

# Inhibition of cytoskeletal protein carbonylation may protect against oxidative damage in traumatic brain injury

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**Abstract.** Oxidative stress is the principal factor in traumatic brain injury (TBI) that initiates protracted neuronal dysfunction and remodeling. Cytoskeletal proteins are known to be carbonylated under oxidative stress; however, the complex molecular and cellular mechanisms of cytoskeletal protein carbonylation remain poorly understood. In the present study, the expression levels of glutathione (GSH) and thiobarbituric acid reactive substances (TBARS) were investigated in PC12 cells treated with H<sub>2</sub>O<sub>2</sub>. Western blot analysis was used to monitor the carbonylation levels of  $\beta$ -actin and  $\beta$ -tubulin. The results indicated that oxidative stress was increased in PC12 cells that were treated with H<sub>2</sub>O<sub>2</sub> for 24 or 48 h. In addition, increased carbonylation levels of  $\beta$ -actin and  $\beta$ -tubulin were detected in H<sub>2</sub>O<sub>2</sub>-treated cells. However, these carbonylation levels were reduced by pretreatment with aminoguanidine, a type of reactive carbonyl species chelating agent, and a similar trend was observed following overexpression of proteasome  $\beta$ 5 via transgenic technology. In conclusion, the present study results suggested that the development of TBI may cause carbonylation of cytoskeletal proteins, which would then undermine the stability of cytoskeletal proteins. Thus, the development of TBI may be improved via the inhibition of cytoskeletal protein carbonylation.

## Introduction

Traumatic brain injury (TBI) is a common cause of mortality and disability in adults worldwide (1,2). Neurological dysfunction and cell death due to TBI are a result of primary injury associated with the direct physical disruptions of various pathways or tissues, as well as secondary injury

associated with delayed biochemical changes that are induced by trauma (3,4). A series of progressive physiological and pathological changes result in severe secondary injury in TBI patients. Oxidative stress is widely known to serve a significant role in the pathogenesis of TBI (5,6), which causes the impairment of cognition, motor function and neurological behavior. However, the complicated molecular and cellular mechanisms underlying TBI remain poorly understood.

A major component of TBI is diffuse axonal injury (DAI), which refers to the manifestation of microstructural cellular trauma and various resulting neurochemical reactions, leading to secondary neuronal death (7). The transportation of axoplasm depends on the normal formation of cytoskeleton proteins. Protein carbonylation is commonly observed in cells exposed to oxidants, resulting in protein aggregation and dysfunction (8), which may precede cellular senescence and cell death. In addition, protein carbonylation is an important event in the context of proteostasis due to its non-enzymatic nature, frequent occurrence and irreversible effects (9). It is well known that the normal transport of axoplasm relies on the normal cytoskeleton; however, the association between the axonal injury and protein carbonylation is not fully understood. Previous studies revealed that the cytoskeleton includes  $\beta$ -actin,  $\beta$ -tubulin and neurofilaments, which are the principal target proteins of carbonylation in neurological disease (10,11). As disease progresses from the inflammatory to the neurodegenerative phase, an inappropriate removal of oxidized cytoskeletal proteins may occur (11). Furthermore, a previous study demonstrated that the levels of carbonyl proteins were increased in TBI rats (12). Actin is involved in the manifold cellular processes, and is thus a sensitive target protein to this oxidative modification (8). The increase in the actin content of carbonyl groups detected *in vivo* indicates drastic oxidative modification leading to significant functional impairments (13).

The aim of present study was to investigate the effectiveness of inhibiting carbonylation of cytoskeletal proteins in regulating oxidative damage in TBI. The protein carbonylation of two cytoskeletal proteins,  $\beta$ -actin and  $\beta$ -tubulin, was detected following the exposure of PC12 cell lines to H<sub>2</sub>O<sub>2</sub>. Furthermore, the carboxylation of these two cytoskeletal proteins was measured after pretreatment of the PC12 cell lines with aminoguanidine (AG), as well as overexpression of proteasome, in order to compare and provide the underlying

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mechanisms of cytoskeletal protein carbonylation mediating the development of brain injury following trauma.

