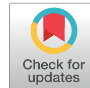

BIOMOLECULES & THERAPEUTICS

Original Article
Biomol Ther 29(3), 321-330 (2021)

Protective Role of Transduced Tat-Thioredoxin1 (Trx1) against Oxidative Stress-Induced Neuronal Cell Death via ASK1-MAPK Signal Pathway

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Abstract

Oxidative stress plays a crucial role in the development of neuronal disorders including brain ischemic injury. Thioredoxin 1 (Trx1), a 12 kDa oxidoreductase, has anti-oxidant and anti-apoptotic functions in various cells. It has been highly implicated in brain ischemic injury. However, the protective mechanism of Trx1 against hippocampal neuronal cell death is not identified yet. Using a cell permeable Tat-Trx1 protein, protective mechanism of Trx1 against hydrogen peroxide-induced cell death was examined using HT-22 cells and an ischemic animal model. Transduced Tat-Trx1 markedly inhibited intracellular ROS levels, DNA fragmentation, and cell death in H_2O_2-treatment HT-22 cells. Tat-Trx1 also significantly inhibited phosphorylation of ASK1 and MAPKs in signaling pathways of HT-22 cells. In addition, Tat-Trx1 regulated expression levels of Akt, NF-κB, and apoptosis related proteins. In an ischemia animal model, Tat-Trx1 markedly protected hippocampal neuronal cell death and reduced astrocytes and microglia activation. These findings indicate that transduced Tat-Trx1 might be a potential therapeutic agent for treating ischemic injury.

Key Words: Tat-Trx1, ASK1, ROS, Apoptosis, Ischemia, Protein therapy

INTRODUCTION

There is some evidence that oxidative stress plays a crucial role in neuronal disorders including brain ischemic injury. Production of large amounts of reactive oxygen species (ROS) can trigger oxidative stress and lead to dysfunction of cells due to DNA and protein damage. Finally, overproduction of ROS could lead to cell death. Although ROS have beneficial roles in regulating cellular signaling pathways, overproduction of ROS is involved in brain ischemic injury (Floyd, 1990; Li *et al*., 2011; Mates *et al*., 2012; Sinha *et al*., 2013; Leak *et al*., 2015; Li and Zhang, 2015). Therefore, some studies have suggested that inhibiting overproduction of ROS is important to prevent neuronal death in brain ischemic injury (Ginsberg *et al*., 2003; Sugawara and Chan, 2003).

Thioredoxin 1 (Trx1), a small (12 kDa) protein, is one of cellular redox enzymes ubiquitously expressed in mammalian cells. Trx1 has a variety of biological functions, including regulating cell growth and apoptosis as an antioxidant protein (Haendeler *et al*., 2002; Kamimoto *et al*., 2010). Several studies have shown that Trx1 protein can prevent cells against oxidative stress such as superoxide and hydrogen peroxide (H_2O_2)-induced toxicity and apoptosis induced by ROS (Saitoh *et al*., 1998; Ueda *et al*., 2002; Yoshida *et al*., 2003; Nadeau *et al*., 2007). Although Trx1 protein plays beneficial roles in cells as an antioxidant protein, the protective mechanism of Trx1 against ischemic injury remains unclear.

Mitogen activated protein kinases (MAPKs) including ex-

Open Access https://doi.org/10.4062/biomolther.2020.154

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Received Sep 3, 2020 **Revised** Nov 23, 2020 **Accepted** Dec 10, 2020
Published Online Jan 13, 2021

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tracellular signal-regulated kinase (ERK), c-Jun *N*-terminal kinase (JNK), and some proteins in p38 signaling pathways are involved in cell growth, proliferation and apoptosis (Ang *et al*., 2016). MAPKs signaling pathways are activated by ROS. They are pivotal signaling pathways that can lead to neuronal cell death (Jellinger and Stadelmann, 2001; Kim and Choi, 2010; Yu *et al*., 2016; Zhang *et al*., 2016). Apoptosis-signal regulating kinase 1 (ASK1) is located at the upstream of MAPKs. It can phosphorylate MAPKs (JNK and p38). ASK1 is known as an important component in oxidative stress-induced apoptosis. The ASK1-MAPK cascade has emerged as a key cell death pathway in response to oxidative stress (Ichijo *et al*., 1997; Shiizaki *et al*., 2013).

Protein transduction domain (PTD) is known as an effective tool for delivering proteins into cells. Thus, PTDs including Tat PTD have been used to delivery therapeutic proteins into cells and tissues (Schwarze *et al*., 1999; Wadia and Dowdy, 2002; van den Berg and Dowdy, 2011). Many studies have reported protective effects of various transduced PTD fusion proteins against cell death both *in vitro* and *in vivo* (Embury *et al*., 2001; Kubo *et al*., 2008; Shin *et al*., 2014; Kim *et al*., 2015; Kim *et al*., 2018; Yeo *et al*., 2018, 2019). In this study, we showed that Tat-Trx1 protein transduced into neuronal cells and brain tissues of an ischemia mice model significantly protected against hippocampal neuronal cell death.

MATERIALS AND METHODS

Materials and HT-22 cell culture

Ni^{2+}-nitrilotriacetic acid Sepharose Superflow was purchased from Qiagen (Valencia, CA, USA). PD-10 columns were purchased from Amersham (Brauncschweig, Germany). Fetal bovine serum (FBS) and antibiotics (streptomycin and penicillin) were obtained from Gibco BRL (Grand Island, NY, USA). Dulbecco's modified Eagle's medium (DMEM) was obtained from Lonza/BioWhittaker (Walkersville, MD, USA). 2′,7′-Dichlorofluorescein diacetate (DCF-DA) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Histidine antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The indicated antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). Unless otherwise stated, all other agents were of the highest grade available.

Mouse hippocampal HT-22 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and antibiotics (100 μg/mL streptomycin, 100 μg /mL penicillin) at 37°C in a humidity chamber with 5% CO_2 and 95% air.

