neuroprotective effects of Tat-HSP70 against neuronal damage in two different systems: an *in vitro* study using oxidative stress damage induced by H_2O_2 in NSC34 cells and an *in vivo* study using ischemia/reperfusion damage induced by occlusion of the aorta in rabbits.

2. Materials and methods

2.1. Construction of Tat-HSP70 and penetration efficiency into NSC34 cells

2.1.1. Construction of expression vectors

TAT expression vector was prepared as previously described (Shin et al., 2014). The cDNA sequence for human HSP70 was amplified by polymerase chain reaction (PCR) using the following primer sequences: forward; 5′-CTC GAG ATG GCC AAA GCC-3′ and reverse; 5′-GGA TCC CTA ATC TAC CTC CTC-3'. The PCR products were excised, eluted (Expin Gel; GeneAll Biotechnology Co., Ltd., Seoul, Korea), and ligated into a TA cloning vector (pGEM®-T easy vector; Promega Corporation, Madison, WI, USA) according to the manufacturer's protocol. The purified TA vector containing human HSP70 cDNA was ligated into the TAT expression vector to produce a TAT-HSP70 fusion protein. In a similar fashion, a control HSP70 that expressed the HSP70 protein without the TAT peptide was constructed. To produce the TAT-HSP70 and control HSP70 proteins, the plasmid was transformed into *Escherichia coli* BL21 cells. The transformed bacterial cells were grown in 100 mL of lysogeny broth (LB) media at 37 °C to an OD_{600} value of 0.4–0.5, and then induced with 0.5-mM isopropyl β-D-1-thiogalactopyranoside (IPTG) followed by incubation at 37 °C for 6 h. The harvested cells were lysed by sonication and purified using Ni^{2+}-nitrilotriacetic acid (Ni-NTA) Sepharose affinity (Qiagen, Inc.) and PD-10 columns (GE Healthcare, Chicago, IL, USA). The concentrations of the purified protein were estimated using Bradford assay (Bradford, 1976).

Equal amounts of proteins were analyzed using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Analyzed proteins were electrotransferred to a polyvinylidene difluoride (PVDF) membrane, and then the membrane was blocked with tri-buffered saline (25 mM Tris-HCl, 140 mM NaCl, 0.1% Tween 20, pH 7.5) containing 5% non-fat dry milk. The membrane was probed using antibodies against polyhistidine tag (1:2,000, His-probe, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Proteins were identified using chemiluminescent reagents as recommended by the manufacturer (Amersham, Franklin Lakes, NJ, USA).

2.1.2. Culture of NSC34 cells

NSC34 cells, purchased from Bio-Medical Science Co., Ltd (Seoul, South Korea), were maintained and subcultured every 3–5 days as described previously (Yoo et al., 2017). The medium was changed every 2 days, and the cells were grown for up to 7 days, allowing them to differentiate into motor neuron-like cells with increased neurite formation (Eggett et al., 2000).

2.1.3. Transduction of Tat-HSP70 proteins into NSC34 cells

Transduction of Tat-HSP70 proteins into NSC34 cells was done as described previously with a few modifications (Yoo et al., 2017). Cells were treated with control-HSP7 or Tat-HSP70 at various concentrations (0 μM, 0.25 μM, 0.5 μM, 0.75 μM, 1.0 μM) for 1 h or at 1 μM concentration for different time periods (0 min, 15 min, 30 min, 45 min, and 60 min). The cells were then treated with trypsin-ethylenediaminetetraacetic acid (trypsin-EDTA) for 1 min and washed with phosphate-buffered saline (PBS) to eliminate the proteins attached to the cellular membranes. Cells were lysed with ice-cold RIPA buffer (Thermo Scientific, IL, USA), and the lysates were centrifuged at 13,000 × *g* for 10 min at 4 °C. The protein concentration of the supernatant was quantified using Bradford assay. Equal amounts of proteins were separated by SDS-PAGE and analyzed by Western blot using a rabbit anti-polyhistidine antibody (1:1,000, His-probe, Santa Cruz Biotechnology) and a goat anti-rabbit secondary antibody (1:10,000, Vector Laboratories, Burlingame, CA, USA).

To demonstrate the morphology of the transduced Tat-HSP70 protein in NSC34 cells, immunocytochemistry was done using polyhistidine probes as described previously (Yoo et al., 2017). Cells were cultured on coverslips and treated with 1 μM Tat-HSP70 or control-HSP70 protein. After 1 h of incubation at 37 °C, the cells were washed with PBS twice and fixed with 4% paraformaldehyde for 5 min. The cells were treated with PBS containing 3% bovine serum albumin and 0.1% Triton X-100 (PBS-BT) at 25 °C for 30 min, then washed with PBS-BT. The cells were incubated with rabbit anti-polyhistidine antibody (1:2000) at 25 °C for 1 h and subsequently with AlexaFluor 488-conjugated anti-rabbit IgG (1:15,000, Invitrogen, Carlsbad, CA, USA) at 25 °C for 1 h. The cell nuclei were stained with 1 μg/mL of 4′,6-diamidino-2-phenylindole (DAPI) (Roche Applied Science, Mannheim, Germany) for 2 min. The stained cells were analyzed using a confocal fluorescence microscope with a confocal laser-scanning system (Bio-Rad MRC-1024ES, 4BIOROD, CA, USA).

2.2. Protective effects of Tat-HSP70 against H_2O_2-induced damage in NSC34 cells

2.2.1. Cell viability assay and DNA damage

The protective effects of Tat-HSP70 or control-HSP70 on cells exposed to H_2O_2 were assessed by measuring the cell viability using an

MTT assay kit (Cayman Chemical, Ann Arbor, MI, USA). NSC34 cells were plated at a confluence of 90% in a 96-well plate and exposed to various concentrations (0.25–1 μM) of Tat-HS70 or control HSP-70 protein. After incubation with Tat-HS70 or control HSP-70 protein for 1 h, cells were treated with 100 μM H_2O_2 for 1 h. Cell viability was measured at 570 nm using an ELISA microplate reader (Labsystems Multiskan MCC/340), and it was expressed as the percentage of the viable treated cells vs untreated control cells.