## Materials and methods

**Antibodies and reagents.** Purified  $\beta$ -actin,  $\beta$ -tubulin and cytoskeletal protein aggregation kits were purchased from Cytoskeleton, Inc. (Denver, CO, USA). Dulbecco's Modified Eagle's medium (DMEM) and Lipofectamine 2000 reagent were obtained from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Anti- $\beta$ -actin monoclonal antibody was commercially available from Genetex, Inc. (cat. no. GTX109639; Irvine, CA, USA). Anti- $\beta$ -tubulin antibody (cat. no. T4026) and phenylmethylsulfonyl fluoride (PMSF) were obtained from Sigma-Aldrich (St. Louis, MO, USA). The peroxidase-conjugated affinity goat anti-mouse IgG secondary antibody was obtained from Roche Diagnostics (Basel, Switzerland). An expression vector pCMV-HA (cat. no. Trans 18-35; Clontech; Takara Bio Inc., Mountain View, CA, USA) encoding for the full-length  $\beta 5$  subunit cDNA used for transfection was constructed by SyngenTech Co., Ltd. (Shanghai, China). The enhanced chemiluminescence (ECL) assay kit (cat. no. PA112) used in western blotting was commercially available from Tiangen Biotech Co., Ltd. (Shanghai, China).

**Cell culture and oxidative stress induction.** PC12 cells (American Type Culture Collection, Rockville, MD, USA) were routinely cultured in DMEM supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) in a 37°C incubator with an atmosphere of 5% CO<sub>2</sub>. PC12 cells were subjected to oxidative stress by treatment with various concentrations of H<sub>2</sub>O<sub>2</sub> (100, 200 and 300  $\mu$ M) for 48 h, or with 300  $\mu$ M H<sub>2</sub>O<sub>2</sub> for different time durations (24 or 48 h). PC12 cells in the control group were treated with culture medium only.

**Levels of glutathione (GSH) and thiobarbituric acid reactive substances (TBARS).** GSH is one of the most important factors protecting from oxidative attacks by reactive oxygen species, since it functions as a reducing agent and free-radical trapper (14). In addition, TBARS is a marker of lipid peroxidation and oxidative damage (15). Therefore, the present study investigated the levels of GSH and TBARS in PC12 cells that were subjected to H<sub>2</sub>O<sub>2</sub>-induced oxidative stress in order to determine the effect of oxidative stress in these cells.

For TBARS detection, 100  $\mu$ l plasma were incubated with 500  $\mu$ l Tris-HCl (Amresco Inc., Solon, OH, USA) and 500  $\mu$ l 35% trichloroacetic acid (TCA; Merck KGaA, Darmstadt, Germany) for 10 min at room temperature. Next, the samples were incubated with 2 M Na<sub>2</sub>SO<sub>4</sub> and 55 mM TBA solution at 95°C for 45 min, and then cooled on ice for 5 min. Subsequent to treatment with 70% TCA, the samples were centrifuged at 12,000  $\times$  g for 3 min. The TBARS product was measured at 530 nm using a spectrophotometer (Ultrospec 500 Pro; GE Healthcare Life Sciences, Chicago, IL, USA).

For the detection of GSH, 5% TCA, 67 mM sodium/potassium phosphate buffer and 1 mM 5,5'-dithiobis(2-nitrobenzoic acid) (Vicmed, Xuzhou, China) were mixed with 20  $\mu$ l erythrocyte lysate and incubated in the dark at room temperature for 45 min. GSH production was determined at 412 nm using a

spectrophotometer (Shimadzu-1640; Shimadzu Corp., Kyoto, Japan).

**Monomer / polymer ratio of cytoskeletal proteins.** PC12 cells were washed with phosphate-buffered saline (PBS) (3 $\times$ 10<sup>6</sup> cells/ml) and lysis treated a lysis buffer containing 50 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, phosphatase inhibitors (100 mM Na<sub>3</sub>VO<sub>4</sub> and 10 mM NaF) and a protease inhibitor (1 mM PMSF). The cell homogenate was incubated with KCl at a final concentration of 500 mM in the presence of 0.5% v/v Triton X-100 (Sigma-Aldrich, Munich, Germany). After 2 h of incubation, the supernatant (namely the monomer) and the pellet (namely the polymer) were separated by centrifugation at 16,000  $\times$  g for 20 min at 4°C. Subsequently, the same amounts (50  $\mu$ g) of supernatant and pellet were loaded onto separate lanes and separated by 10% SDS-PAGE. The samples were then probed with antibodies against  $\beta$ -tubulin and  $\beta$ -actin. The integrated densities of the protein bands were obtained using ImageJ software (version 1.3; National Institute of Mental Health, Bethesda, MD, USA) to calculate the ratio of monomer to polymer in  $\beta$ -tubulin and  $\beta$ -actin under KCl conditions.