Purification and transduction of Tat-Trx1 proteins into HT-22 cells

Preparation of the Tat expression vector has been described in a previous study (Shin *et al*., 2014; Kim *et al*., 2015). Human Trx1 was amplified by PCR using the sense primer 5′-CTCGAGATGGTGAAGCAGATCG-3′ which contained an *Xho*I restriction site and the antisense primer 5′-GGATCCT-TAGACTAATTCATTAATGGTGG-3′ which contained a *Bam*HI restriction site. The resulting PCR products were ligated into a TA vector (pGEM®-T easy vector; Promega Corporation, Madison, WI, USA) and digested with *Xho*I and *Bam*HI restriction enzymes. Fragments were then ligated into the Tat expression vector to generate Tat-Trx1 protein. Trx1 protein was prepared without the Tat peptide. Subsequently, Tat-Trx1 and Trx1 protein were expressed in *Escherichia coli* BL21 (DE3) cells by adding 0.5 mM isopropyl-β-D-thiogalactoside (Duchefa, Haarlem, the Netherlands) at 37°C for 6 h. Then, Tat-Trx1 and Trx1 protein was purified using a Ni^{2+}-nitrilotriacetic acid Sepharose affinity column and PD-10 column chromatography according to the manufacturer's instructions. Tat-Trx1 and Trx1 protein concentration was determined by the Bradford assay (Bradford, 1976).

To examine Tat-Trx1 protein transduction efficiency, HT-22 cells were cultured in a 60 mm dish plate and exposed to different concentrations of Tat-Trx1 and Trx1 protein (0.5-5 μM) for 1 h or over various time periods (10-100 min) of Tat-Trx1 and Trx1 protein (5 μM). The cells were treated with trypsin-EDTA (Gibco BRL) and washed twice with phosphate-buffered saline (PBS). To determine the intracellular stability of transduced Tat-Trx1 protein, cells were cultured over various time periods (1-24 h) after Tat-Trx1 protein transduction. We confirmed the transduced levels of Tat-Trx1 protein which were measured by Western blot analysis and fluorescence microscopy analysis using an anti-His antibody.

Western blot analysis

After transduction of Tat-Trx1 protein, protein extraction was performed using cell lysis buffer (RIPA; ELPIS BIOTECH, Daejeon, Korea) according to the manufacturer's instructions. Then, equal amount of proteins were loaded into 15% SDS-PAGE and electrotransferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with TBS-T (25 mM Tris-HCl, 140 mM NaCl, 0.1% Tween 20, pH 7.5) buffer containing 5% non-fat dry milk or BSA for 1 h. After being washed with TBS-T buffer, the membrane was incubated with the indicated primary antibodies and horseradish peroxidase-conjugated secondary antibodies. Then the membranes were washed with TBS-T buffer three times and the protein bands were identified using chemiluminescent reagents as recommended by the manufacturer (Amersham, Franklin Lakes, NJ, USA) (Shin *et al*., 2014; Jegal *et al*., 2019).

Confocal fluorescence microscopy analysis

To determine the intracellular distribution of transduced Tat-Trx1 protein in HT-22 cells, we performed confocal fluorescence microscopy as described previously (Shin *et al*., 2014; Yeo *et al*., 2019). HT-22 cells were placed on coverslips and treated with 5 μM of Tat-Trx1 protein for 1 h. The cells were washed with PBS twice and fixed with 4% paraformaldehyde for 5 min. The cells were treated in PBS containing 3% bovine serum albumin and 0.1% Triton X-100 (PBS-BT) at room temperature for 30 min and washed with PBS-BT. The histidine primary antibody was diluted 1:1,500 and incubated at room temperature for 3 h. The Alexa fluor 488-conjugated secondary antibody (Invitrogen, Carlsbad, CA, USA) was diluted 1:1,500 and incubated in the dark for 1 h. Nuclei were stained with 1 μg/mL DAPI (Roche Applied Science, Mannheim, Germany) for 2 min. Then stained cells were analyzed by confocal fluorescence microscopy using a confocal laser-scanning system (MRC1024, Bio-Rad, CA, USA).

Cell viability assay

A cell viability assay was performed using a water-soluble tetrazolium salt-1 (WST-1) cytotoxicity assay (EZ-Cytox cell viability assay kit, Daeil Lab service Co., Seoul, Korea) accord-

ing to the manufacturer's protocols (Shin *et al*., 2014; Jegal *et al*., 2019). HT-22 cells were seeded into 96-well plates and treated with Tat-Trx1 (1-5 μM) and Trx1 for 1 h. Then, the cells were incubated with 700 μM hydrogen peroxide (H_2O_2) for 1 h 30 min. The absorbance was measured at 450 nm using an ELISA microplate reader (Multiskan MCC/340; Thermo Labsystems, Helsinki, Finland). Cell viability was defined as the % of untreated control cells.

Measurement of intracellular reactive oxygen species (ROS) levels

Intracellular ROS levels were determined using 2′,7′-Dichlorofluorescein diacetate (DCF-DA) as described previously (Shin *et al*., 2014; Kim *et al*., 2019; Yeo *et al*., 2019). ROS levels in the HT-22 cells were determined in the presence or absence of Tat-Trx1 protein (5 μM). After 1 h of pre-treatment with Tat-Trx1 protein, the cells were treated with H_2O_2 (700 μM) for 30 min. Following a PBS wash, the cells were treated with 20 μM DCF-DA for 20 min. Then, fluorescent images were obtained by fluorescence microscopy (Eclipse 80i, Nikon, Tokyo, Japan) and the fluorescence intensity was detected with excitation at 485 nm and emission at 538 nm using a Fluoroskan ELISA plate reader (Thermo Labsystems).

TUNEL assay

To examine whether transduced Tat-Trx1 protein protects against H_2O_2-induced DNA damage in cells, HT-22 cells were pretreated with 5 μM Tat-Trx1 protein for 1 h and exposed to 700 μM H_2O_2 for 6 h. DNA fragmentation was determined using a Cell Death Detection Kit (Roche Applied Science, Basel, Switzerland) according to the manufacturer's instructions. Fluorescent images were obtained by fluorescence microscopy (Eclipse 80i, Nikon) and the fluorescence intensity was detected with excitation at 485 nm and emission at 538 nm using a Fluoroskan ELISA plate reader (Thermo Labsystems) (Shin *et al*., 2014; Yeo *et al*., 2019).