To examine the effects of Tat-HSP70 protein on DNA damage, 1 μM of Tat-HSP70 or control-HSP70 protein was treated for 1 h and exposed to 100 μM H_2O_2 for 3 h, as described previously (Yoo et al., 2017), and the cellular damage was confirmed by terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) and a Cell Death Detection kit (Roche Applied Science). Images were obtained using a fluorescence microscope (Nikon eclipse 80i, Tokyo, Japan), and the fluorescence levels were measured using a Fluoroskan enzyme-linked immunosorbent assay (ELISA) plate reader (Labsystems Multiskan MCC/340, Helsinki, Finland) at 485 nm excitation and 538 nm emission wavelengths.

2.2.2. Measurement of intracellular ROS levels

Formation of intracellular ROS was determined by measuring the level of 2′,7′-dichlorofluorescein (DCF) formed from DCF diacetate (DCF-DA) after ROS exposure, as described previously (Yoo et al., 2017). ROS levels were assessed in NSC34 cells in several conditions, such as without any treatment, with only H_2O_2 treatment (100 μM), and with Tat-HSP70 or control-HSP70 protein pretreatment (1 μM). After 1 h of pre-treatment with Tat-HSP70 or control-HSP70 protein, the cells were treated with H_2O_2 (100 μM) for 10 min. Following a PBS wash, the cells were treated with 20 μM DCF-DA for 30 min. Fluorescence levels were measured using a Fluoroskan ELISA plate reader (Labsystems Oy, Helsinki, Finland) at 485 nm excitation and 538 nm emission wavelengths.

2.3. Neuroprotective effects of Tat-HSP70 against ischemic damage in the rabbit spinal cord

2.3.1. Experimental animals

Male New Zealand white rabbits (1.2–1.5 kg) were purchased from the Experimental Animal Center (Cheonan Yonam College, Cheonan, South Korea). Animals were housed in conventional rooms of animal facility in College of Veterinary Medicine in Seoul National University. The protocol in this experiment was made according to the ARRIVE guidelines (Kilkenny et al., 2010) and approved by Institutional Animal Care and Use Committee (IACUC) of Seoul National University (approval number: SNU-170807-10).

2.3.2. Experimental design

To observe the neuroprotective effects and determine optimal dosage of Tat-HSP70 ischemic damage in the spinal cord, the animals were divided into six groups: sham-operated (control), vehicle (10% glycerol) treated ischemic group, 4.5 mg/kg control-HSP70-treated ischemic group, 0.5, 1.5, or 4.5 mg/kg Tat-HSP70-treated ischemic groups. All materials were treated intraperitoneally into the rabbits immediately after reperfusion.

2.3.3. Induction of transient spinal cord ischemia

Spinal cord ischemic surgery was conducted as described in a previous study (Kiyoshima et al., 2003; Yoo et al., 2017). Briefly, the animals were anesthetized with a mixture of 2.5% isoflurane (Baxtor, Deerfield, IL, USA) in 33% oxygen and 67% nitrous oxide. Abdominal aorta was occluded at the subrenal region using a non-traumatic aneurysm clip for 30 min, and reperfusion was confirmed under surgical stereoscope. The body temperature of animals during and after surgery was controlled by a thermometric blanket and a thermal incubator (Mirae Medical Industry, Seoul, South Korea). Control animals were subjected to a sham surgical procedure without occlusion of abdominal aorta.

2.3.4. Physiological monitoring before and after ischemia/reperfusion

To analyze the acute toxic effects of control-HSP70 and Tat-HSP70, arterial blood gases (PaO_2 and $PaCO_2$), pH, and glucose levels were measured using a GEM Premier 3000 gas analyzer (Instrumentation Laboratory, Milan, Italy) before ischemia/reperfusion and 10 min after, as described previously (Yoo et al., 2017).

2.3.5. Neurological assessment

To assess behavioral deficits after ischemia/reperfusion, two blinded observers checked the behaviors according to modified Tarlov criteria (Tarlov, 1957) to ensure objectivity as described in the previous studies (Yoo et al., 2017; Jung et al., 2016) as follows: 0, no voluntary hind-limb function; 1, only perceptible joint movement; 2, active movement but unable to stand; 3, able to stand but unable to walk; or 4, completely normal hind-limb motor function. Behavioral deficits were assessed 24 h and 72 h after reperfusion because the animals showed neurological dysfunction 12–24 h after reperfusion and complete paraplegia within the ensuing 48 h (Moore and Hollier, 1991; Wisselink et al., 1998).

2.3.6. Cresyl violet staining in the spinal cord

For morphological analysis, animals used in the neurological assessment ($n = 5$ in each group) were anesthetized with 2 g/kg urethane (Sigma) and were perfused transcardially, first with 0.1 M PBS (pH 7.4), followed by 4% paraformaldehyde in 0.1 M phosphate-buffer (pH 7.4), as described previously (Yoo et al., 2017). The animals' spinal cords were removed, and the 5–6th lumbar segments (L_5-L_6) of the spinal cord were post-fixed in the same fixative for 12 h. The spinal cord tissues were cryoprotected by infiltration with 30% sucrose overnight, and 30-μm-thick spinal cord sections were cut serially in the coronal plane using a cryostat (Leica, Wetzlar, Germany).

The procedures of cresyl violet staining were conducted, as described in a previous study (Yoo et al., 2015). Five sections per animals were mounted on the silane-coated slide (Muto-Glass, Tokyo, Japan) and rinsed in 1% cresyl violet acetate solution containing glacial acetic acid (Sigma). Before and after staining for 5 min at 25 °C, the sections were washed twice in distilled water. They were then dehydrated and cover-slipped using Canada Balsam (Kanto, Tokyo, Japan).

Cresyl violet-positive cells in all the groups were measured using an image analysis system equipped with a computer-based CCD camera (software: Optimas 6.5®, CyberMetrics, Scottsdale, AZ, USA), in a tissue area observed under 100 × primary magnification. In brief, the cresyl violet-positive neurons present at the center of the ventral horn of the spinal cord were counted. The images were converted to grayscale and cresyl violet-positive neurons were automatically selected according to the intensity of cresyl violet immunostaining. Cell counts were obtained for each animal by averaging the counts from five sections in each group, and the cell number was reported as a percentage of that obtained from the control groups.

