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Oxidative stress mediates hippocampal neuronal apoptosis through ROS/JNK/P53 pathway in rats with PTSD triggered by high-voltage electrical burn

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Background: *The pathogenesis of post-traumatic stress disorder (PTSD) triggered by high-voltage electrical burn (HVEB) remains unclear and the oxidative stress plays a role in this process. The purpose of this study is to investigate the underlying mechanism of oxidative stress mediates hippocampal neuronal apoptosis in rats with PTSD triggered by HVEB.*

Materials and methods: *The PTSD rat model was developed by stimulating with high voltage electricity and screened using behavioural performance including Morris water maze (MWM), elevated plus-maze (EPM) and open-field test (OFT). The reactive oxygen species (ROS) generation was measured by DHE fluorescence staining or flow cytometry. Western blotting assay was used to detect the proteins of p-JNK, JNK, P53, PUMA, Bcl-2 and Bax in hippocampal tissue or HT22 cells treated with electrical stimulation.*

Results: *The serum MDA and 8-OHdG levels were increased ($p < 0.001$), while the activities of SOD and CAT were decreased ($p < 0.001$) significantly in patients with HVEB. Behavioural test results showed that high-voltage electric stimulation induced the PTSD-like symptoms and the ROS-JNK-P53 pathway was involved in the neuronal apoptosis in rats with PTSD induced by HVEB. In vitro experiments further confirmed the electrical stimulation induced neuronal apoptosis through ROS/JNK/P53 signalling pathway and the antioxidant NAC could rescued the ROS generation, activation of JNK/P53 proteins and improved the cell apoptosis rate in HT22 cells. Finally, the JNK inhibitor SP600125 could significantly inhibited the percentage of HT22 cell apoptosis induced by electrical stimulation ($p < 0.001$).*

Conclusions: *These results indicated that oxidative stress mediates hippocampal neuronal apoptosis through ROS/JNK/P53 pathway in rats with PTSD triggered by HVEB.* (Folia Morphol 2024; 83, 2: 300–313)

Keywords: high voltage electric burn, P53 protein, oxidative stress, brain injury, PTSD

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INTRODUCTION

High-voltage electrical burn (HVEB) is a rare yet destructive class of injury that has been considered as a serious public health issue worldwide [6, 17, 28, 41]. The high-voltage alternating current causes a series of damages such as muscle tonic contraction and intracellular ion disturbances through thermal and chemical effects, resulting in progressive damage to the body [9, 18, 36]. Post-traumatic stress disorder (PTSD), a mental health disorder, is triggered by a frightening and/or traumatic event — whether experienced or witnessed. The clinical symptoms of PTSD may include recurrence of traumatic events, avoidance of traumatic cues, continuous hypervigilance, selective forgetting, emotional numbness, nightmares and severe anxiety [1, 25, 26]. It is a state of post-traumatic psychological imbalance, which can easily cause intense psychological pain and severe damage to social functions of patients, and is the main manifestation of stress disorder [32, 37]. As the HVEB is a terrifying life event, researches have shown that 41% of electrical burn patients have the post-traumatic stress response, and 7.6% of them have been diagnosed as PTSD [30, 48]. To our knowledge, there is no accurate statistical data on the incidence of electrical burn patients complicated by mental disorders in China and even in the world. At the present stage, researches on the pathogenesis of PTSD triggered by high-voltage electric burn are still in its infancy, and the exact molecular mechanism remains unclear.

