Alcohol and Alcoholism, 2020, 55(4) 357–366
doi: 10.1093/alcalc/agaa031
Advance Access Publication Date: 4 May 2020
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Neuroprotective Effects of Grape Seed Procyanidins on Ethanol-Induced Injury and Oxidative Stress in Rat Hippocampal Neurons

Wenyang Jin, Mizhu Sun, Bingbing Yuan, Runzhi Wang, Hongtao Yan, and Xiaomeng Qiao*

Department of Forensic Medicine, School of Basic Medical Sciences, Zhengzhou University, Zhengzhou 450001, China

*Corresponding author: No. 100, Science Avenue, Zhengzhou, Henan 450001, China. E-mail: xiaomeng416520@126.com

Received 25 November 2019; Revised 25 March 2020; Accepted 4 April 2020

Abstract

Aims: Ethanol is a small molecule capable of interacting with numerous targets in the brain, the mechanisms of which are complex and still poorly understood. Studies have revealed that ethanol-induced hippocampal neuronal injury is associated with oxidative stress. Grape seed procyanidin (GSP) is a new type of antioxidant that is believed to scavenge free radicals and be anti-inflammatory. This study evaluated the ability and mechanism by which the GSP improves ethanol-induced hippocampal neuronal injury.

Methods: Primary cultures of hippocampal neurons were exposed to ethanol (11, 33 and 66 mM, 1, 4, 8, 12 and 24 h) and the neuroprotective effects of GSP were assessed by evaluating the activity of superoxide dismutase (SOD), the levels of malondialdehyde (MDA) and lactate dehydrogenase (LDH) and cell morphology.

Results: Our results indicated that GSP prevented ethanol-induced neuronal injury by reducing the levels of MDA and LDH, while increasing the activity of SOD. In addition, GSP increased the number of primary dendrites and total dendritic length per cell.

Conclusion: Together with previous findings, these results lend further support to the significance of developing GSP as a therapeutic tool for use in the treatment of alcohol use disorders.

INTRODUCTION

Ethanol is a lipid-soluble and water-soluble molecule that easily passes through the blood-brain barrier and damages the central nervous system (CNS), thereby disrupting brain function and behavioral phenotype (Karamanos *et al*., 2014; Rivera *et al*., 2019). Studies have shown that excessive drinking causes anxiety, depression and cognitive impairments both in human (Gallagher *et al*., 2018; Silva-Pena *et al*., 2019) and animal models (Ji *et al*., 2018). Intense ingestion of ethanol induces oxidative stress and produces reactive oxygen species (ROS) such as hydrogen peroxide, superoxide anion, hydroxyl radical and lipid peroxides, and also activates neuroinflammatory and apoptotic pathways (Das and Vasudevan, 2007), all of which are involved in human cognition and affections.

The hippocampus is a key region involved in spatial learning, contingency and declarative memory, which regulates emotions and learning (Lindquist *et al*., 2013). The hippocampus converts short-term procedural memory into long-term procedural memory, thus programming and storing new information (Dougherty *et al*., 2008). Moreover, in the developing brain, the hippocampus is one of the most sensitive regions to ethanol (Haes *et al*., 2010). There are a large amount of unsaturated fatty acids that are easily oxidized in hippocampus, and the antioxidant content is relatively low, thus it is easily damaged by free radicals (Bhattacharya *et al*., 2020). Free radicals directly damage carbohydrates, lipids, DNA and proteins (Dizdaroglu and Jaruga, 2012; Salah *et al*., 2019). Under normal physiological conditions in the body, free radical levels are in dynamic equilibrium. However, ethanol induces excess production of free radicals and the lipid peroxide myeloperoxidase through enzymatic or non-enzymatic pathways, disrupting the balance between antioxidant defense mechanisms and the intracellular production of free

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radicals to induce oxidative stress (Karadayian *et al*., 2015). Thus, excessive decomposition products damage membrane fluidity and permeability, as well as ion transport and barrier functions, leading to changes in cell metabolism, function and structure, and even death (Varoni *et al*., 2015). Ethanol inhibits the release of superoxide dismutase (SOD) in the hippocampus, and changes the levels of malondialdehyde (MDA) and lactate dehydrogenase (LDH), thus inducing abnormalities in the hippocampal morphology and structure and eventually leading to memory, learning and behavioral impairments (Soleimani *et al*., 2016; Hasanein *et al*., 2017).

Antioxidants that present in neuronal tissue are potential candidates for prevention or treatment of disorders involving oxidative damage. Grape seed procyanidin (GSP), which is extracted from French Pinot Noir red grape seeds, has long been recognized to posse myriads of properties, including antioxidant, anti-inflammatory, anticarcinogenic, platelet aggregation inhibiting and metal chelating capabilities, etc. GSP is rich source of monomeric phenolic compounds that scavenge hydroxyl and other radicals, such as superoxide radicals (Balu *et al*., 2005; Wang *et al*., 2017). Moreover, GSP protects against the neurotoxic effect of ethanol by reducing the crosslinking of cellular proteins and repairing damaged nerve fibers, promoting normal signal transmission between neurons to improve impaired learning and memory (Li *et al*., 2015). In addition, GSP has been shown to improve symptoms such as irritability, fatigue and hypomnesia (Mao *et al*., 2015). Although GSP has proven to be a healthy food ingredient, little is known about the molecular mechanisms underlying the neuroprotective effects of this compound, particularly its ability to reduce ethanol-induced neurotoxicity.

In the present study, therefore, an *in vitro* experimental model of ethanol treatment was used to investigate the ability of GSP to counteract the ethanol-induced neurotoxicity. In particular, primary cultures of rat hippocampal neurons were exposed to 11, 33 and 66 mM ethanol for 1, 4, 8, 12, 24 h, and the neuroprotective effects of GSP on ethanol-induced injury and oxidative stress were assessed by evaluating cell morphology, the antioxidant barrier such as SOD and the main marker of oxidative stress (LDH and MDA) levels. These results will provide a theoretical basis for further explorations of the mechanisms by which ethanol damages the CNS and potential treatment for ethanol use disorders.

MATERIALS AND METHODS

Animals

Male and female Sprague–Dawley (SD) rats (born within 24 h) were purchased from the Experimental Animal Center of Henan province (license: SCXK(YU):2015-0004), and housed under specific pathogen free conditions. All animal procedures were approved by the Institutional Animal Care Committee of Zhengzhou University.