2.3.7. Biochemical assessments in spinal cord tissue

For the biochemical assessments of the malondialdehyde (MDA), glutathione peroxidase (GPx), Cu, Zn-superoxide dismutase (SOD1), and Mn-SOD (SOD2) activities in the spinal cord tissue, the animals in the control group, the vehicle-treated ischemic group, the control-HSP70-treated ischemic group, and 1.5 mg/kg Tat-HSP70-treated ischemic group ($n = 10$ in each group) were anesthetized with 2 g/kg urethane (Sigma). Then, 24 h and 72 h after ischemia/reperfusion, the spinal cord tissues were obtained from the L_5-L_6 levels. The spinal cord tissue samples were stored at −80 °C till further analysis, as described in a previous study (Yoo et al., 2017). The spinal cord samples were homogenized in chilled PBS, and then centrifuged at 10,000 × *g* at 4 °C for 10 min. The tissue protein concentration was determined using a

standard commercial kit (Bio-Rad Laboratories, Hercules, CA, USA). Tissue MDA (Cayman Chemical Company, Ann Arbor, MI, USA), GPx (Cayman Chemical Company), SOD1 (Cusabio, Hubei, China), and SOD2 (Cusabio) activities were measured by commercially available kits.

2.4. Statistical analysis

The data were expressed as the mean of the experiments performed for each experimental investigation. In order to determine the protective effects of Tat-HSP70 or control-HSP70 protein against H_2O_2-induced oxidative stress and ischemic damage in rabbits, mean differences were analyzed statistically by a one-way analysis of variance (ANOVA) followed by Bonferroni's post-hoc test using GraphPad Prism 5.01 software (GraphPad Software, Inc., La Jolla, CA, USA).

3. Results

3.1. Confirmation of Tat-HSP70 and control-HSP70 protein expression and effective delivery of proteins into the NSC34 cells

3.1.1. Confirmation of Tat-HSP70 and control-HSP70 protein expression

The purified and expressed HSP70 proteins were confirmed by Western blot analysis using polyhistidine probes. The Western blot results showed that the Tat-HSP70 and control-HSP70 proteins were of approximately 72 kDa and 70 kDa, respectively (Fig. 1B).

3.1.2. Confirmation of Tat-HSP70 delivery into NSC34 cells

Intracellular delivery of Tat-HSP70 protein into NSC34 cells was confirmed by Western blot analysis using polyhistidine probe after treatment with various concentrations of Tat-HSP70 or control-HSP70. Treatment with 0.25–1 μM of control-HSP70 protein did not show any polyhistidine expression at 1 h after treatment, while polyhistidine expression was found at 0.5 μM Tat-HSP70 treatment and increased in a dose-dependent manner when treated with 1 μM Tat-HSP70 at 1 h after treatment (Fig. 1C).

Weak expression of polyhistidine was found at 30 min after 0.75 μM Tat-HSP70 treatment, and thereafter, the polyhistidine expression gradually increased up to 60 min after treatment. However, treatment with 0.75 μM control-HSP70 did not show any polyhistidine expression 60 min after treatment (Fig. 1D).

Transduced Tat-HSP70 or control-HSP70 was confirmed by immunocytochemical staining for polyhistidine. Polyhistidine positive structures were not detected in control or 1 μM control-HSP70-treated groups at 1 h after treatment. However, in the Tat-HSP70-treated group, intracellular polyhistidine expression was mainly found in the cytosol at 1 h after treatment (Fig. 1E).

3.2. Protective effects of Tat-HSP70 against H_2O_2-induced oxidative damage in NSC34 cells

The neuroprotective effects of the control-HSP70 or Tat-HSP70 were evaluated using MTT assay to observe cell viability after exposure to 100 μM H_2O_2 for 1 h. Treatment with 100 μM H_2O_2 for 1 h significantly reduced the cell viability to 44.9% of the viability seen in the control group. Pre-treatment with control-HSP70 followed by exposure to 100 μM H_2O_2 showed similar cell viability (39.3–41.7% vs. control group) as that of the 100 μM H_2O_2 treated group. Cell viability was significantly increased with 0.5 μM Tat-HSP70 treatment (75.4% vs. control group) compared to that in the 100 μM H_2O_2 treated group. Cell viability was maintained by 1 μM Tat-HSP70 treatment (Fig. 2A).

Cell death induced by DNA damage was assayed by TUNEL staining in the NSC34 cells 3 h after exposure to H_2O_2. In the 100 μM H_2O_2 treated group, strong TUNEL-positive cells were found in the NSC34 cells and reactive fluorescence intensity was significantly elevated to 4.4 fold of the intensity seen in the control group. In the control-HSP70-treated group 1 h before exposure to 100 μM H_2O_2, TUNEL-positive cells were abundantly observed, and the fluorescence intensity was similar to that of the 100 μM H_2O_2 treated group. In the Tat-HSP70-treated group 1 h before exposure to 100 μM H_2O_2, only a few TUNEL positive cells were observed and the fluorescence intensity dramatically decreased to 49.7% of the intensity seen in the 100 μM H_2O_2 treated group (Fig. 2B).

ROS levels were measured by the fluorescence generated due to the conversion of DCF-DA to DCF in the NSC34 cells. In the control group, very weak or no fluorescent cells were found in the NSC34 cells. In the 100 μM H_2O_2 treated group, strong fluorescence was in NSC34 cells, and the fluorescence intensity was prominently increased in NSC34 cells compared to that in the control group. In the control-HSP70-treated group, similar level of fluorescent intensity was seen in the NSC34 cells compared to that in the 100 μM H_2O_2 treated group. In the Tat-HSP70-treated group, the fluorescent cells nearly disappeared, and fluorescence intensity was significantly decreased compared to that of the 100 μM H_2O_2 treated group (Fig. 2C).

3.3. Neuroprotective effects of Tat-HSP70 against ischemic damage in spinal cord

3.3.1. Changes in physiological parameters

Administration of Tat-HSP70 or control-HSP70 did not show any significant changes in the levels of arterial blood gases (PaO_2 and $PaCO_2$), pH, and glucose. These parameters were evaluated before and 10 min after ischemia/reperfusion (Table 1).