Oxidative stress is a status of imbalance between cellular generation of reactive oxygen species (ROS) and the antioxidant defence systems, causing to cellular injury. Researches have suggested the link between oxidative stress and high-voltage electric burn injury in patients or animal models [9, 10, 27, 31]. Severe life stress events, such as HVEB, could stimulate the increase of sympathetic nerve-adrenergic medulla responsiveness, leading to the dysfunction of the hypothalamic-pituitary-adrenal axis (HPA axis) of the nervous system and contribute to the pathogenesis of PTSD [33, 39, 43]. The main reason for oxidative stress triggered by high-voltage electric burns is that the electrothermal effects can directly cause the cell damage of multiple organs. The ROS generation, 8-oxo-deoxyguanosine (8-OHdG) and the levels of end product of lipid peroxides (malondialdehyde, MDA) are most commonly used markers for oxidative stress studies. Meanwhile, the activities of SOD and CAT represent the main antioxidant enzymes in the body. The p53 protein is a key mediator of the DNA damage response and tumour suppression and plays an important role in the cellular response to oxidative stress [16, 38, 42]. Studies have found that the oxidative stress can activate P53 protein through ROS-JNK pathway [50]. P53 is involved in apoptosis and is also related to cell death pathways such as programmed cell necrosis and autophagy [7, 15]. When the body is in a continuous and high-level oxidative stress state, the P53 protein on the one hand triggers the overexpression of P53-inducible gene 3 (PIG3) and proline oxidase (proline oxidase) to reduce the mitochondrial membrane Potential, aggravate oxidative stress damage, leading to DNA damage and apoptosis [49]. On the other hand, it promotes apoptosis by inducing pro-apoptotic proteins (PUMA and Bax) [8]. Imaging studies suggest that the continuous reduction of hippocampal volume in PTSD patients might be related to the activation of P53 protein by high levels of oxidative stress to trigger neuronal apoptosis [11, 23]. However, the role of oxidative stress and its underlying mechanism in the pathogenesis of PTSD triggered by High-voltage electrical burn need to be further investigated.

In this study, the PTSD rat model triggered by HVEB and the HT22 cell model stimulating with electrical current were used to detect the oxidative stress biomarkers and the proteins related to the ROS/JNK/ /P53 pathway and the neuronal apoptosis. The research will provide an experimental basis for further elucidating the mechanism of high-voltage electrical burn-induced PTSD and screening the potential intervention targets in clinic.

MATERIALS AND METHODS

Participants and serum samples

This study included 30 patients with HVEB and 30 subjects of healthy control. The patients with HVEB were recruited from the department of burns and plastic surgery, the First Hospital of Hebei Medical University. The informed consent was obtained from all participants in accordance with the protocol approved by the Research Ethics Committee of the First Hospital of Hebei Medical University.

The demographic characteristics of the participants in the study were shown in Table 1. A fasting blood sample (3 mL) was collected from each subject, and placed in a test tube (BD Biosciences, NJ, USA), and placed vertically for 30 min at room temperature.

Table 1. Demographic characteristics of the samples

Demographics	Control (n = 30)	HVEB (n = 30)	p-value
Gender			
Male	27 (90 %)	25 (83.3 %)	
Female	3 (10 %)	5 (16.7 %)	0.706
Age (years)	35.90 ± 11.96	36.90 ± 9.42	0.72
BMI	23.39 ± 2.50	23.35 ± 2.34	0.948
TBSA %		11.50 (6.75, 20.00)	
MDA [ng/mL]	1206.58 ± 501.94	2362.02 ± 900.73*	< 0.001
SOD [U/mL]	261.49 ± 57.36	151.01 ± 53.97*	< 0.001
8-OHdG [ng/mL]	35.95 ± 11.02	87.30 ± 22.02*	< 0.001
CAT [U/mL]	475.34 ± 132.16	216.45 ± 82.95*	< 0.001

HVEB — high-voltage electrical burn; BMI — body mass index; TBSA — total bod surface area; MDA — malondialdehyde; SOD — superoxide dismutase; 8-OHdG — 8-oxo--deoxyguanosine; CAT — catalase. ***p < 0.001 vs. the control group. Data are shown as mean ± standard deviation.

Table 2. Neuropsychological symptoms of patients with the high-voltage electrical burns during the acute phase

Neuropsychological	Incidence % (n = 30)
Anxiety	58%
Sleep difficulties	60%
Depressed mood	49%
Memory/concentration difficulties	62%
Avoidance	29%
Hypervigilance	15%
Hyperarousal nightmares	31%
Social withdrawal	18%
Suicidal ideations	5%
Dizziness	12%
Headaches	17%
Phantom limb pain	11%
Flashbacks	20%
Avoidance hypervigilance	25%
Flashbacks	21%

Blood samples were centrifuged at 3000 g for 10 minutes at room temperature and then at 16,000 g for 10 minutes at 4°C. The serum was aliquoted and stored at –80°C until use, avoiding repeated freeze/ /thaw cycles. The neuropsychological symptoms of patients with high-voltage burn were evaluated during the acute phase in Table 2.