Materials

GSPs (Solarbio, Beijing, China, SP8520), NAD-ADH Reagent Multiple Test Vial (Sigma-Aldrich, USA), anti-neuron-specific enolase (NSE) Antibody (Boster Biological Technology, China, #BM4495), the SOD kit, MDA kit, LDH assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China, A001-3, A003-1 and A020-2, respectively), anti-brain microtubule-associated proteins 2 (MAP2) (Abcam, UK, ab11267), Goat anti-mouse IgG H&L (Alexa Fluor® 488) (Abcam, UK, ab150117) and DAPI (Solarbio, China, C0065) were used in the present study.

Primary culture of hippocampal neurons

Primary cultures of hippocampal neurons were prepared from 24-h-old rats born from SD dams. The hippocampi were dissected free of meninges and chopped into 1 mm^3 pieces. The hippocampal chunks were digested by 0.025% (w/v) trypsin at 37°C for 10 min and then resuspended in Dulbecco's modified eagle medium (DMEM) supplemented 10% fetal bovine serum in order to stop trypsin activity. After centrifugation at 14,000*g* for 5 min, the supernatant was discarded and the cell pellet was resuspended in DMEM. Hippocampal cultures were maintained in serum-free neurobasal medium with supplements of 0.1 mM glutamine, 10 μg/ml gentamicin, and 2% B27. Cells were counted and then transferred into 12-well culture slides coated with poly-L-lysine at a density of 1.0×10^5 cells per well and grown. Cultures were incubated at 37°C in a humidified atmosphere of 5% carbon dioxide (CO_2)/95% air. Cytosine arabinoside (10 μM; Sigma-Aldrich, USA) was added within 24 h of plating to prevent glial cell proliferation. After 3 days in culture, half of the culture medium was replaced with fresh medium. Hippocampal neurons were chosen for experiments after 7 days of *in vitro* incubation.

Immunocytochemistry and microscopy

The purity of neuron cultures was assessed by immunocytochemistry using anti-NSE antibodies. Briefly, cells were washed with PBS (pH 7.4) and fixed in 4% paraformaldehyde for 30 min. They were permeabilized with 0.35% Triton X-100 for 20 min, then endogenous peroxide activity was quenched with 3% hydrogen peroxide for 10 min, and non-specific binding was blocked with goat serum for 30 min, then incubated with primary rabbit anti-NSE antibody (1:100) overnight at 4°C. The next day, cells were incubated with the goat anti-rabbit secondary antibody for 1 h and horseradish enzyme-labeled streptavidin working fluid for 30 min. Cells were then stained with DAB solution for about 1 min when they appeared brown. Hematoxylin was used to stain the nuclei, and an optical microscope (Olympus, Tokyo, Japan) a 10× objective was used to observe and obtain images.

Exposure to ethanol and pharmacological treatment

After 7 days of *in vitro* incubation, cultures were treated with ethanol. Hippocampal neurons were randomly divided into control group and ethanol-treated low-dose group (11 mM, similar to the blood ethanol concentration of 50 mg/dl), medium-dose group (33 mM, 150 mg/dl) and high-dose group (66 mM, 300 mg/dl) (Romero *et al*., 2010). All cells were further cultured for 1, 4, 8, 12 and 24 h. The concentration of ethanol in the medium was determined using the kit NAD-ADH Reagent Multiple Test Vial. The supernatant was collected to detect the LDH, MDA and SOD levels ($n = 6$/group). Hippocampal neuronal injury was evaluated based on the results of MAP2 immunofluorescence staining.

When exerting cytoprotective effect (antioxidant and immunomodulation), the effective working concentration of GSP is generally not higher than 10 μg/ml (Narita *et al*., 2011; Ali Shah *et al*., 2013; Fu *et al*., 2019). Due to the different structures of proanthocyanidins extracted from plants, their ability to bind to cell membrane receptors varies, and their effective neuroprotective concentrations are also different. No cytotoxicity was observed in GSP at concentrations of ≤5 μg/ml (Bai *et al*., 2014), so two concentrations of GSP

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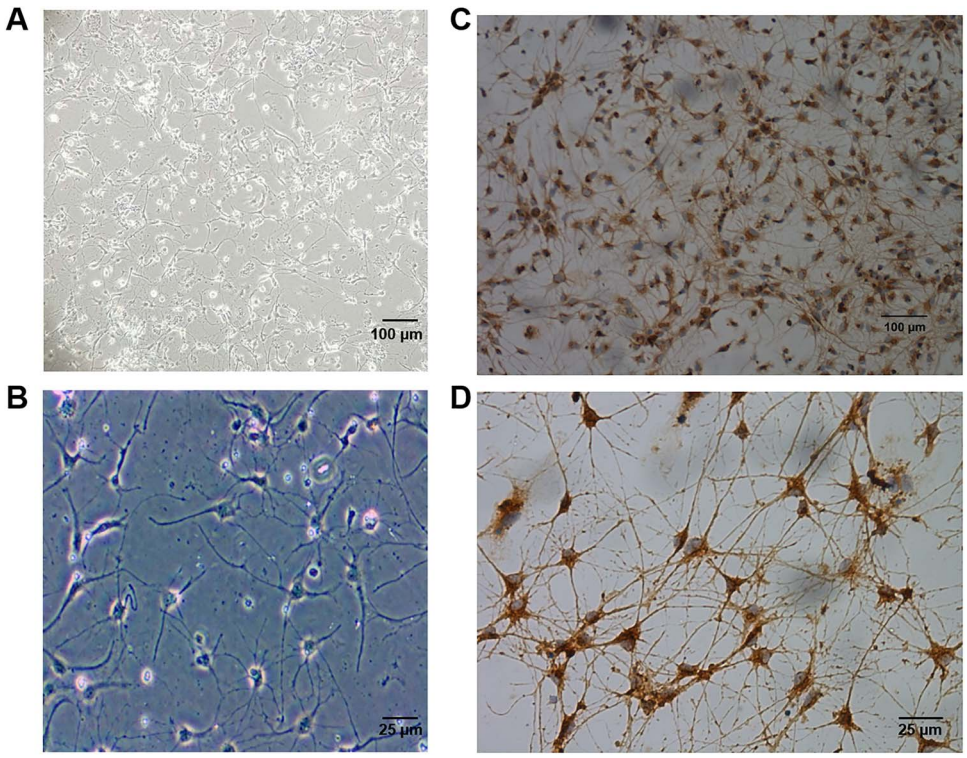

Fig. 1. The morphology of hippocampal neurons after 7 days of culture. (**A**) Normally cultured hippocampal neurons under an inverted microscope. Scale bar = 100 μm. (**B**) Neurons at high magnification. Scale bar = 25 μm. (**C**) Representative images of NSE (brown) immunostaining with hematoxylin (light blue) re-staining in hippocampal neuronal cells on the seventh day. Scale bar = 100 μm. (**D**) NSE positive cells at high magnification. Scale bar = 25 μm.