3.3.2. Changes in neurological score

Neurological scores were determined by Tarlov's criteria at 24 h and 72 h after ischemia/reperfusion. In the control group, the animals showed normal behavior and movements with a neurological score of 4. In the vehicle-treated group, the animals displayed impaired walking with extended hindlimb and showed neurological scores of 1 and 0.5 at 24 h and 72 h after ischemia/reperfusion, respectively. In the control-HSP70- and 0.5 mg/kg Tat-HSP70-treated group, the animals could not stand up due to hindlimb extension and showed similar neurological score compared to that in the vehicle-treated group. The animals treated with 1.5 and 4.5 mg/kg of Tat-HSP70 could stand and walk and showed a significant increase in neurological scores to 2.7–3.3 at 24 h and 72 h after ischemia/reperfusion, compared to that in the vehicle-treated group. There were no significant differences in the neurological scores between the 1.5 and 4.5 mg/kg Tat-HSP70-treated group (Fig. 3A).

3.3.3. Changes in cresyl violet positive cells

In the control group, abundant cresyl violet positive neurons were found in the ventral horn of spinal cord, and the number of cresyl violet positive neurons was around 26.04 per section. In the vehicle-treated group, a few cresyl violet positive neurons were detected in the ventral horn, and the number was significantly lower, by 4.12 per section, compared to that of the control group at 72 h after ischemia/reperfusion. In the control-HSP70- and 0.5 mg/kg Tat-HSP70-treated groups, the number of cresyl violet positive neurons was similar to that of the vehicle-treated group. In the 1.5 and 4.5 mg/kg Tat-HSP70-treated groups, some cresyl violet positive neurons were observed in the ventral horn. In these groups, the number was significantly increased compared to that of the vehicle-treated group and was 14.68 and 16.6 per section, respectively (Fig. 3B).

3.4. Neuroprotective mechanisms of Tat-HSP70 against ischemic damage in spinal cord

To investigate the role of Tat-HSP70 against ischemic damage in the spinal cord, the activities of MDA, GPx, SOD1, and SOD2 were measured using the spinal cord homogenates at 24 h and 72 h after