Measurement of MAPKs and apoptotic protein expression

HT-22 cells were incubated in the absence or presence of Tat-Trx1 (5 μM) for 1 h, and then treated with H_2O_2 for various times. The expression of Akt, MAPKs and apoptotic protein expression levels were determined by Western blotting using indicated specific antibodies. The bands were quantified by Image J software (NIH, Bethesda, MD, USA) (Shin *et al*., 2014).

Experimental animals

Male gerbils (65-75 g; 6 months old) obtained from the Experimental Animal Center, at Hallym University (Chuncheon, Korea) were housed at a temperature of 23ºC, with humidity of 60%, and exposed to 12 hour periods of light and dark with free access to food and water. All experimental procedures involving animals and their care conformed to the Guide for the Care and Use of Laboratory Animals of the National Veterinary Research & Quarantine Service of Korea and were approved by the Institutional Animal Care and Use Committee of Soonchunhyang University (Cheonan, Korea) [SCH 15-0002-3].

The transient forebrain ischemia model was performed as described previously (Shin *et al*., 2014; Yeo *et al*., 2019). Briefly, the animals were anesthetized, common carotid arteries were isolated, freed of nerve fibers, and occluded with non-traumatic aneurysm clips. Complete interruption of blood flow was confirmed by observing the retinal artery using an ophthalmoscope. After 5 min occlusion, the aneurysm clips were removed. The restoration of blood flow (reperfusion) was observed directly under the ophthalmoscope.

To explore the protective effects of Tat-Trx1 protein against ischemic damage, the animals were divided into 5 groups (each n=10): control sham group, vehicle-treated group, Tat-Trx1-treated group, Trx1-treated group and Tat peptide-treated group. The Tat-Trx1 proteins, Trx1 proteins and Tat peptide (2 mg/kg) were administered intraperitoneally 30 min before ischemia-reperfusion. The brains from each group were harvested and the levels of 4-hydroxynonenal (4-HNE) and endogenous Trx1 proteins were determined by Western blot analysis using 4-HNE (Santa Cruz, CA, USA) and Trx1 (Cell Signaling Technology) antibodies. Also, intracellular ROS level was determined using a ROS assay kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions.

Immunohistochemical analysis

Immunohistochemistry was performed as described in previous studies (Shin *et al*., 2014; Yeo *et al*., 2019). Brain tissue samples were obtained at 7 days after ischemia-reperfusion. To examine the protective effects of transduced Tat-Trx1 protein against ischemic damage, the sections were incubated in 10% normal goat serum in PBS for 30 min and the sections were stained with a histidine antibody, Cresyl violet (CV), Fluoro-Jade B (F-JB), ionized calcium-binding adapter molecule 1 (Iba-1) and glial fibrillary acidic protein (GFAP).

The positive neuronal cell number and intensity of immunoreactivity were calculated using an image analyzing system equipped with a computer based CCD camera (software: Optimas 6.5, CyberMetrics, Scottsdale, AZ, USA). The staining intensity of the immunoreactive structures was evaluated as the relative optical density (ROD). A ratio of the ROD was calibrated as % (Shin *et al*., 2014; Yeo *et al*., 2019).

Statistical analysis

Data are expressed as the mean ± SEM of three experiments. Differences between groups were analyzed by ANOVA followed by a Bonferroni's post-hoc test. Statistical significance was considered at $p<0.05$.

RESULTS

Construction and purification of Tat-Trx1 fusion protein

We constructed a Tat-Trx1 fusion protein for Trx1 protein transduction by fusing human Trx1 gene in frame with Tat-peptide and His-tag. As a negative control, Trx1 gene was fused to His-tag alone (Fig. 1A). After the protein was overexpressed by IPTG indcution, Tat-Trx1 and Trx1 proteins were purified by Ni-NTA and PD-10 chromatography. By SDS-PAGE, one single band was detected for purified Tat-Trx1 or Trx1 protein at their expected molecular weights. Furthermore, both purified Tat-Trx1 and Trx1 proteins were identified by Western blotting using an anti-His antibody (Fig. 1B).

Transduction of Tat-Trx1 protein into HT-22 cells

Transduction of Tat-Trx1 protein was examined in HT-22 cells. These cells were treated with Tat-Trx1 protein (0.5-5 μM) for 1 h or with Tat-Trx1 protein (5 μM) at different time intervals