**AG pretreatment.** AG is a nucleophilic hydrazine and nontoxic small molecule, which exerts pharmacological effects indicating that it has antioxidant properties (16). The protective effects of AG have been investigated in several experimental animal models, including oxidative stress-induced lung and liver injuries (17), and ischemia/reperfusion injury (18). AG appears to have useful properties that may block the protein carbonylation in PC12 cell lines exposed to H<sub>2</sub>O<sub>2</sub>; therefore, AG pretreatment was conducted prior to H<sub>2</sub>O<sub>2</sub> exposure in the present study. PC12 cells in the experimental group were treated with 0.5 mM AG (AB120123; Abcam, Cambridge, MA, USA) for 30 min, while PC12 cells in the control group were treated with culture medium only. After 300  $\mu$ M H<sub>2</sub>O<sub>2</sub> treatment for 48 h, the changes in the carbonylation level were determined using western blot analysis.

**Transfection.** Prior to H<sub>2</sub>O<sub>2</sub> treatment, cells were suspended at 5 $\times$ 10<sup>5</sup> cells/ml in DMEM supplemented with 10% fetal bovine serum and transfected with an empty vector or the  $\beta 5$  plasmid using the Lipofectamine 2000 reagent, according to the protocol provided by the manufacturer. At 2 days after transfection, the expression of  $\beta 5$  in the transfected cells was examined by western blot analysis.

**Detection of carbonylation levels of  $\beta$ -tubulin and  $\beta$ -actin.** A pull-down/western blot method was used to determine the extent of protein carbonylation. Briefly, biotinylation of protein carbonyls was performed through reaction of cells with biotin hydrazide (Bioworld Technology, Inc., St. Louis Park, MN, USA) in the presence of sodium cyanoborohydride (Santa Cruz Biotechnology, Santa Cruz, CA, USA). An aliquot (200  $\mu$ g) of these protein homogenates was kept for western blot analysis, while streptavidin-agarose (Thermo Fisher Scientific Inc.) was added to the remainder homogenate in order to isolate the biotinylated proteins. Next, SDS sample buffer was used to elute the proteins from the avidin agarose beads, followed by western blot analysis.

**Western blot analysis.** At 2 days after transfection, PC12 cells were washed with phosphate-buffered saline (PBS) and treated with a lysis buffer containing 50 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, phosphatase inhibitors (100 mM  $\text{Na}_3\text{VO}_4$  and 10 mM NaF) and a protease inhibitor (1 mM PMSF). The lysates were centrifuged at  $11,000 \times g$  for 10 min at  $4^\circ\text{C}$ , and the supernatant was collected. The protein concentrations were then quantitated using the Lowry protein assay method. An equal amount of sample (50  $\mu\text{g}$ ) was subjected to 10% SDS-PAGE and was blotted onto polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). Subsequently, the samples were blocked in PBS-Tween 20 (PBST) with 5% nonfat dry milk, and the membranes were incubated with primary antibodies against  $\beta$ -tubulin (1:1,000) and  $\beta$ -actin (1:5,000) at appropriate dilutions in PBST overnight at  $4^\circ\text{C}$ . The membranes were then washed three times with PBST solution, followed by incubation with goat anti-mouse secondary antibody (1:3,000) in PBST. Subsequently, the proteins were probed with horseradish peroxidase (HRP)-phytohemagglutinin-L and HRP-concanavalin A lectin. Visualization of the results was performed by fluorography using an ECL assay system.

**Statistical analysis.** Densitometric analysis of the western blot results was performed by the ImageQuant TL control center (GE Healthcare Life Sciences). Statistical analysis was performed using GraphPad Prism version 5 (GraphPad Software, Inc., La Jolla, CA, USA). One-way analysis of variance was used to compare the parameters measured in the different study groups.