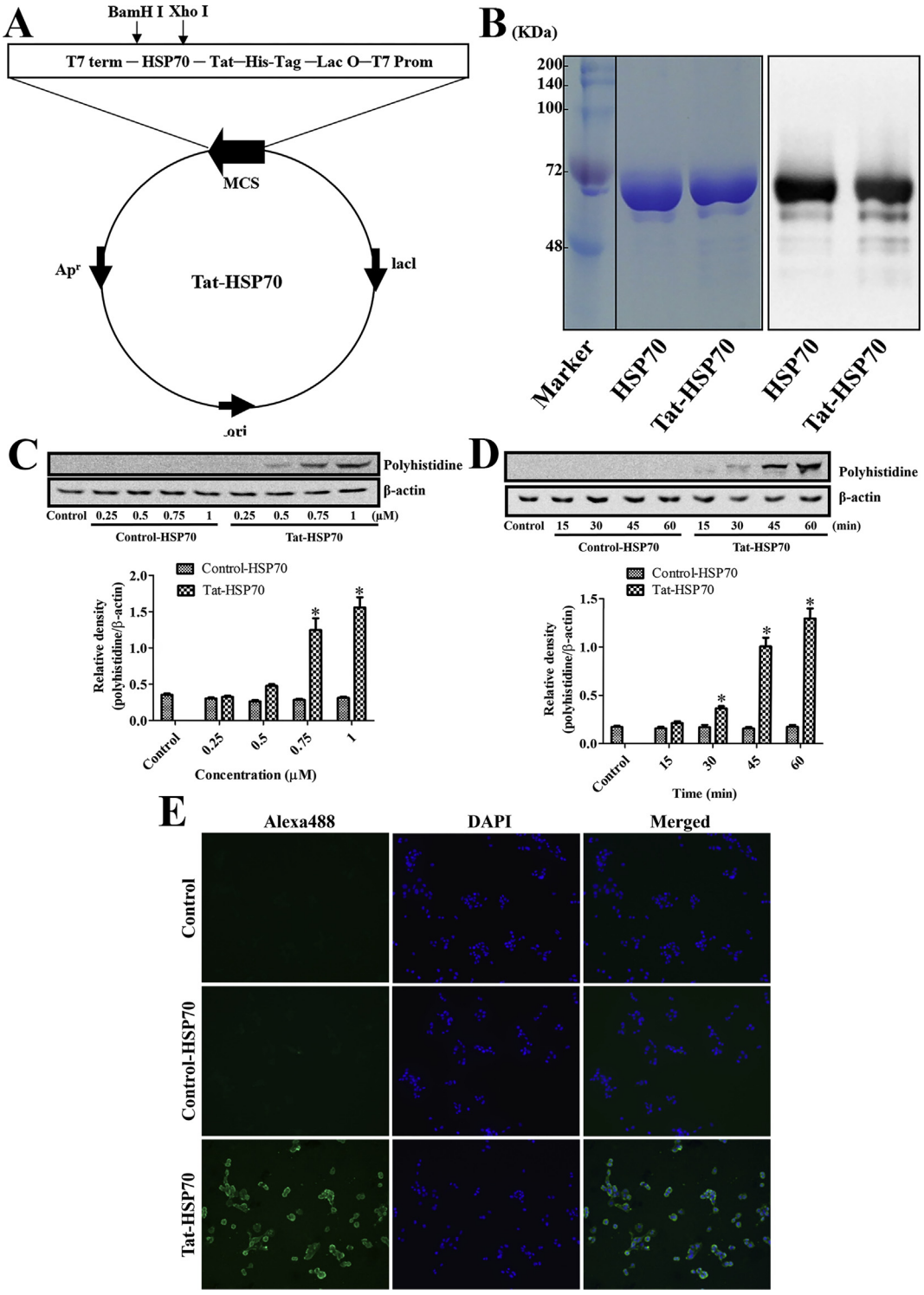

Fig. 1. Generation and confirmation of expressed control-HSP70 and Tat-HSP70 fusion proteins, and efficiency of concentration- and time-dependent transduction of control-HSP70 and Tat-HSP70 fusion proteins into NSC34 cells. (A) Diagrammatic representation of control-HSP70 and Tat-HSP70 vector. (B) Confirmation of the expression of control-HSP70 and Tat-HSP70 fusion protein by Western blot analysis using polyhistidine probes. (C) Transduction efficiency when cells are treated with various (0.25–1 μM) concentrations of control-HSP70 and Tat-HSP70 is determined 1 h after treatment by Western blot probing using polyhistidine probes. (D) Time-dependent transduction efficiency is assessed by Western blot analysis using polyhistidine probes at 1 h after 1 μM control-HSP70 and Tat-HSP70 treatment. (C and D) Data was analyzed by one-way analysis of variance followed by a Bonferroni's post-hoc test ($^{a}p < 0.05$, significantly different from the control group; $^{b}p < 0.05$, significantly different from the vehicle-treated group; $^{c}p < 0.05$, significantly different from the control-HSP70-treated group). The data are shown as mean with standard deviation. (E) Microphotographs obtained after immunocytochemistry using polyhistidine probes at 1 h after treatment with 1 μM control-HSP70 and Tat-HSP70. Scale bar = 20 μm.

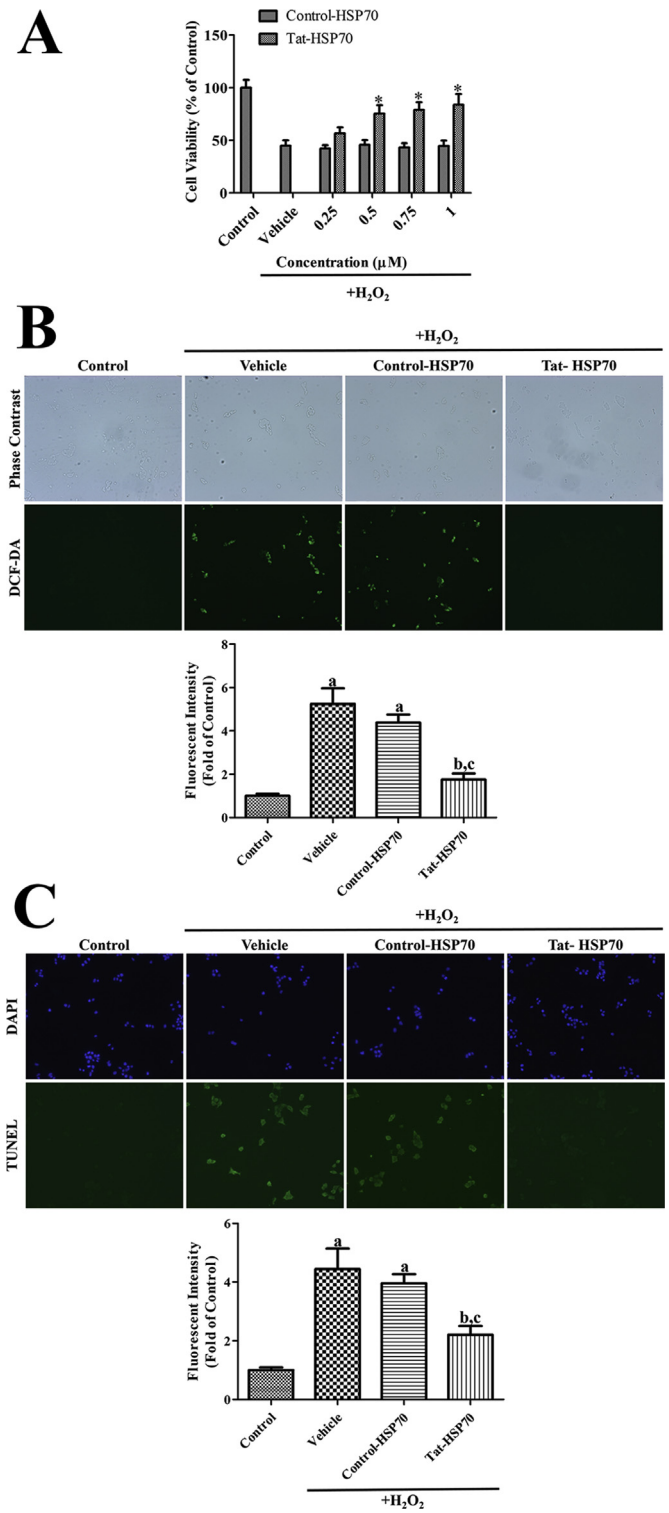

Fig. 2. Neuroprotective effects of control-HSP70 and Tat-HSP70 proteins against oxidative damage induced by 100 μM H_2O_2 in NSC34 cells. (A) Concentration-dependent (0.25–1.0 μM) cell viability of control-HSP70 and Tat-HSP70 proteins exposed to 100 μM H_2O_2 is assessed by MTT assay in NSC34 cells. (B and C) Immunofluorescent staining for DCF-DA and TUNEL to measure ROS formation and DNA fragmentation in NSC34 cells exposed to 100 μM H_2O_2 after treatment with 1 μM of control-HSP70 and Tat-HSP70, respectively. Scale bar = 50 μm (B and C). The data were analyzed using one-way analysis of variance followed by a Bonferroni's post-hoc test ($^a p < 0.05$, significantly different from the control group; $^b p < 0.05$, significantly different from the vehicle-treated group; $^c p < 0.05$, significantly different from the control-HSP70-treated group). The data are shown as mean with standard deviation.

ischemia/reperfusion. MDA levels were significantly increased in the vehicle-treated group compared to the control group at 24 h and 72 h after ischemia/reperfusion. Administration of control-HSP70 did not show any remarkable changes in the MDA levels at 24 h and 72 h after ischemia/reperfusion, while 1.5 mg/kg Tat-HSP70 treatment showed significant reduction of MDA levels at 24 h and 72 h after ischemia/reperfusion (Fig. 4).