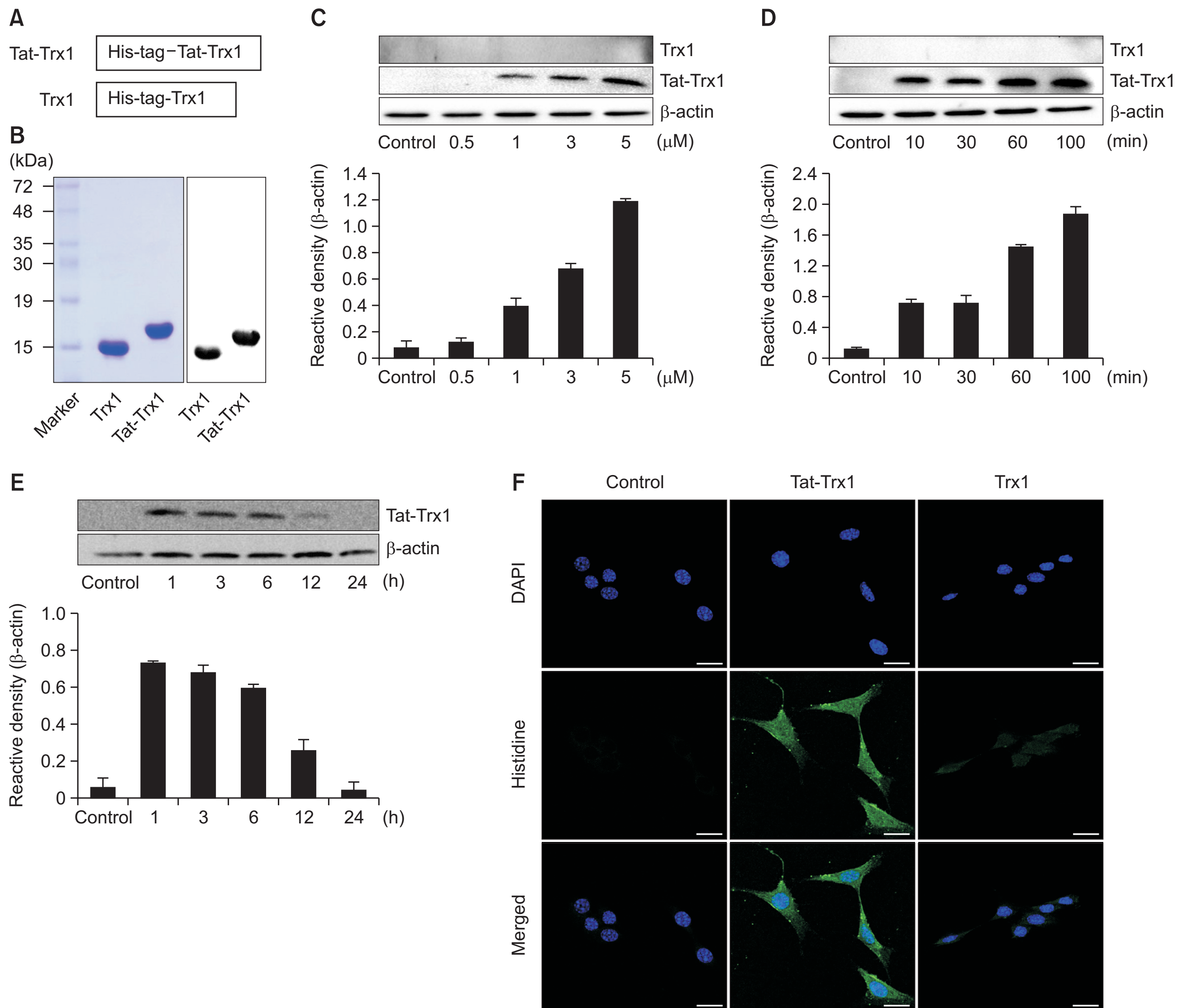

Fig. 1. Purification and transduction of Tat-Trx1 protein into HT-22 cells. (A) Diagrams of the expressed Tat-Trx1 proteins. (B) Purified Tat-Trx1 and Trx1 proteins were identified by 15% SDS-PAGE and detected by Western blot analysis using an anti-histidine antibody. (C) Transduction of Tat-Trx1 proteins into HT-22 cells. HT-22 cell culture media were treated with Tat-Trx1 protein at different doses (0.5-5 μM) or with the Trx1 protein for 1 h. (D) The cell culture media were treated with Tat-Trx1 protein (5 μM) or Trx1 protein for different time periods (10-100 min). (E) Intracellular stability of transduced Tat-Trx1 protein. HT-22 cell culture media were incubated for 24 h after transduction of Tat-Trx1 protein for 1 h. Transduction of Tat-Trx1 protein was measured by Western blotting and the intensity of the bands was measured by a densitometer. (F) The localization of transduced Tat-Trx1 protein was examined by confocal fluorescence microscopy. Scale bar=20 μm. The bars in the figure represent the mean ± SEM obtained from 3 independent experiments.

(10-100 min). Then, transduced proteins were determined by Western blotting. As shown in Fig. 1C and 1D, a dose- and time-dependent increase in the amount of transduced Tat-Trx1 protein was detected in HT-22 cells. However, Trx1 protein without a Tat-peptide was not transduced in HT-22 cells.

We further confirmed the stability and distribution of transduced Tat-Trx1 protein by Western blotting and confocal fluorescence microscopy. As shown in Fig. 1E, transduced Tat-Trx1 protein levels were significantly increased in HT-22 cells at 12 h compared to those in controls. Immunofluorescence staining showed that transduced Tat-Trx1 protein was distributed in the cytoplasm and nucleus of cells (Fig. 1F). These results indicate that Tat-Trx1 protein transduced into HT-22 cells and persisted for 12 h.

Effects of Tat-Trx1 protein on H_2O_2-induced cell viability

After pretreatment with Tat-Trx1 protein at different doses for 1 h, HT-22 cells were exposed to 700 μM of H_2O_2 and their viability was determined using a WST-1 assay. As shown in Fig. 2A, only 51% of cells survived in H_2O_2 only exposed cells. However, cells pretreated with Trx1 protein and Tat peptide did not show significant decrease of cell viability after exposure to H_2O_2. Tat-Trx1 protein significantly increased the survival of HT-22 cells up to 92% compared to H_2O_2 only exposed cells.