## Results

**Increased oxidative stress and cytoskeletal protein carbonylation in PC12 cells treated with  $\text{H}_2\text{O}_2$ .** Upon treatment of PC12 cells with increasing concentrations of  $\text{H}_2\text{O}_2$ , the level of GSH was reduced compared with that in the untreated control group (Fig. 1A). The GSH content in PC12 cells was reduced to 40% compared with the untreated control, and after treatment with GSH for 24 or 48 h, the GSH content decreased to ~50 and 30%, respectively, which indicated a clear time and dose-dependent effect. By contrast, the level of TBARS increased steadily with increasing concentrations of  $\text{H}_2\text{O}_2$ , and was ~3-fold higher than the control group after treatment with 300  $\mu\text{M}$   $\text{H}_2\text{O}_2$  for 48 h (Fig. 1B). These results suggested that  $\text{H}_2\text{O}_2$  induced oxidative stress.

Subsequently, the  $\beta$ -actin and  $\beta$ -tubulin carbonylation was investigated. A significant increase ( $P<0.05$ ) was observed in the proportion of carbonylated  $\beta$ -actin and  $\beta$ -tubulin (Fig. 1C and D) in  $\text{H}_2\text{O}_2$ -treated cells compared with the untreated control cells, in a dose-dependent manner. Therefore, these results indicated that the increase in oxidative stress following TBI may disturb the balance of the reactive carbonyl species metabolism and lead to carbonylation of cytoskeletal proteins.

**Stability of cytoskeletal proteins in PC12 cells exposure to  $\text{H}_2\text{O}_2$  was affected by protein carbonylation.** Since protein carbonylation is known to affect cytoskeleton stability, it can be suggested that the observed changes in cytoskeletal protein carbonylation in PC12 cells treated with  $\text{H}_2\text{O}_2$

have physiological consequences. It has been reported that carbonylation of tubulin leads to the disassembly and instability of microtubules, while actin filaments are easily depolymerized upon carbonylation (19). Thus, in the present study, a series of assays were performed to examine the functional consequences of protein carbonylation. As shown in Fig. 2A and B, the results identified that the monomer/polymer ratios of  $\beta$ -actin and  $\beta$ -tubulin were significantly ( $P<0.05$ ) increased with  $\text{H}_2\text{O}_2$  treatment, which suggested dysfunction in the formation of polymers by the carbonylated  $\beta$ -actin and  $\beta$ -tubulin. These findings indicated that cytoskeletal proteins could not form stable polymer conditions in PC12 cells exposed to  $\text{H}_2\text{O}_2$ , providing evidence of the functional disturbance in cytoskeletal proteins under oxidative stress conditions.

**Inhibition of protein carbonylation upon pretreatment with AG in PC12 cells exposed to  $\text{H}_2\text{O}_2$ .** The administration of AG at 0.5 h prior to  $\text{H}_2\text{O}_2$  treatment resulted in reduction in the level of protein carbonylation when compared with that in the control group (Fig. 3A). In addition, the pretreatment with AG was found to also block the proportion of carbonylated  $\beta$ -actin and  $\beta$ -tubulin in PC12 cells exposed to  $\text{H}_2\text{O}_2$ . The quantification of the western blot analysis is shown in Fig. 3B, with a significant difference ( $P<0.05$ ) observed between the  $\text{H}_2\text{O}_2$ -treated and  $\text{H}_2\text{O}_2$ +AG-treated groups.

**Overexpression of proteasome  $\beta 5$  in PC12 cells exposed to  $\text{H}_2\text{O}_2$  blocks protein carbonylation.** It has been reported that reduced proteasomal activity contributes to the accumulation of carbonylated proteins during chronic experimental autoimmune encephalomyelitis in C57BL/6 mice (11). The proteasome serves a critical role in protein degradation and signal transduction following cellular stress or tissue injury (20). Overexpression of proteasome  $\beta 5$  assembled subunit increases the amount of proteasome and confers ameliorated response to oxidative stress and higher survival rates (21). In order to reveal the effect of proteasome  $\beta 5$  on the protein carbonylation, plasmids of proteasome  $\beta 5$  were transfected into PC12 cells. Upregulation of proteasome  $\beta 5$  in was first observed in the transfected PC12 cells (Fig. 4A and B). In addition, the level of protein carbonylation was increased compared with the control group. The western blot analysis results revealed that the overexpression of proteasome  $\beta 5$  in PC12 cells also blocked the proportion of carbonylated  $\beta$ -actin and  $\beta$ -tubulin following cell exposure to  $\text{H}_2\text{O}_2$  (Fig. 4C and D).