Animals

Fifty healthy male SD rats, weighing 200–250 g, were purchased from the Experimental Animal Centre of Hebei Medical University. The experimental animals were kept in a clean-grade animal room with a temperature of (24 ± 3)°C, a relative humidity of (40 ± ± 5)%, and natural light. The experimental protocol was reviewed and approved by the Experimental Animal Ethics Committee of the First Hospital of Hebei Medical University. The SD rats were randomly divided into control group (without electric stimulation), Single-Prolonged Stress (SPS) group and PTSD with high voltage electric burn (HVEB) group. Ten rats in the control group were selected as the sham shock, ten rats in the SPS group were selected as positive control group, and the others were given high-voltage electrical stimulation as the HVEB group to establish a high-voltage electrical PTSD model. After the high-voltage electrical stimulation, the rats in the three groups were subjected to a 5-day behavioural test using the Morris water maze automatic recorder. Behavioural tests (water maze test, open field test and elevated plus-maze test) were performed after two weeks of high-voltage electric stimulation.

Preparation of high-voltage electric burn triggered PTSD animal model

The laboratory humidity was at 35~45% RH and the temperature was at 24~26°C. The rats were supine on the insulating table after they were anesthetized with 10 mL/kg of 3% pentobarbital via intraperitoneal (ip) injection. Their limbs were fixed, and send it between the high-voltage output circuits. Fix the current inlet electrode plate on the rat's head and the outlet electrode plate in the hair removal area of the left lower extremity, after confirming that the high-voltage line is connected correctly, turn on the 220 V power supply, turn on the voltage regulator, and turn the voltage regulator button. When the voltage rises to 2 kV, the switch is turned on and the current is actually passed (1.92 ± 0.24) A. Power on for 3 seconds, immediately disconnect the switch and release the electrode plate. Check the vital signs of the rat, and immediately perform cardiac compression resuscitation for respiratory and cardiac arrest. Immediately after the electric shock, the rats showed muscle spasms all over the body, and the skin of the current outlet and inlet was browned, and some of them became black and carbonized. The model was successfully replicated. The control group was subjected to a sham electric experiment. Before the experiment, the switch was turned off to ensure that no current passed through. The rest of the process

was replicated with the animal model of high-voltage electric burn. The rats received high-voltage electric stimulation were treated with low-dose meloxicam (1 mg/kg, subcutaneous injection, Shandong, China) as the analgetic once daily for 3 days. After two weeks of high-voltage electric stimulation, the behavioural tests of the three groups of rats were performed to screen the rats with PTSD, including Morris water maze test, open field test and elevated plus-maze apparatus.

PTSD model of Single-Prolonged Stress (SPS)

The protocol of SPS model used in this study was described previously [21]. Rats were restrained in clear plexiglass restraint tubes for 2 hours, and then immediately swam in clear acrylic cylinders (24 cm in diameter, 50 cm in height) in 24°C water for 20 minutes. Then rats were placed back to recovery for 15 minutes, and exposed to diethyl ether until they became unconsciousness, all under bright white light conditions.

Behavioural Test

Survival of rats after high-voltage electrical stimulation: Except for 10 rats in control group and 10 rats in SPS group as positive control, 11 rats of the remaining 30 rats in HVEB group had died, and the mortality rate was 36%. After two week of high--voltage electric stimulation, the behavioural tests for the three groups of rats were performed, including Morris water maze test, open field test and elevated plus-maze test. Each group selected 6 animals for the subsequential experiment.

Morris Water Maze (MWM) Test

The rats in the control group, SPS group and the HVEB group were randomly selected, and the experiment included a concealed platform test. In the concealed platform test, the rats were placed into the water from one of the four quadrants facing the pool wall. Each time the experimental rats swim for 60 seconds to find the hidden platform. Record the swimming path and the time to find the hidden platform in the pool, which is the escape latency (EL). If the rat cannot find the platform within 60 seconds, guide it to the stage and stand for 10 seconds. Analysis software was used to analyse percentage of time spent in correct quadrant of MWM and latency to finding a hidden platform of rats. Test the spatial learning and memory abilities of rats.

Open Field (OF) Test

Randomly selected the rats in the control group, SPS group and HVEB group, and recorded the distance and time of central movement of each rat in the central area of the open field for 20 min. This experiment is used to assess the animal's alertness level, anxiety state and ability to adapt to the environment.