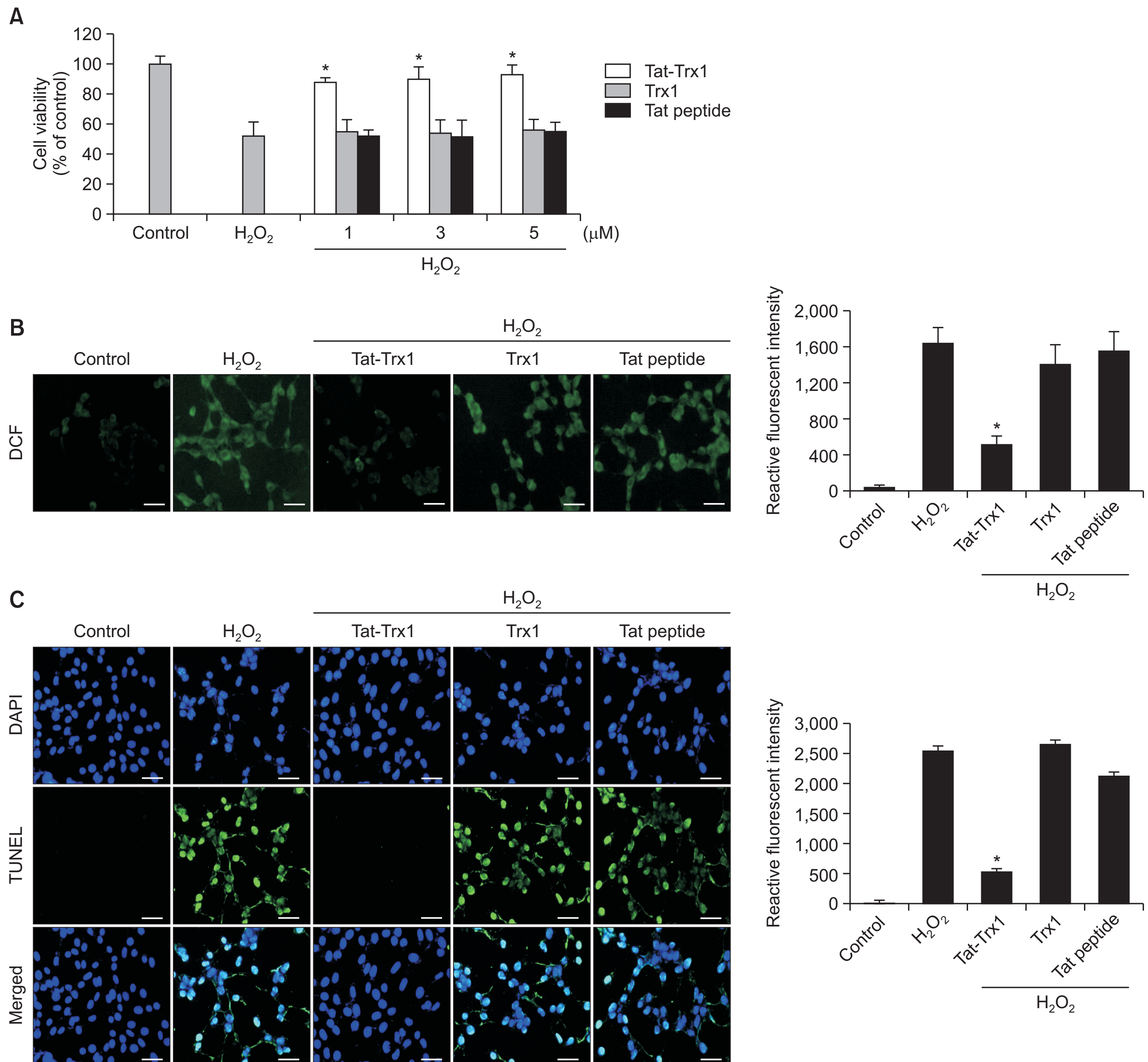

Fig. 2. Effects of transduced Tat-Trx1 protein on cell viability in response to oxidative stress. (A) Effect of transduced Tat-Trx1 protein on cell viability. HT-22 cells were pretreated with Tat-Trx1 protein (1-5 μM) for 1 h and exposed to H_2O_2 (700 μM) for 1 h 30 min. Cell viabilities were estimated using a colorimetric assay using WST-1. Effects of Tat-Trx1 protein on H_2O_2-induced ROS production and DNA damage. Treatment with Tat-Trx1 protein (5 μM) and Trx1 protein was followed by 1 h treatment with H_2O_2 (700 μM). Intracellular ROS levels were measured by (B) DCF-DA staining and (C) DNA fragmentation was detected by TUNEL staining. The fluorescence intensity was measured by ELISA plate reader; scale bar=50 μm. The bars in the figure represent the mean ± SEM obtained from 3 independent experiments. *$p<0.05$ compared with H_2O_2-treated cells.

To determine effects of Tat-Trx1 protein on oxidative stress, ROS generation and DNA fragmentation in H_2O_2 exposed HT-22 cells were assessed. As shown in Fig. 2B and 2C, levels of ROS generation and DNA fragmentation were markedly reduced in Tat-Trx1 protein treated cells as compared with H_2O_2 only exposed cells. However, they showed no significant difference between H_2O_2 alone treated cells and Trx1 protein or Tat peptide treated cells. These results indicate that transduced Tat-Trx1 protein can inhibit cell death caused by oxidative stress by decreasing ROS generation and DNA fragmentation.

Protective mechanism of Tat-Trx1 against oxidative stress-induced cell death

The cascade of ASK1 and MAPKs has emerged as a key cell death pathway in response to oxidative stress (Ichijo *et al.*, 1997; Shiizaki *et al.*, 2013). We determined effects of Tat-Trx1 on ASK1 and MAPKs signaling pathways. ASK1 and MAPKs (p38, JNK, and EKR) were markedly activated in H_2O_2 only exposed HT-22 cells compared to those in control cells. In