## Discussion

TBI is a serious health concern often resulting in mortality, while it may lead to severe neurological dysfunction in the case of survival (1,2,22). It has been reported that axonal injury is widely observed in the development of TBI (7). However, examining the clinical effectiveness of neuroprotective agents is challenging.

Free radical-induced oxidative damage reactions, and in particular membrane lipid peroxidation, are among the best validated secondary injury mechanisms in preclinical TBI models. Antioxidants have been demonstrated to alleviate the

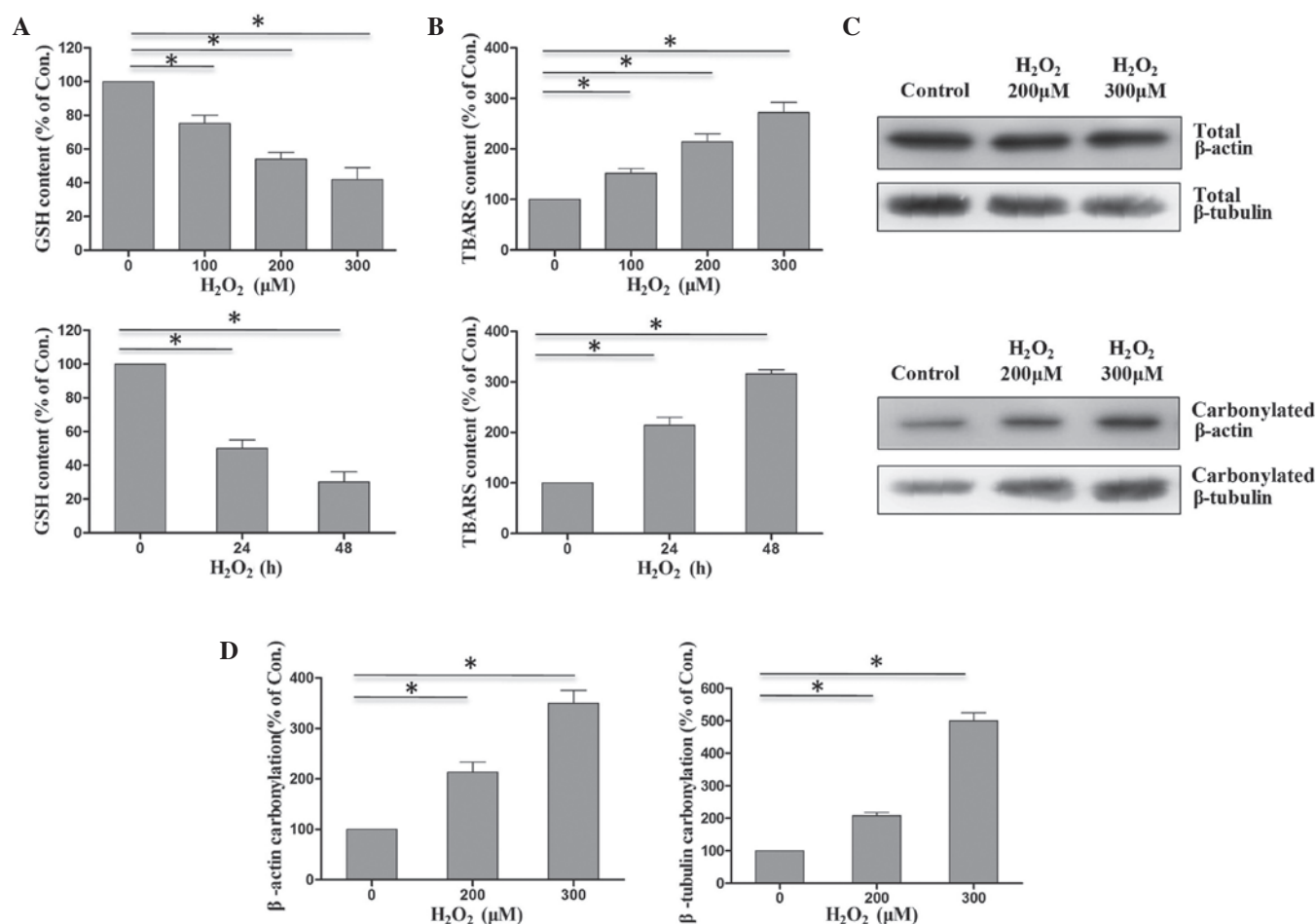


Figure 1. Oxidative indexes and cytoskeletal protein carbonylation in PC12 cells following treatment with H<sub>2</sub>O<sub>2</sub>. (A) GSH and (B) TBARS levels in PC12 cells following exposure to H<sub>2</sub>O<sub>2</sub> at different concentrations, or different time durations. Each value represents the mean  $\pm$  standard error of three experiments. (C) Protein expression levels of the total and carbonylated  $\beta$ -actin and  $\beta$ -tubulin in PC12 cells treated with or without H<sub>2</sub>O<sub>2</sub>, as measured by western blot analysis. (D) Quantification of the levels observed in the western blots. \* $P < 0.05$ . GSH, glutathione; TBARS, thiobarbituric acid reactive substances; Con, control.

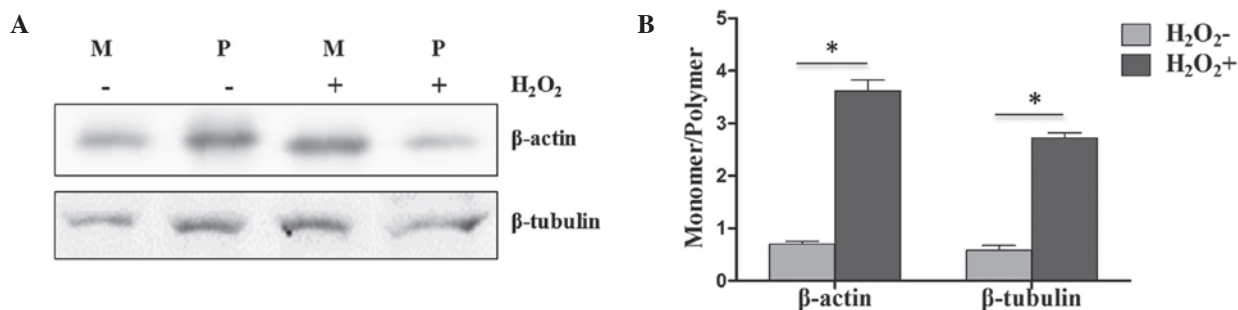