(2.5 and 5.0 mg/l) were selected to detect the protective effect of GSP in ethanol-induced hippocampal injury. Similarly, GSP and equal amount of PBS were added to the culture medium of ethanol exposure group and control group (n = 6/group), respectively, and cultured for 24 h. The effect of GSP on LDH, MDA and SOD levels and the protective effect on hippocampal neurons were tested.

The levels of LDH, MDA and SOD in supernatants

MicroELISA assay was used to measure the levels of LDH according to the manufacturer's instructions (n = 6/group), which formed a red–brown coordination complex with maximum absorbance at 450 nm when reacting with the pyruvate dinitrobenzene hydrazine substance.

Thiobarbituric acid (TBA) assay was used to measure the levels of MDA. The supernatants were mixed with operating fluid II, reagent blank control or standard solution. The TBA solution was mixed with operating fluid I, and the mixtures were incubated in a water bath at 95°C for 80 min and then cooled. Finally, the absorbance was measured at 532 nm.

The water-soluble tetrazolium salt (WST-1) assay was used to measure the activity of SOD according to the manufacturer's instructions. The supernatant concentration with SOD inhibition rate of 40–60% was selected in the preliminary experiment. The absorbance was measured at 450 nm.

Immunofluorescence staining and morphological analysis

The morphological changes of hippocampal neurons were observed with MAP2 fluorescence staining at 4, 8, 12, 24 h after ethanol treatment and at 8, 24 h after GSP and ethanol interaction. Briefly, cells were fixed in 4% paraformaldehyde in PBS for 30 min and permeabilized with 0.1% Triton X-100 in PBS for 15 min at room temperature. After blockade with 5% normal goat serum for 30 min, cells were incubated with mouse monoclonal anti-MAP-2 antibody, a marker for neuronal soma and dendrites (1:1000), at 4°C overnight. Cells were then incubated with anti-mouse IgG Alexa Fluor® 488 conjugate (1:500) for 1 h at 37°C, later washed in PBS and counterstained with DAPI for 3 min. Laser scanning confocal microscopy (Olympus FluoView FV1000, Japan) with a 40× objective was used to observe the morphology of hippocampal neurons.

Hippocampal neurons were examined in different randomly selected microscopic fields on a minimum of three dishes for each experiment, and at least three experiments for each condition. MAP-2 positive cells were quantified with Image J software. Image-pro plus 6.0 was used for quantitative measurements of number of primary dendrites and total length of dendritic arborization per cell (Gao *et al.*, 2010). Briefly, after the target neuron was identified, the dendrites were traced to the end, and the complete overview of the dendrites at all levels was outlined. The software was then used to measure the total dendrite length of the target neuron.

Statistical analysis

All data were presented as the means ± SD. The statistical analysis was performed using SPSS for Windows, version 18.0 (SPSS Inc., Version X, New York, USA). Comparisons between multiple groups were carried out by an analysis of variance (ANOVA) followed by Tukey's *post-hoc* test. The accepted level of significance for all tests was $P < 0.05$.

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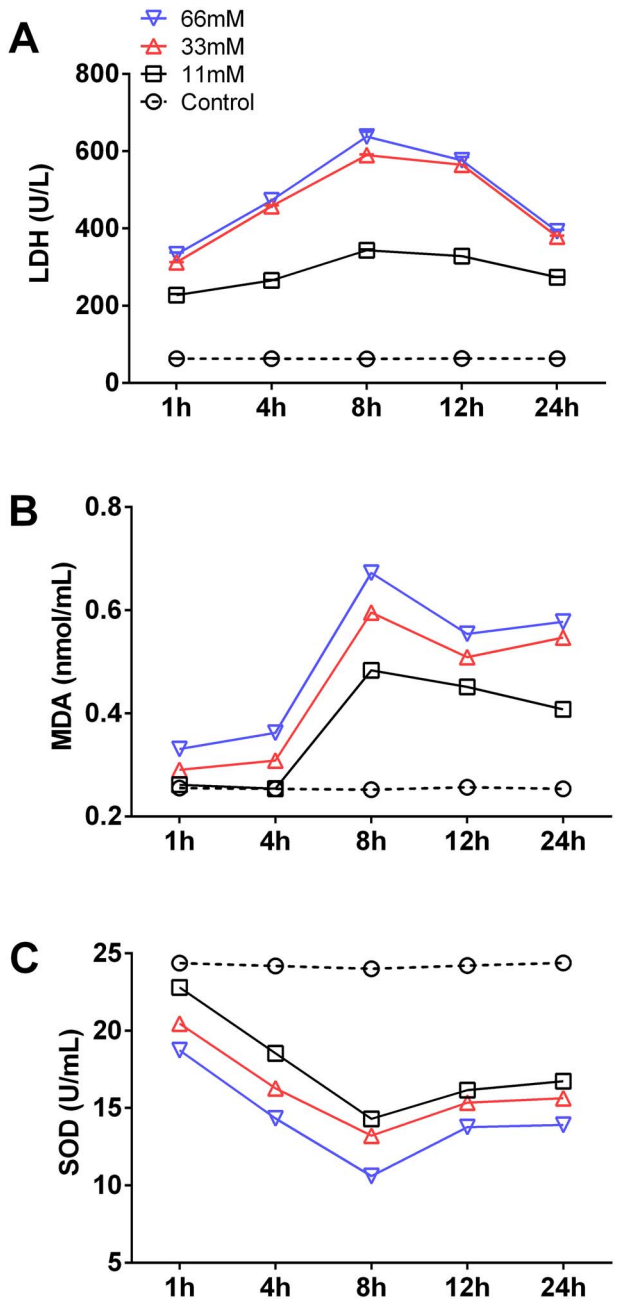

Fig. 2. The levels of LDH (**A**), MDA (**B**) and SOD (**C**) in the supernatant of hippocampal cell cultures exposed to ethanol. They were so sensitive to ethanol that they changed significantly compared to the control group at each point in the ethanol treatment ($P < 0.0001$, **** not shown in the figure).