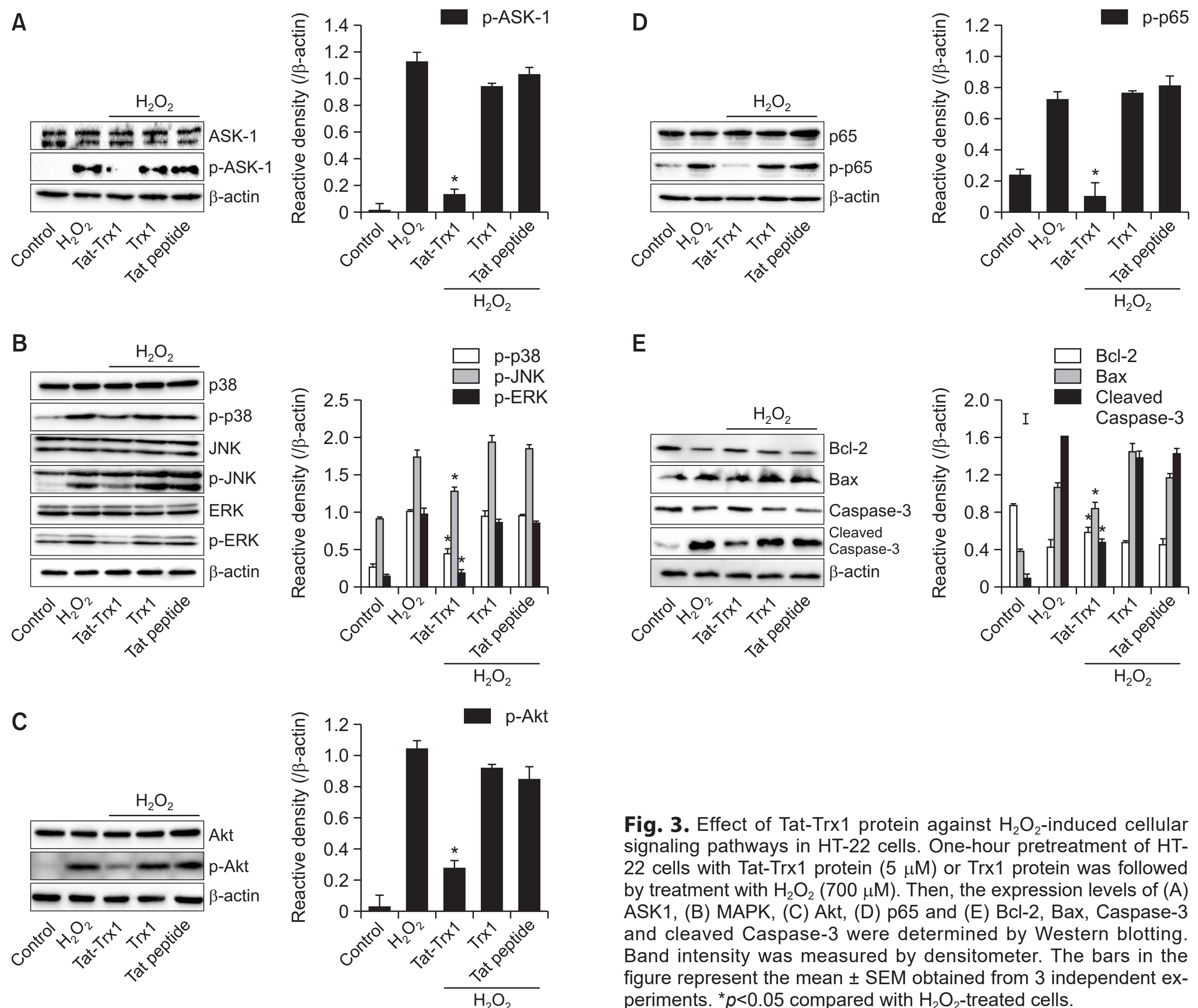

Fig. 3. Effect of Tat-Trx1 protein against H_2O_2-induced cellular signaling pathways in HT-22 cells. One-hour pretreatment of HT-22 cells with Tat-Trx1 protein (5 μM) or Trx1 protein was followed by treatment with H_2O_2 (700 μM). Then, the expression levels of (A) ASK1, (B) MAPK, (C) Akt, (D) p65 and (E) Bcl-2, Bax, Caspase-3 and cleaved Caspase-3 were determined by Western blotting. Band intensity was measured by densitometer. The bars in the figure represent the mean ± SEM obtained from 3 independent experiments. **p*<0.05 compared with H_2O_2-treated cells.

cells treated with Trx1 protein or Tat peptide, phosphorylation levels of ASK1 and MAPKs were not significantly changed. In contrast, Tat-Trx1 protein markedly reduced H_2O_2-induced phosphorylation of ASK1 and MAPKs (Fig. 3A, 3B).

To explore cellular mechanisms underlying the protective effect of Tat-Trx1 protein, we investigated expression levels of Akt, p65, and apoptotic related proteins in H_2O_2 exposed HT-22 cells. Phosphorylation levels of Akt and p65 were reduced in Tat-Trx1 protein treated cells. However, their levels were unchanged in Trx1 protein or Tat peptide treated cells (Fig. 3C, 3D). Tat-Trx1 protein increased expression levels of Bcl-2 and Caspase-3 in H_2O_2 treated HT-22 cells. In contrast, expression levels of Bax and cleaved Caspase-3 showed opposite patterns compared to Bcl-2 and Caspase-3 expression (Fig. 3E). These results indicate that Tat-Trx1 protein can inhibit HT-22 cell death by modulating the expression of ASK1, MAPKs, and apoptotic proteins.

Protective effect of Tat-Trx1 protein against ischemic injury

To investigate effects of Tat-Trx1 protein against ischemic injury in an animal model, we performed immunohistochemistry. As shown in Fig. 4A, Tat-Trx1 protein significantly protected neuronal cell death in the hippocampal CA1 region. However, both Trx1 protein and Tat peptide treated groups showed a similar pattern compared with vehicle treated group. We also examined whether Tat-Trx1 protein inhibited the activation of microglia and astrocytes using F-JB, Iba-1, and GFAP staining, respectively (Fig. 4B). In the vehicle-, Trx1 protein, and Tat peptide protein-treated groups, F-JB, Iba-1, and GFAP fluorescence signals were intensively detected in the hippocampal CA1 region. In contrast, intensive fluorescence signals were markedly reduced in Tat-Trx1 protein treated group. These results indicate that Tat-Trx1 protein could protect against neuronal cell damage resulting from ischemic injury by decreasing microglia and astrocyte activation.

Effects of Tat-Trx1 protein on oxidative stress in an ischemic injury model

To examine whether Tat-Trx1 protein could inhibit ischemia-induced oxidative stress, we performed DHE and 4-HNE to determine ROS generation and lipid peroxidation levels in