Figure 2. Solubility of cytoskeletal proteins in H<sub>2</sub>O<sub>2</sub>-treated PC12 cells was augmented upon incubation of KCl. (A) M and P levels of  $\beta$ -actin and  $\beta$ -tubulin detected by western blotting. (B) Densitometric analysis detected an increase in the ratio of M/P (for both  $\beta$ -actin and  $\beta$ -tubulin) following H<sub>2</sub>O<sub>2</sub> treatment. Values are represented as the mean  $\pm$  standard error of triplicate experiments. \* $P < 0.05$ . M, monomer; P, polymer.

occurrence of second injury following TBI (6). Cytoskeletal proteins, such as GFAP,  $\beta$ -actin and  $\beta$ -tubulin, are easily carbonylated in cells when exposed to oxidants (14). The present study aimed to identify the effect of H<sub>2</sub>O<sub>2</sub> exposure, which simulates the oxidative stress conditions observed in TBI, on the cytoskeletal proteins in PC12 cells. When the PC12 cells were cultured with H<sub>2</sub>O<sub>2</sub> in different concentrations, the increased expression of TBARS and the decreased

expression of GSH indicated increased oxidative stress induced by the increasing H<sub>2</sub>O<sub>2</sub> concentrations. Furthermore, the carbonylation levels of  $\beta$ -actin and  $\beta$ -tubulin were found to be significantly increased in PC12 cells exposed to H<sub>2</sub>O<sub>2</sub> for 24 and 48 h. In neurodegenerative disorders, the cytoskeletal proteins are known to be particularly susceptible to carbonylation (23). The present study confirmed that cytoskeletal proteins, including  $\beta$ -actin and  $\beta$ -tubulin, were the target