RESULTS

Effects of ethanol on the levels of LDH, MDA and SOD in hippocampal neuron supernatants

After 7 days of culture, hippocampal neurons were mature and the neural fiber network was formed (Fig. 1A and B). The percentage of NSE positive cells (tan cytoplasm and neurites, light blue nucleus) was more than 95% (Fig. 1C and D). At this time, ethanol was added to the cell culture medium to a final concentration of 11, 33 and 66 mM, respectively.

After 1, 4, 8, 12 and 24 h treatment with ethanol, the levels of LDH and MDA, the activity of SOD in the supernatant were measured. For LDH (Fig. 2A), two-way ANOVA demonstrated a significant effect of EtOH [$F_{(3, 20)} = 61256$, $P < 0.0001$] and time [$F_{(4, 80)} = 4883$, $P < 0.0001$] with a significant interaction between them [$F_{(12, 80)} = 862.9$, $P < 0.0001$]. *Post-hoc* analysis showed that the levels of LDH in each ethanol-treated group were significantly higher than those in the control group (shown in Table 1). Surprisingly, LDH levels peaked after 8 h of ethanol treatment and then decreased, but were still significantly higher than those in the control group. Similarly, the MDA levels were significantly higher in the ethanol-treated groups than in the control groups [$F_{\text{Ethanol (3, 20)}} = 122.0$, $P < 0.0001$; $F_{\text{Time (4, 80)}} = 73.83$, $P < 0.0001$; $F_{\text{Ethanol} \times \text{Time (12, 80)}} = 9.33$, $P < 0.0001$] (Fig. 2B). The higher the ethanol concentration was, the higher the MDA levels were, but they reached saturation at 8 h of ethanol treatment. The statistical data were shown in Table 2.

The SOD activity reflects the antioxidant levels, which was significantly decreased in the ethanol-treated groups than in the control groups [$F_{\text{Ethanol (3, 20)}} = 9671$, $P < 0.0001$; $F_{\text{Time (4, 80)}} = 2359$, $P < 0.0001$; $F_{\text{Ethanol} \times \text{Time (12, 80)}} = 254.2$, $P < 0.0001$] (Fig. 2C). Under different concentrations of ethanol, the activity of SOD was lowest at 8 h. The statistical data are shown in Table 3.

Effects of ethanol on the morphology of hippocampal neurons

Morphological changes induced by EtOH in hippocampal neurons were investigated by MAP-2 fluorescence staining (Fig. 3A and B). For the number of neurons, two-way ANOVA revealed a significant effect of ethanol [$F_{(3, 80)} = 6.518$, $P < 0.001$] and time [$F_{(3, 80)} = 7.741$, $P < 0.001$] with a significant interaction [$F_{(9, 80)} = 2.422$, $P < 0.05$] (Fig. 3C). There was no significant difference in the number and morphology of neurons in the control group. Normal hippocampal neurons were spindle-shaped and oval. The nucleus was stained blue by DAPI, with complete structure and clear boundary. Neurons extended one or more rough filamentous projections to connect to each other. *Post-hoc* analysis showed that 11 and 33 mM ethanol had little effect on the number of hippocampal neurons ($P > 0.05$). However, there was a significant neurons loss after 66 mM EtOH treatment (12 h: control 17.67 ± 3.27 vs. 66 mM EtOH 10.17 ± 2.79, $P < 0.05$; 11 mM EtOH: 17.33 ± 4.46 vs. 66 mM EtOH 10.17 ± 2.79, $P < 0.05$; 24 h: control 18.0 ± 2.76 vs. 66 mM EtOH 8.5 ± 3.08, $P < 0.001$; 11 mM EtOH: 15.67 ± 3.72 vs. 66 mM EtOH 8.5 ± 3.08, $P < 0.05$).

Similarly, two-way ANOVA demonstrated a significant effect of EtOH [$F_{(3, 80)} = 17.94$, $P < 0.0001$] and time [$F_{(3, 80)} = 11.63$, $P < 0.0001$] with a significant interaction between them [$F_{(9, 80)} = 3.32$, $P < 0.01$] (Fig. 3D). 11 and 33 mM EtOH did not affect the dendrites of per neuron. 66 mM EtOH (12 h: control 3.83 ± 0.75 vs. 66 mM EtOH 1.17 ± 1.17, $P < 0.01$; 11 mM EtOH: 3.67 ± 0.82 vs. 66 mM EtOH 1.17 ± 1.17, $P < 0.01$; 24 h: control 3.67 ± 0.82 vs. 66 mM EtOH 0.17 ± 0.41, $P < 0.0001$; 11 mM EtOH: 3.67 ± 1.51 vs. 66 mM EtOH 0.17 ± 0.41, $P < 0.0001$) significantly decreased the number of dendrites per cell. Meanwhile, EtOH also significantly reduced the dendritic length of hippocampal neurons (Supplementary Fig. 1A).

GSP attenuates the effects of ethanol on the LDH, MDA and SOD in hippocampal neuron supernatants

LDH, MDA and SOD were sensitive to GSP. Since the effects of ethanol on neuron density and dendrites were not significant during

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Table 1. The level of LDH in the supernatant of hippocampal neurons after ethanol exposure (U/L)

Group	Time				
	t1 (1 h)	t2 (4 h)	t3 (8 h)	t4 (12 h)	t5 (24 h)
Control	62.53 ± 2.06	62.86 ± 1.818	62.20 ± 1.35	63.03 ± 1.82	62.70 ± 1.90
11 mM	227.43 ± 6.83[b]	265.25 ± 4.02[b]	343.60 ± 5.18[b]	328.3 ± 0.84[b]	273.6 ± 1.86[b]
33 mM	312.3 ± 0.6[b,c]	457.6 ± 6.83[b,c]	590.06 ± 4.25[b,c]	564.83 ± 0.42[b,c]	379.0 ± 5.94[b,c]
66 mM	333.3 ± 4.31[b,c,d]	472.62 ± 0.01[b,c,d]	637.95 ± 13.01[b,c,d]	576.5 ± 8.65[b,c,d]	392.62 ± 3.86[b,c,d]

[a]Values were represented as mean ± SD, $n = 6$ for each group.
[b]$P < 0.0001$ versus control group.
[c]$P < 0.0001$ versus 11 mM ethanol group.
[d]$P < 0.0001$ versus 33 mM ethanol group.