Spinal cord ischemia significantly decreased GPx activities in the spinal cord homogenates compared to the control group at 24 h and 72 h after ischemia/reperfusion. In addition, GPx activities were further decreased 72 h after ischemia compared to the 24 h post-ischemic group. In the control-HSP70- and Tat-HSP70-treated groups, similar GPx activities were detected in the spinal cord homogenates of vehicle-treated group at 24 and 72 h after ischemia (Fig. 4).

SOD1 activities in the vehicle-treated group were significantly increased in the spinal cord homogenates compared the control group at 24 h after ischemia/reperfusion, and similar SOD1 levels were observed at 72 h after ischemia/reperfusion. In the control-HSP70-treated group, SOD1 activities showed similar pattern compared to the vehicle-treated group at 24 h and 72 h after ischemia/reperfusion. In the Tat-HSP70-treated group, SOD1 activities were similar to those of the vehicle-treated group, 24 h after ischemia/reperfusion, while SOD1 activities were significantly higher than those in the vehicle-treated group, 72 h after ischemia/reperfusion (Fig. 4).

Spinal cord ischemia did not cause any significant changes in SOD2 activities of spinal cord ischemia. In addition, the administration of control-HSP70 or Tat-HSP70 also showed no remarkable changes in the SOD2 activities compared to the control or vehicle-treated group at 24 h and 72 h after ischemia/reperfusion (Fig. 4).

4. Discussion

HSPs are necessary for various biological processes, including protein folding and modulation of cellular processes. Among all the known HSPs, HSP70 is highly expressed in the motor neurons of the spinal cord (Shabbir et al., 2015), which are very susceptible to excitotoxic damage that triggers neuronal death (David et al., 2009; Kuzhandaivel et al., 2011; Mandir et al., 2000). Several reports have shown indirect evidence that HSP70 might have neuroprotective effects against ischemic damage in the spinal cord by developing ischemic tolerance (Carmel et al., 2004; Cizkova et al., 2004). In the present study, we expressed Tat-HSP70 and control-HSP70 fusion proteins with polyhistidine tags and confirmed their expression by Western blot using polyhistidine probes. We observed the expression of polyhistidine at 70 kDa and 72 kDa, respectively, suggesting that Tat-HSP70 and control-HSP70 fusion proteins are effectively expressed.

We also investigated the transduction potentials of Tat-HSP70 or control-HSP70 into NSC34 cells (mouse neuroblastomas × motor neurons from embryonic mouse spinal cords), which shows similar behavior as the developing motor neurons (Cashman et al., 1992). After treatment, Tat-HSP70 was effectively transduced into NSC34 cells in a concentration- and time-dependent manner, while treatment of the cells with various concentrations of control-HSP70 did not show any expression of polyhistidine 60 min post harvesting. We also confirmed the cytoplasmic expression of polyhistidine 1 h after Tat-HSP70, not control-HSP70, treatment. These results suggest that Tat-HSP70 protein is effectively transduced into the NSC34 cells in a concentration- and time-dependent manner, while the control HSP70 is not.

In this study, we also observed the neuroprotective effects of Tat-HSP70 or control-HSP70 against oxidative stress induced by H_2O_2 in NSC34 cells based on the results of DCF-DA, TUNEL staining, and MTT assays. We saw that treatment with H_2O_2 significantly increased the ROS formation and cell damage in NSC34 cells, which is consistent with the results of our colleague's study (Yoo et al., 2017). Treatment with Tat-HSP70 significantly reduced ROS formation and cell death after exposure to H_2O_2 in NSC34 cells in a concentration dependent manner,

Table 1
Physiological parameters before and after ischemic surgery. Note that there are no remarkable changes of pH, $PaCO_2$, PaO2, and glucose levels before and after ischemic surgery in control, vehicle-treated, control-HSP70-treated, and Tat-HSP70 treated groups.

	pH	$PaCO_2$ (mmHg)	PaO_2 (mmHg)	Glucose (mM)
Pre-ischemia				
Control	7.41 ± 0.02	37.7 ± 4.09	108.4 ± 9.08	6.43 ± 1.19
Vehicle	7.38 ± 0.04	37.1 ± 4.25	106.0 ± 8.76	6.28 ± 1.35
Control-HSP70 (4.5 mg/kg)	7.42 ± 0.05	37.3 ± 4.70	105.2 ± 8.83	6.32 ± 1.08
Tat-HSP70 (0.5 mg/kg)	7.39 ± 0.04	36.8 ± 4.27	104.9 ± 7.96	6.42 ± 1.26
Tat-HSP70 (1.5 mg/kg)	7.42 ± 0.04	37.3 ± 5.02	108.1 ± 8.60	6.37 ± 1.23
Tat-HSP70 (1.5 mg/kg)	7.41 ± 0.04	37.4 ± 4.77	106.5 ± 8.19	6.38 ± 1.51
Reperfusion 10 min				
Control	7.38 ± 0.04	37.1 ± 4.63	107.1 ± 8.93	6.31 ± 1.36
Vehicle	7.35 ± 0.09	39.6 ± 6.25	109.4 ± 10.3	6.79 ± 1.74
Control-HSP70 (4.5 mg/kg)	7.37 ± 0.08	39.3 ± 5.96	109.8 ± 9.94	6.92 ± 1.51
Tat-HSP70 (0.5 mg/kg)	7.37 ± 0.08	40.1 ± 7.08	108.1 ± 10.3	6.88 ± 1.63
Tat-HSP70 (1.5 mg/kg)	7.38 ± 0.07	37.9 ± 5.05	109.7 ± 10.9	7.01 ± 1.47
Tat-HSP70 (4.5 mg/kg)	7.37 ± 0.09	38.5 ± 4.88	111.2 ± 10.6	6.96 ± 1.64

while control-HSP70 treatment did not show any remarkable effects at any concentration of control-HSP70. This result is consistent with a previous study done using astrocyte culture, which showed that overexpression of HSP70 shows neuroprotective effects after oxygen and glucose deprivation by reducing the expression of pro-inflammatory genes (Kim et al., 2015a). In addition,