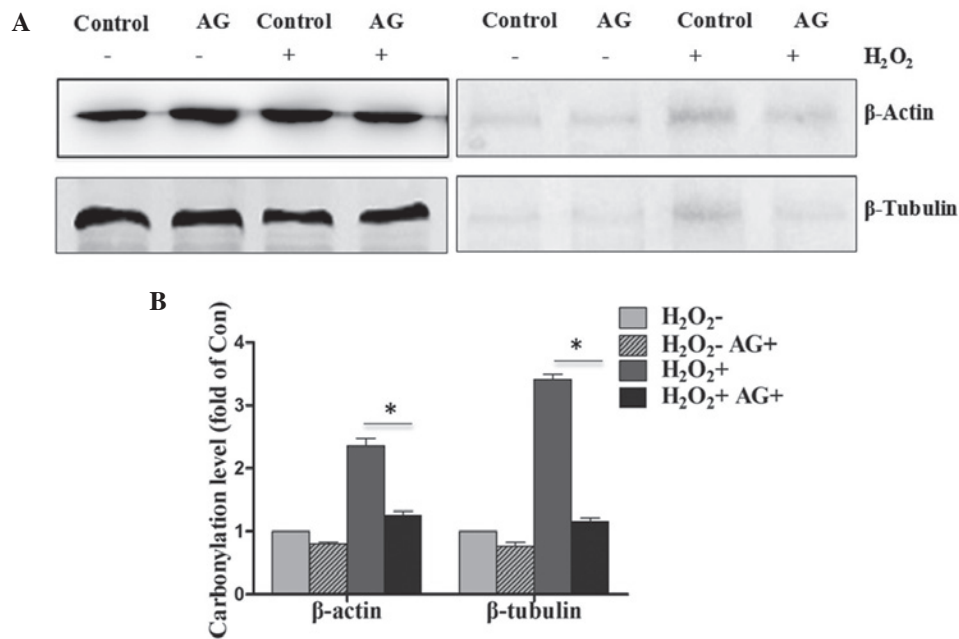


Figure 3. Pretreatment with AG blocks the H<sub>2</sub>O<sub>2</sub>-induced protein carbonylation in PC12 cells exposed to H<sub>2</sub>O<sub>2</sub>. (A) Carbonylation levels of  $\beta$ -actin and  $\beta$ -tubulin in PC12 cells pretreated with AG and then exposed to H<sub>2</sub>O<sub>2</sub>, as measured by western blot analysis. Data are representative of three independent experiments. (B) Quantification of the levels shown in the western blots. Each value represents the mean  $\pm$  standard error of triplicate experiments. \*P<0.05. AG, aminoguanidine; Con, control.