Table 2. The level of MDA in the supernatant of hippocampal neurons after ethanol exposure (nmol/ml)

Group	Time				
	t1 (1 h)	t2 (4 h)	t3 (8 h)	t4 (12 h)	t5 (24 h)
Control	0.255 ± 0.003	0.254 ± 0.005	0.252 ± 0.008	0.257 ± 0.004	0.254 ± 0.004
11 mM	0.262 ± 0.020	0.254 ± 0.007	0.484 ± 0.009[b]	0.451 ± 0.029[b]	0.408 ± 0.118[b]
33 mM	0.290 ± 0.024	0.308 ± 0.009	0.596 ± 0.004[b,f]	0.509 ± 0.042[b]	0.547 ± 0.052[b,g]
66 mM	0.331 ± 0.001	0.362 ± 0.014[e,f]	0.672 ± 0.026[b,c]	0.554 ± 0.166[b,f]	0.578 ± 0.112[b,c]

[a]Values were represented as mean ± SD, $n = 6$ for each group.
[b]$P < 0.0001$ versus control group.
[c]$P < 0.0001$ versus 11 mM ethanol group.
[d]$P < 0.0001$ versus 33 mM ethanol group.
[e]$P < 0.01$ versus control group.
[f]$P < 0.01$ versus 11 mM ethanol group.
[g]$P < 0.001$ versus 11 mM ethanol group.

Table 3. The activity of SOD in the supernatant of hippocampal neurons after ethanol exposure (U/ml)

Group	Time				
	t1 (1 h)	t2 (4 h)	t3 (8 h)	t4 (12 h)	t5 (24 h)
Control	24.37 ± 0.013	24.18 ± 0.372	24.00 ± 0.497	24.20 ± 0.410	24.38 ± 0.047
11 mM	22.80 ± 0.126[b]	18.54 ± 0.287[b]	14.31 ± 0.193[b]	16.15 ± 0.082[b]	16.73 ± 0.037[b]
33 mM	20.45 ± 0.121[b,c]	16.27 ± 1.108[b,c]	13.21 ± 0.186[b,c]	15.34 ± 0.115[b,c]	15.64 ± 0.164[b,c]
66 mM	18.74 ± 0.133[b,c,d]	14.33 ± 0.141[b,c,d]	10.60 ± 0.394[b,c,d]	13.77 ± 0.179[b,c,d]	13.91 ± 0.054[b,c,d]

[a]Values were represented as mean ± SD, $n = 6$ for each group.
[b]$P < 0.0001$ versus control group.
[c]$P < 0.0001$ versus 11 mM ethanol group.
[d]$P < 0.0001$ versus 33 mM ethanol group.

the first and fourth hours, 2.5 and 5.0 mg/l GSP were added to the culture medium during eighth and 24th hours. The results showed that GSP significantly attenuated the effects of ethanol on LDH both during eighth and 24th hours, and the greater the concentration of GSP, the stronger the effect. Ethanol increased the levels of LDH (Fig. 4A and B) and MDA (Fig. 4C and D) ($P < 0.0001$), while GSP significantly reduced the effects of ethanol ($P < 0.0001$). In contrast, ethanol reduced the SOD levels (Fig. 4E and F) ($P < 0.0001$), while GSP partially restored it ($P < 0.0001$).

GSP repairs hippocampal neurons damaged by ethanol

The morphology of hippocampal neurons was examined after 24 h of ethanol and GSP interaction (Fig. 5A and B). GSP had no effect on ethanol-induced reduction of MAP2 positive cell number [$F_{\text{Ethanol}\ (3,\ 60)} = 30.16$, $P < 0.0001$; $F_{\text{GSP}\ (2,\ 60)} = 2.88$, $P = 0.06$; $F_{\text{Ethanol} \times \text{Time}}\ (6,\ 60) = 3.71$, $P < 0.01$] (Fig. 5C). GSP itself did not affect the morphology of normal hippocampal neurons ($P > 0.05$). However, there was a significant effect of ethanol [$F_{(3,\ 60)} = 20.64$, $P < 0.0001$] and GSP [$F_{(2,\ 60)} = 7.01$, $P < 0.01$] on primary dendrites. Exposure to 33 ($P < 0.05$ vs. control + PBS) and 66 mM ($P < 0.001$ vs. 11 mM EtOH + PBS) ethanol for 24 h decreased primary dendrite numbers in individual cells (Fig. 5D). About 5.0 mg/l GSP partially repaired hippocampal neuronal dendrites damaged by 66 mM ethanol ($P < 0.01$ vs. 66 mM EtOH + PBS), manifested by increased number of dendrites per cell. Meanwhile, 2.5 mg/l GSP significantly increased the mean dendritic length of neurons damaged by 66 mM ethanol ($P < 0.01$), and 5.0 mg/l GSP significantly increased the mean dendritic length per