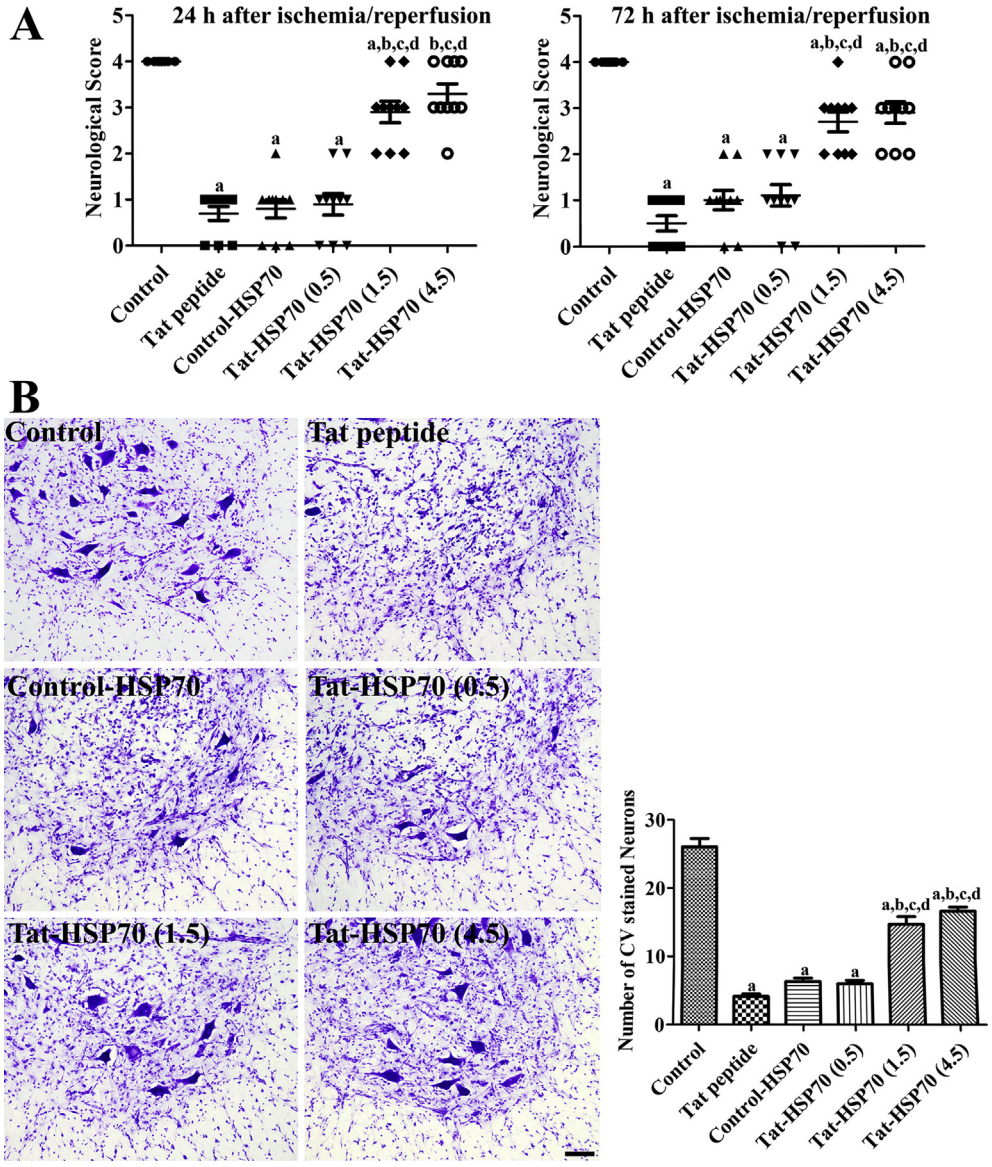

Fig. 3. Effects of control-HSP70 and Tat-HSP70 on the behavior and morphology of rabbits after spinal cord ischemia. (A) Neurological scores based on behavioral and postural defects are assessed by Tarlov's criteria, 24 h and 72 h after ischemia/reperfusion [$n = 10$ per group; $^{a}p < 0.05$, significantly different from the control group, $^{b}p < 0.05$, significantly different from the vehicle-treated group; $^{c}p < 0.05$, significantly different from the control-HSP70-treated group; $^{d}p < 0.05$, significantly different from the Tat-HSP70 (0.5) group]. The bars indicate mean ± standard deviation. (B) Photomicrographs showing cresyl violet stained neurons in the ventral horn of spinal cord 72 h after ischemia/reperfusion. The number of cresyl violet-immunoreactive neurons compared that of the control group per section in all the groups is also shown [$n = 5$ per group; $^{a}p < 0.05$, significantly different from the control group; $^{b}p < 0.05$, significantly different from the vehicle-treated group; $^{c}p < 0.05$, significantly different from the control-HSP70-treated group; $^{d}p < 0.05$, significantly different from the Tat-HSP70 (0.5) group]. The data are shown as mean with standard deviation.