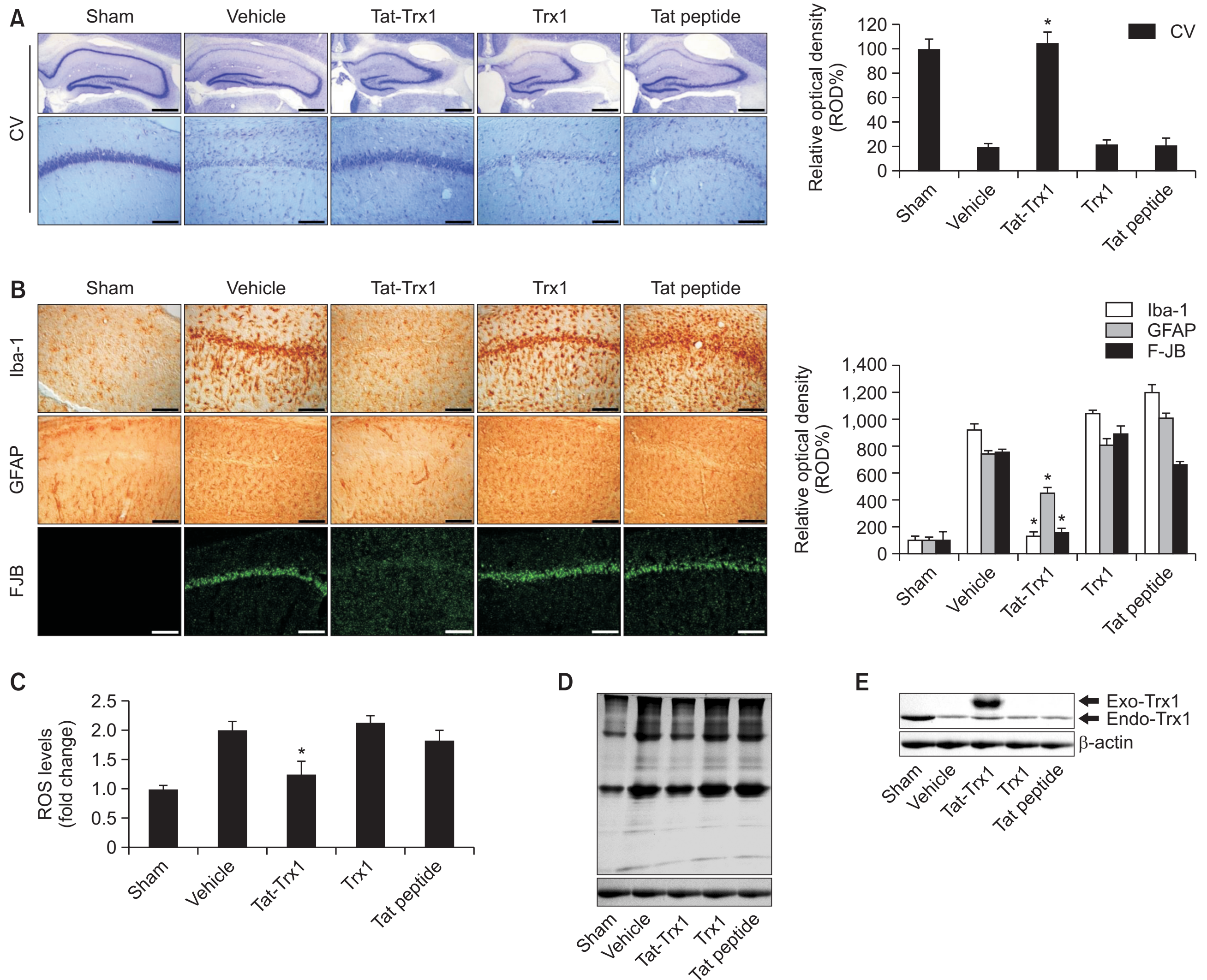

Fig. 4. Protective effects of transduced Tat-Trx1 protein on ischemic injury. Gerbils were treated with single injections Tat-Trx1 (2 mg/kg) proteins and killed after 7 days (n=10 per groups). Then, the effects of transduced Tat-Trx1 protein on neuronal cell viability after ischemic insults using immunostaining. (A) The hippocampus was stained with CV and (B) Iba-1, GFAP, F-JB in sham-, vehicle-, Tat-Trx1-, Trx1- and Tat peptide-treated animals 7 days after I/R. Relative numeric analysis of CV-, Iba-1-, GFAP- and F-JB-positive neurons in CA1 region. Scale bar=400 and 50 μm. The brains from each group were harvested. (C) The intracellular ROS level was measured using an ROS assay kit. (D, E) Analysis of HNE and endogenous Trx1 protein levels. The levels of 4-HNE and endogenous Trx1 protein in the brain were analyzed by Western blot analysis using a 4-HNE and Trx1 antibody. *$p<0.05$, significantly different from the vehicle group.

an ischemic injury animal model. As shown in Fig. 4C and 4D, levels of ROS and lipid peroxidation were significantly increased in vehicle-, Trx1 protein-, and Tat peptide-treated groups compared with the sham control group. In contrast, Tat-Trx1 protein treated group reduced ischemia-induced ROS and lipid peroxidation levels. We also determined endogenous Trx1 protein expression levels. Compared with the vehicle group, endogenous Trx1 protein levels were similar in other treatment groups (Fig. 4E). These results indicate that Tat-Trx1 protein plays a role in reducing ischemia-induced cell damage by inhibiting oxidative stress in an ischemic injury animal model.

DISCUSSION

Thioredoxin 1 (Trx1) is a multifunctional protein with MW of 12 kDa. It is expressed in all living cells including prokaryotic and eukaryotic cells. Trx1 has two redox-active cysteine residues within a conserved active site having a sequence of Cys-Gly-Pro-Cys. It plays key roles in cellular growth, regulation of gene expression, and apoptosis (Susanti *et al*., 2014; Booze *et al*., 2016). Several studies have shown that Trx1 protein can act as an antioxidant protein and inhibit cell death caused by oxidative stress (Saitoh *et al*., 1998; Ueda *et al*., 2002; Yoshida *et al*., 2003; Nadeau *et al*., 2007). In addition, several reports have shown that oxidative stress induced by ROS contributes to various ischemia-induced tissue injuries including those to the brain, heart, and liver (Floyd, 1990; Chan, 2001;

Sugawara and Chan, 2003; Leak *et al*., 2015). However, functional roles and precise protective mechanisms of Trx1 protein on ischemia remain unclear.

We showed that Tat-Trx1 protein transduced into HT-22 cells and markedly inhibited HT-22 cell death, ROS generation, and DNA fragmentation caused by oxidative stress. Recent studies have shown that knockdown of Trx1 can decrease astrocyte cell viability in an oxygen glucose deprivation/reperfusion (OGD/R)-induced cell model, suggesting that Trx1 can protect astrocyte cells from oxidative stress by exerting anti-oxidant effects (Wang *et al*., 2016). Overexpression of Trx1 protein can significantly protect against progressive β-cell failure in a T2DM animal model (Stosic-Grujicic *et al*., 2008). Overexpression of Trx1 protein can also reduce hyperglycemia in pancreatic islets of an STZ-induced T1DM animal model by triggering an anti-oxidant effect (Yamamoto *et al*., 2008). These results suggest that Trx1 protein can protect pancreatic β-cell function against oxidative stress in both T2DM and T1DM. In addition, Trx1 protein can act as a radical scavenger under oxidative stress conditions and DNA damage is markedly higher in Trx1 knockdown human PKO cells after exposure of *N*-Nitroso-*N*-methylurea (NMU) (Das and Das, 2000; Kim *et al*., 2013). In this study, we also demonstrated that Tat-Trx1 protein could inhibit HT-22 cell death caused by oxidative stress, indicating that this protein could act an antioxidant protein.