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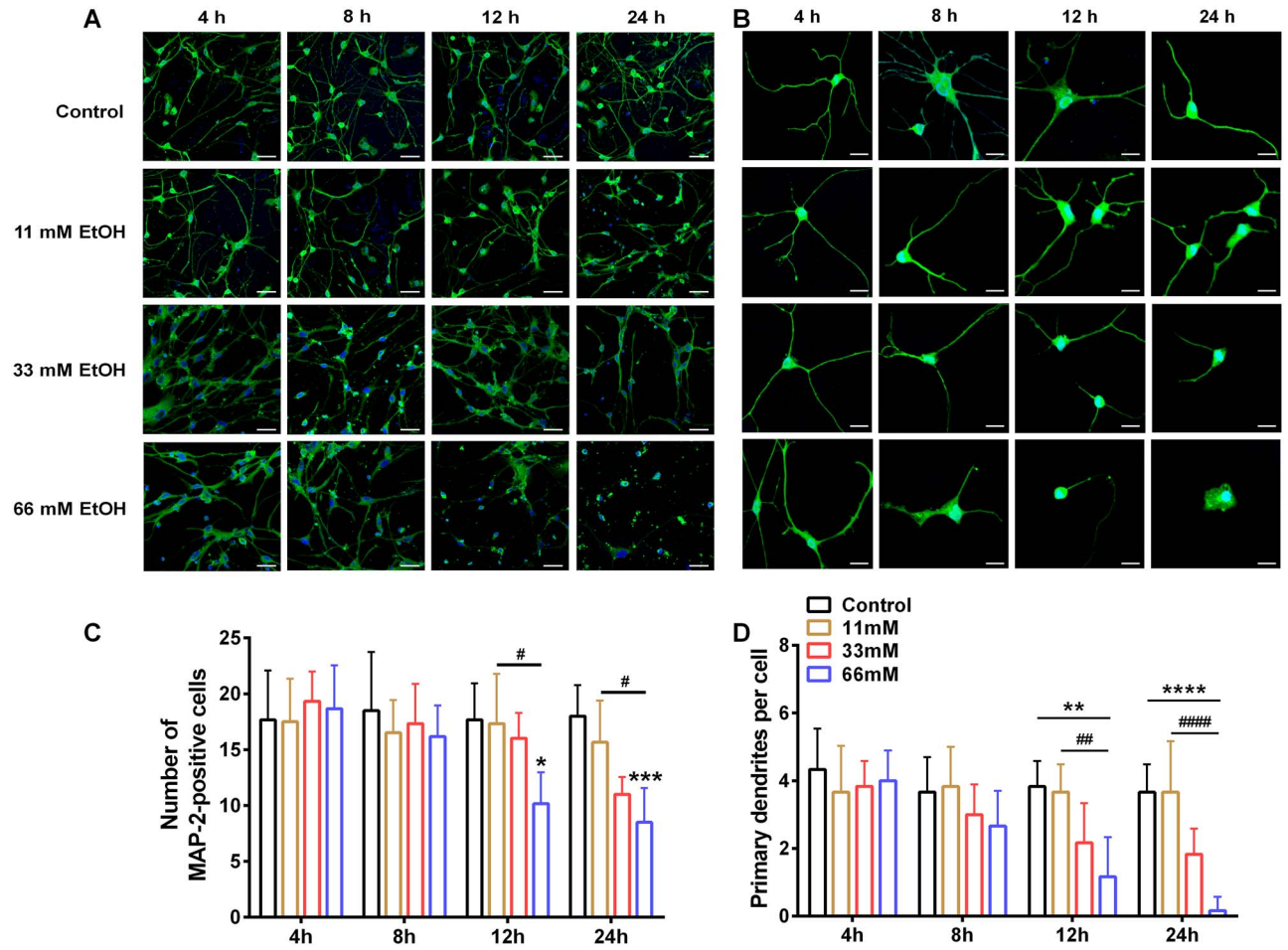

Fig. 3. Ethanol reduced the number of MAP-2 positive cells and induced morphological changes. (**A**) Representative images of MAP-2 and DAPI immunostaining in hippocampal neuronal cells incubated in the absence (control) or presence of ethanol. Scale bar = 50 μm. (**B**) Neurons at high magnification. Scale bar = 10 μm. Number of MAP-2 positive cells (**C**) and number of primary dendrites per cell (**D**) were evaluated. $*P < 0.05$, $**P < 0.01$, $****P < 0.0001$ different from control group at each time point; $^{\#\#}P < 0.01$, $^{\#\#\#\#}P < 0.0001$ different from the 11 mM ethanol group at each time point.

cell damaged by 33 ($P < 0.01$) and 66 mM ethanol ($P < 0.001$) (Supplementary Fig. 1B).

DISCUSSION

Our study shows that ethanol enhanced the oxidative stress response and induced hippocampal neuron injury. In this case, hippocampal neurons produced large amounts of oxygen free radicals (Hwang *et al.*, 2017; Ikram *et al.*, 2019), which led to lipid peroxidation and produce large amount of harmful products MDA and LDH (markers of lipid peroxidation and cell damage, respectively). Meanwhile, the activity of antioxidant enzyme SOD significantly reduced. As a highly effective natural antioxidant, GSP significantly attenuated the oxidative stress induced by ethanol, which reduced the release of MDA and LDH, and increased the activity of SOD. Based on its pharmacological function, GSP repaired the damaged hippocampal neurons through increasing the number and length of primary dendrites.

Ethanol has previously been shown to produce morphological and functional changes of neurons in the hippocampus and other brain regions (Guadagnoli *et al.*, 2016; Louth *et al.*, 2018). The maintenance of neural morphology depends on microtubules and microfilaments in the cytoskeleton (Roberto and Varodayan, 2017). MAP-2 is a neuron-specific cytoskeletal protein involved in microtubule assembly, which stabilizes microtubule growth by cross-linking with intermediate filaments and other microtubules (Dehmelt and Halpain, 2005). In this study, exposure to 66 mM ethanol for 12 and 24 h after 7 days of development of hippocampal neurons *in vitro* resulted in fewer neurons. Furthermore, survived neurons showed a fewer dendrites per cell and shorter individual dendrites. Indeed, an inhibitory effect of ethanol on the assembly and organization of the cytoskeleton components was described in hippocampal neurons as a result of prenatal exposure to ethanol (Romero *et al.*, 2010; Tomasini *et al.*, 2016). Growing evidence shows that ethanol increases intracellular free radical production in hippocampal cell cultures, which disrupts the dynamic balance between the accumulation of ROS and antioxidant enzyme activity. Under the present experimental conditions, oxidative stress could be the potential mechanism underlying the ethanol-induced neurotoxicity. When the oxidative balance is disrupted by ethanol, molecules throughout the cell, including proteins, lipids, etc., are affected, thus damaging the nervous system and inducing cognitive and memory impairments as a result of neurotoxicity and eventually leading to the occurrence of neurodegenerative lesions (Ramezani *et al.*, 2012).

The mitochondrion is the target organelle of oxidative stress and one of the main sources of free oxygen (Akbar *et al.*, 2016). When ethanol acts on neurons in the CNS, the mitochondrion swells and