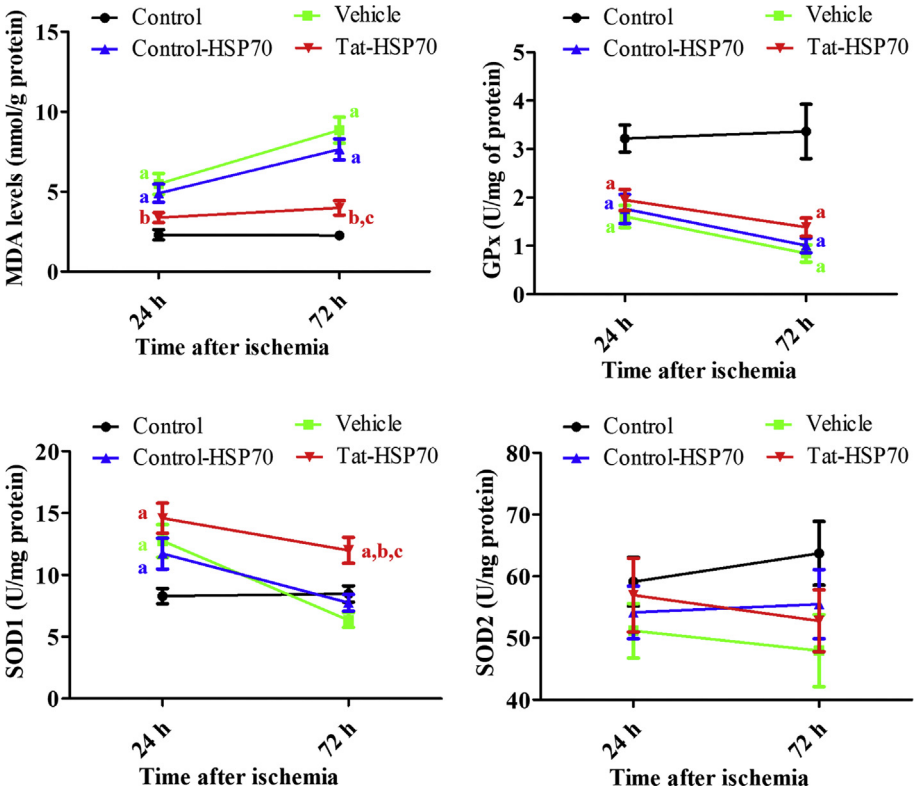

Fig. 4. Effects of control-HSP70 and Tat-HSP70 on the levels of malondialdehyde (MDA), glutathione peroxidase (GPx), Cu, Zn-superoxide dismutase (SOD1), and Mn-superoxide dismutase (SOD2) in the spinal cord after ischemia in rabbit. The data were analyzed by one-way analysis of variance followed by a Bonferroni's post-hoc test ($^{a}p < 0.05$, significantly different from the control group; $^{b}p < 0.05$, significantly different from the vehicle-treated group; $^{c}p < 0.05$, significantly different from the control-HSP70-treated group). The data are shown as mean with standard deviation.

geranylgeranylacetone, an inducer of HSP70, significantly ameliorates H_2O_2-induced ROS generation and upregulates the Bax gene in human neuroblastoma cells (Kim et al., 2015b). In contrast, pharmacological inhibition of HSP70 by VER155008 shows similar neurotoxicity in the HEK and SH-SY5Y cells compared with kainic acid (Shabbir et al., 2015).

In this study, we observed the neuroprotective effects of Tat-HSP70 or control-HSP70 against spinal cord ischemic damage in rabbits based on behavioral and morphological assessment. Treatment with Tat-HSP70 or control-HSP70 did not significantly affect the physiological parameters, including pH, pO_2, pCO_2, and glucose levels, 10 min before and after ischemia/reperfusion. Spinal cord ischemia induced abnormal posture and impaired ability to stand or walk in rabbits with neurological score below 1.0. In addition, spinal cord ischemia significantly decreased the cresyl violet-positive cells in the ventral horn of spinal cord. Administration of control-HSP70 demonstrated similar behavioral score and morphology in the ventral horn of the spinal cord compared to the vehicle-treated group. In contrast, administration of Tat-HSP70 significantly improved the neurological score and increased the number of cresyl violet-positive cells in the ventral horn of spinal cord. Induction of HSP70 by hyperbaric preconditioning and low-frequency pulse electromagnetic fields attenuates ischemic liver damage (Wu et al., 2018) and spinal cord damage (Wang et al., 2019), respectively. In addition, inhibition of HSP70 with a neutralizing anti-HSP70 antibody attenuates the beneficial effects of hyperbaric preconditioning against ischemic damage in liver (Wu et al., 2018).

To elucidate the neuroprotective role of Tat-HSP70 in spinal cord ischemia, we evaluated MDA levels as well as GPx, SOD1, and SOD2 activities in the spinal cord homogenates at 24 h and 72 h after ischemia/reperfusion. MDA is believed to be one of the detrimental and critical byproducts of ROS and lipid peroxidation after ischemia/reperfusion in the spinal cord (Gokce et al., 2016; Liu et al., 2015). Transient spinal cord ischemia significantly increased the MDA levels and decreased GPx activities in the spinal cord. This result is consistent with previous studies, which show that MDA levels are significantly increased in the spinal cord after ischemia in rabbit (Yoo et al., 2017) and rat (Akar et al., 2017). This result suggests that lipid peroxidation was significantly increased in the spinal cord after ischemia/reperfusion and may cause the neuronal death in the ventral horn of spinal cord. In the present study, administration of Tat-HSP70 significantly increased SOD1 activities 72 h after ischemia/reperfusion, while SOD1 activities in other groups were significantly decreased and showed similar levels compared to that in the control group 72 h after ischemia/reperfusion. However, the GPx and SOD2 activities did not change significantly in all groups. These results suggest that administration of Tat-HSP70 prevented neuronal damage by reducing lipid peroxidation and increasing the SOD1 activities, not SOD2 or GPx activities in the spinal cord homogenates. According to a recent report, HSP70 modulates SOD1 expression in mice, while its depletion significantly decreases SOD1 activity and resulted in lower levels of SOD1 protein compared to that in the wild-type littermates (Choi et al., 2005). In contrast, overexpression of HSP70 significantly increased GPx and glutathione reductase activities in Madin-Darby canine kidney (MDCK) cells after exposure to glucose deprivation conditions (Guo et al., 2007). In addition, HSP70β prevents neuronal damage induced by ischemic and oxidative damage in the hypothalamus of transgenic mice overexpressing HSP70β by increasing GPx and glutathione reductase activities (Chen et al., 2009). However, in the present study, we did not observe any significant changes in GPx activities in the ischemic spinal cord after Tat-HSP70 treatment. This discrepancy may be associated with target tissues after ischemic damage.

5. Conclusion

In this study, we expressed Tat-HSP70 and control-HSP70 proteins to compare the efficiency of its transduction into NSC34 cells, and observed amelioration of H_2O_2-induced oxidative stress and cell death in the Tat-HSP70-treated group compared to vehicle- or control-HSP70-treated groups. In addition, administration of Tat-HSP70, not control-HSP70, prevents behavioral impairment and neuronal damage induced during spinal cord ischemia by reducing lipid peroxidation and elevating SOD1 activities in the spinal cord. These results suggest that Tat-

HSP70 can act as an efficient neuroprotectant in reducing the neuronal damage induced by spinal cord ischemia, which occurs during aortic repair surgery and *en bloc* spondylectomy.

Competing interests

All authors have approved the manuscript for submission, and we have no conflicts of interest to disclose.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuint.2019.104477.

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