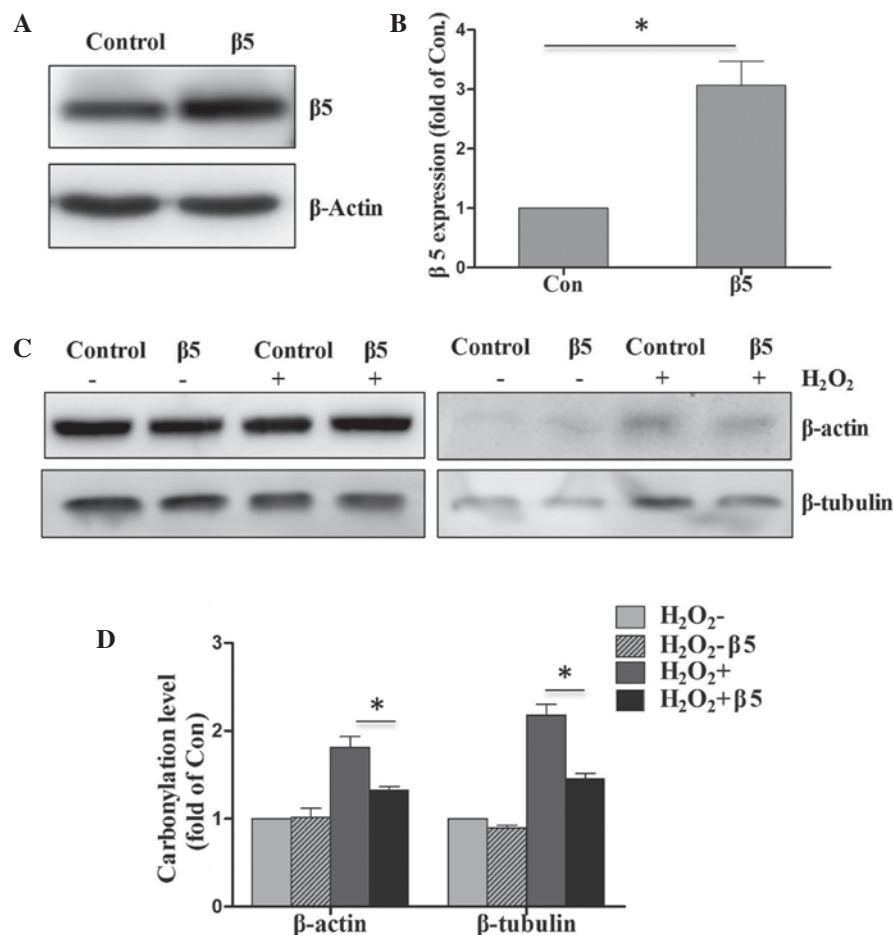


Figure 4. Overexpression of proteasome  $\beta$ 5 blocked the H<sub>2</sub>O<sub>2</sub>-induced protein carbonylation in PC12 cells. (A) Upregulation of proteasome  $\beta$ 5 in PC cells following transfection was detected by western blot analysis. These data are representative of three independent experiments. (B) Quantification of the expression shown in the western blots. (C) Carbonylation levels of  $\beta$ -actin and  $\beta$ -tubulin in cells overexpression proteasome  $\beta$ 5, which were exposure to H<sub>2</sub>O<sub>2</sub>, was measured by western blot analysis. These data are representative of three independent experiments. (D) Quantification of the  $\beta$ -actin and  $\beta$ -tubulin levels shown in the western blots. Each value represents the mean  $\pm$  standard error of triplicate experiments. \*P<0.05.

carbonylated proteins when cells were under oxidative stress. Furthermore, carbonylation of cytoskeletal proteins has been reported to cause loss of function. The current study results revealed that, when PC12 cells were cultured with H<sub>2</sub>O<sub>2</sub>, the monomer/polymer ratios of  $\beta$ -actin and  $\beta$ -tubulin were significantly increased, which indicated that cytoskeletal  $\beta$ -actin and  $\beta$ -tubulin could not form stable polymer conditions in PC12 cells exposed to H<sub>2</sub>O<sub>2</sub>, providing evidence of functional disturbance in cytoskeletal proteins under oxidative stress. Thus, it can be further postulated that carbonylation will cause instability of cytoskeletal proteins, thus leading to the axonal injury in TBI.

In the current study, the carbonylation levels of  $\beta$ -actin and  $\beta$ -tubulin were blocked following pretreatment with AG and by overexpression of proteasome  $\beta$ 5 in PC12 cells. The principal function of the proteasome is targeted degradation of intracellular proteins. The results also revealed that proteasome  $\beta$ 5 serves an important role in the development of TBI. It has been reported that PA28 $\alpha$  overexpression is sufficient to upregulate 11S proteasomes (24), enhance proteasome-mediated removal of misfolded and oxidized proteins, as well as protect against oxidative stress in cardiomyocytes; thus, it can results in an increase in proteasomal degradation of abnormal cellular proteins (25). The results of the present study, thus, indicate the potential value of AG and proteasome  $\beta$ 5 in healing axonal injury in TBI.

In conclusion, the current study suggested that cytoskeletal proteins carbonylation was involved in the development of TBI. These findings suggested that the blocking of oxidative stress-induced carbonylation of cytoskeletal proteins may have a therapeutic value in the treatment of TBI. However, other factors may also be associated with the development of TBI, which requires further investigation.

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