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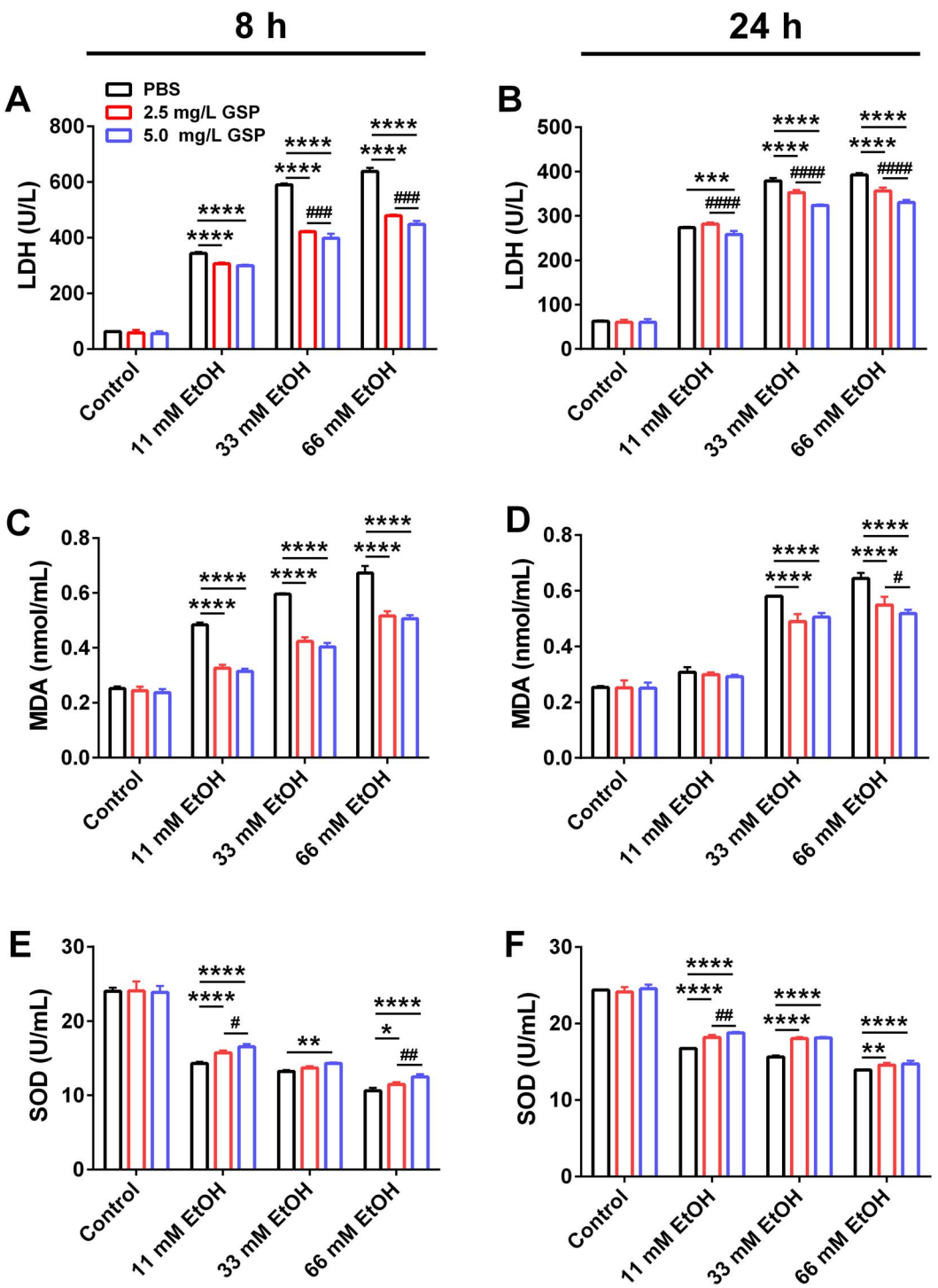

Fig. 4. GSP attenuated the effects of ethanol on the LDH, MDA and SOD in hippocampal neuron supernatants. The level of LDH after 8 h (**A**) and 24 h (**B**) of ethanol and GSP interaction. The level of MDA after 8 h (**C**) and 24 h (**D**) of ethanol and GSP interaction. The level of SOD after 8 h (**E**) and 24 h (**F**) of ethanol and GSP interaction. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ vs. different concentration of ethanol + PBS group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, $^{\#\#\#\#}P < 0.0001$ vs. different concentration of ethanol +2.5 mg/l GSP group.

becomes dysfunctional, resulting in the excess production of free radicals, which induce cytotoxicity and eventually cell death (Tapia-Rojas *et al.*, 2017). SOD is an enzyme that catalyzes the degradation of hydrogen peroxide and peroxide anions. Increased SOD activity is considered an adaptive response to oxidative stress. Acute alcoholism reduces the activity of copper- and zinc-SOD in the cytoplasm and mitochondria of hippocampal neurons (Scolaro *et al.*, 2012). Ethanol leads to lipid peroxidation of polyunsaturated fatty acids and phospholipids, resulting in the production of biologically active aldehydes such as 4-hydroxybenzenes and MDA. Therefore, the levels of MDA

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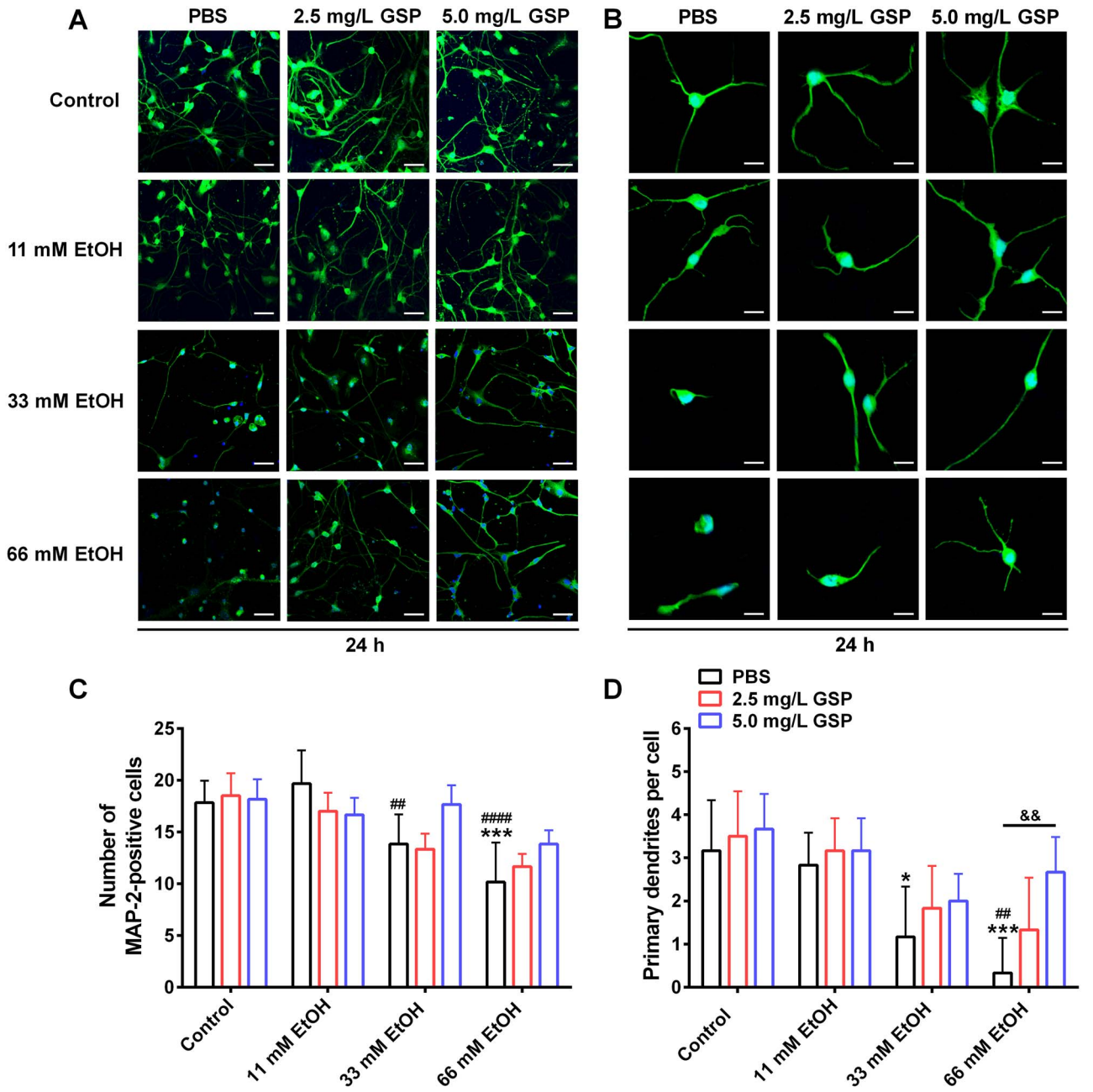