Several studies have demonstrated that excessive ROS play a key role in ischemic injury and that ROS are associated with the induction of MAPKs, NF-κB, and Akt activation in neuronal cells (Kwon *et al*., 2011; Zhu *et al*., 2017; Jia *et al*., 2018). It has been well described that Trx1 can regulate the redox functions of various signal molecules involved in transduction pathway including NF-κB, p53, ASK-1, Akt, and MAPKs (Meuillet *et al*., 2004; Kaimul Ahsan *et al*., 2005; Nakamura *et al*., 2006; Fujino *et al*., 2007). We showed that Tat-Trx1 protein markedly inhibited against H_2O_2-induced activation of ASK-1, MAPKs, Akt, and NF-κB in HT-22 cells. Wu *et al*. (2015) have shown that inhibition of Trx1 with siRNA can increase phosphorylation levels of ASK1, JNK, and p38 expression in middle cerebral artery occlusion (MCAO) rats, suggesting that Trx1 can protect against neuronal cell death *via* inhibition of the ASK1-JNK/p38 signaling pathway. It is well known that ASK1 is a member of the MAP3 kinase family that can activate JNK and p38 kinase pathways. It is activated by various stimuli including oxidative stress (Ichijo *et al*., 1997; Shiizaki *et al*., 2013). Lee *et al*. (2012) have shown that phosphorylation of ASK1 is increased in MPP^+ or H_2O_2 treated SH-SY5Y cells and MPTP exposed mice dopaminergic cells. Other studies also have suggested that inhibiting MAPKs and NF-κB activation is a plausible therapeutic strategy against ischemic injury (Yu *et al*., 2016; Li *et al*., 2017). Akt plays a critical role in cell survival and protects against apoptosis. Oxidative stress-induced phosphorylation of Akt (ser-473) can activate apoptosis and subsequent neuronal cell death (Yano *et al*., 2001). Furthermore, other studies have demonstrated that oxidative stress-induced cellular damage is mediated by activation of PI3K/Akt signaling pathway in neuronal cells (Angeloni *et al*., 2011; Mo *et al*., 2012).

Next, we examined effects of Tat-Trx1 protein against H_2O_2-induced apoptotic cell death. Tat-Trx1 protein markedly inhibited Bax and cleaved Caspase-3 expression, whereas Tat-Trx1 protein increased Bcl-2 and Caspase-3 expression in H_2O_2 exposed HT-22 cells. In Trx1 knockdown EMT6 cells, cleaved Caspase-3 expression is markedly increased, meaning that knockdown of Trx1 can increase apoptosis and cell death (Yoo *et al*., 2013). Zhang *et al*. (2004) have shown that activation of ASK1 by TNF can activate pro-apoptotic proteins, leading to cell death in Trx knockdown endothelial cells. Although the precise mechanism remains to be elucidated, Tat-Trx1 protein protects against H_2O_2-induced HT-22 cell death by inhibiting ASK1 and MAPKs activation and by regulating the apoptotic signaling pathway.

We further investigated the effect of Tat-Trx1 protein on ischemic insults using an animal ischemia model. Tat-Trx1 protein markedly inhibited neuronal cell death and reduced astrocytes and microglia activation in the ischemic animal model. In a previous study, we have shown that various PTDs fused with proteins can transduce into animal ischemia model brain and inhibit neuronal cell death (Shin *et al*., 2014; Yeo *et al*., 2019). Other studies have also demonstrated that microglial and astrocyte activation markedly increased during ischemic injury. Thus, the activation of microglial and astrocytes is considered as a key phenomenon of ischemic neuronal injury (Ito *et al*., 2001; Chen and Swanson, 2003; Angeloni *et al*., 2011). Overexpression of Trx1 protein can also reduce infarct size in a mouse model of ischemia reperfusion, suggesting that Trx1 protein may be a useful therapeutic tool for ischemia reperfusion injury (Tao *et al*., 2004). Janac *et al*. (2006) have shown that occlusion of common carotid artery (CCA) can increase neuronal damage in the hippocampus, striatum, and neocortex known to be involved in the control of locomotor and stereotypic activities. Several studies have reported that chronic diffuse cerebral ischemia models are important for understanding the correlation between chronic cerebral hypoperfusion and cognitive functions. Chronic cerebral hypoperfusion causes white matter degeneration and neuronal degradation of the hippocampal CA1 regions and triggers oxidative stress in animal models. These brain damages can lead to spatial learning and memory impairment (Shibata *et al*., 2004; Yoshizaki *et al*., 2008; Miki *et al*., 2009; Luo *et al*., 2015). Other studies have shown significantly impairment in spatial learning and memory in the occlusion of CCA group compared with the sham group and suggested that further studies are warranted to understand mechanisms of brain dysfunction and impairment and to assess the efficacy of therapeutic agents for treating neurological disorders (Mehla *et al*., 2018; Mansour *et al*., 2019). Although further experiments are needed to understand behavioral, spatial learning, and memory impairment, we showed that Tat-Trx1 protein plays a key role in hippocampal neuronal cell death *in vitro* and *in vivo*. The exact mechanisms of neuronal damage in ischemia remain to be explored.

In summary, we demonstrated that Tat-Trx1 protein transduced into HT-22 cells and significantly inhibited oxidative stress-induced cell death. In addition, Tat-Trx1 protein prevented hippocampal neuronal cell death in an animal ischemia model. Our resutls suggest that Tat-Trx1 protein may represent a potential therapeutic strategy against brain ischemic injury.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

This research was supported by Basic Science Research Program (2018R1D1A3B07049265 & 2019R1A6A1A11036849) through the National Research Foundation of Korea (NRF) funded by the Ministry of Education.

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