Fig. 5. Effects of GSP on ethanol-induced morphological changes of hippocampal neurons. (**A**) Representative images of hippocampal neurons after 24 h of ethanol and GSP interaction. Scale bar = 50 μm. (**B**) Neurons at high magnification. Scale bar = 10 μm. (**C**) Number of MAP-2 positive cells. (**D**) Number of primary dendrites per cell. $*P < 0.05$, $***P < 0.001$ vs. control + PBS group; $^{\#\#}P < 0.01$, $^{\#\#\#\#}P < 0.0001$ vs. control +2.5 mg/l GSP group; $^{\&\&}P < 0.01$ vs. 66 mM EtOH + PBS group.

and SOD in the supernatant of hippocampal neurons were evaluated in the present study. The results showed that ethanol increased the level of MDA but decreased the level of SOD, especially at 8 h after administration of ethanol, after which the level of SOD increased and the level of MDA decreased slightly. We hypothesized that ethanol induced oxidative stress in neurons, but after 8 h, this response reached saturation, and neurons somehow developed self-protective mechanisms against the neurotoxicity of ethanol. LDH is a glycolytic enzyme that catalyzes the dehydrogenation of lactic acid to pyruvate. As one of the most sensitive indicators of cell damage, LDH in the cerebrospinal fluid is significantly increased (Islam, 2017). In the present study, ethanol induced an increase in LDH, reaching a peak at 8 h after administration of ethanol.

Since oxidative stress is a key factor in alcoholic encephalopathy, the use of antioxidants and/or reagents to induce antioxidant systems is a promising way to alleviate oxidative damage. In the recent years, procyanidins have received increasing attention in many fields such as nutrition, medicine and health due to its excellent antioxidant capacity and a broad spectrum of safety (Gonzalez-Abuin *et al.*, 2015; Ottaviani *et al.*, 2018). The GSP, one of procyanidins, contains many phenolic hydroxyl groups. When these groups are oxidized, they release H^+ to prevent free radical chain reactions by

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competitively binding with reactive free radicals to reduce the consumption of antioxidants. Simultaneously, these groups have electron-delocalized and resonant structures that substantially increase the activity of antioxidant enzymes, such as SOD (Gong *et al*., 2016), which enhance the antioxidant capacity of cells, effectively prevent ethanol-induced neurotoxicity and repair nerve fibers to normalize neurotransmission (Dong, 2015). GSP may play the antioxidant role by affecting signaling pathways and protein functions. Studies have found that GSP display the protective effect against ethanol-mediated toxicity through the regulation of antioxidant enzymes and ethanol metabolism systems via enhancing the phosphorylation levels of ERK, JNK and p38 MAPK in ethanol-treated cells and rats (Bak *et al*., 2016). GSP effectively reduces lipid peroxide, glutathione and peroxidase levels to protect the brain from ethanol-triggered oxidative DNA damage in mouse brain cells (Guo *et al*., 2007). Consistent with the above studies, our results showed that GSP significantly enhanced the SOD activity and decreased the level of MDA or LDH at 8 and 24 h after administration of ethanol, suggesting that the neuroprotective effects of GSP against damage induced by ethanol might through decreasing lipid peroxide generation and stimulating antioxidant enzyme. Moreover, although GSP did not regenerate hippocampal neurons damaged by ethanol, it significantly increased the number of dendrites and total dendritic length per cell. Therefore, GSP represents a potential clinical nutritional therapy for ethanol-related neurological diseases.

In conclusion, GSP effectively improves ethanol-induced hippocampal neuronal toxicity by increasing SOD activity and decreasing the levels of MDA and LDH. Moreover, GSP repairs neuronal morphology destroyed by ethanol by increasing the number of dendrites and total dendritic length per cell. Given its superior neuroprotective effects, GSP may be used as a therapeutic tool for the treatment of alcohol use disorders.

SUPPLEMENTARY MATERIAL

Supplementary material is available at *Alcohol and Alcoholism* online.

ACKNOWLEDGEMENTS

We would like to thank Weiqi Zhang (Department of Forensic Medicine of Zhengzhou University) for technical support for cultured hippocampal neurons. We are very grateful to Dr Fangyuan Yin from Xi'an Jiaotong University for linguistic assistance.

FUNDING

This work was supported by a grant from the Science and Technology Research Project of Henan province (No. 162102310144).

CONFLICT OF INTEREST STATEMENT

The authors declare that they do not have any conflict of interest.

AUTHOR CONTRIBUTION

W.J., M.S. and B.Y. performed the experiments. W.J. and R.W. assisted with data analysis and interpretation of findings. H.Y. and X.Q. were responsible for the study concept and design. W.J. wrote the first version of the manuscript. X.Q. contributed significantly to the final version of the manuscript.

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