Elevated Plus-maze (EPM) Test

An elevated plus labyrinth device, located in a dark test room, was used to measure anxiety. The device had a maze of two open arms (50 cm × 10 cm) and two closed arms (50 cm × 10 cm × 40 cm) connected by a central square (10 cm × 10 cm) and elevated 50 cm above the floor centimetre. The maze was made of opaque black plexiglass, and a camera was installed above the maze to transmit video to a computer monitor. At the beginning of the experimental study, the rats were placed in the central area facing the open arms, and the number of open/closed arm entries (OA and CA, respectively) and open/closed arm time (OT/CT) were recorded for each rat.

Cell culture

HT-22 cells were obtained from Life Technologies (Waltham, MA, USA) and cultured in complete Dulbecco's Modification of Eagle's Medium (DMEM, Gibco, Invitrogen GmbH, Karlsruhe, Germany) supplemented with 10% fetal bovine serum, 1% penicillin and streptomycin at 37°C, 5% CO_2, and 95% saturated humidity. When the cells reach the logarithmic growth phase, they are subcultured in proportion.

Establishment of electrical stimulation cell model

The HT22 cells were seeded onto 24-wellplates (the number of cells per well is 5×10^4) and divided into control group and electrical stimulation group. The control group was added with normal culture medium, and the electrical stimulation group was given different voltage stimulation conditions (0, 25, 50, 75, and 100 V) for 5 s with the 2 × 5 mm carbon graphite electrodes. Cell Counting Kit-8 (CCK-8, Dojindo Laboratories, Japan) measured the cell viability as instructed. Choose the optimal electrical voltage to prepare the electrical stimulation cell model.

Treatment of HT22 cells with N-acetylcysteine

N-acetyl-L-cysteine (NAC) is a commonly used antioxidant. The HT22 cells were divided into control group and experimental group. Normal culture me-

dium (without NAC) was added to the cells of control group, and the different concentrations of NAC (0.25, 0.5, 1, 2, and 4 mmol/L) were added to the cells of experimental group, and the cells were incubated for 24 h. Then the cell activity was determined by CCK-8 assay.

Cell grouping

The HT22 cells were seeded in a 96-well cell culture plate (the number of cells was 1×10^5 per well). The HT22 cells were divided into four groups, including control group, ES group (50 V), NAC treatment group (1 mmol/L) and ES+NAC group. Each group had at least 3 replicate wells.

Detection of oxidative stress biomarkers

The levels of oxidative stress biomarkers, including Superoxide dismutase (SOD), Malondialdehyde (MDA), 8-oxo-deoxyguanosine (8-OHdG) and catalase (CAT) in serum of patients or animals were detected using an enzyme-linked immunosorbent assay (ELISA) assay kits and Colorimetric Assay Kits (Elabscience Wuhan, China).

Dihydroethidium (DHE) staining

The hippocampal tissue sections were stained with Dihydroethidium (DHE, ThermoFisher, Waltham, MA, USA) to detect the ROS generation in the hippocampus from rats.

Determination of the apoptotic rate of HT22 cells

Annexin V-FITC and propidium iodide (PI) (Biyuntian, Shanghai, China) fluorescence were used to analyse the percentage of apoptosis. HT22 cells were seeded in a six-well plate at a ratio of 2×10^5 cells/ /well, and processed as described above. The cells were washed twice with cold phosphate buffered saline (PBS), centrifuged at 4°C for 5 minutes, discarded the supernatant, added 100 μL 1× Binding Buffer, and blown to a single cell suspension. Next, 5 μL Annexin V-FITC and 5 μL PI Staining Solution were added to each tube, incubated at 25°C for 10 min in the dark, following which 400 μL 1× Binding Buffer was added, mixed and the sample was stained for 1 h. Finally the cells were detected using flow cytometry (Kraemer Blvd, Brea, CA, USA).

Determination of ROS generation

The levels of intracellular ROS were detected by Oxidation-sensitive fluorogenic probe, 2′,7′-Dichlorofluorescin Diacetate (DCFH-DA) (Biyuntian Biotechnology Company, Shanxi, China). The processed cells were collected by conventional centrifugation and the supernatant was discarded, PBS was added to resuspend the cells. Then, the cells were washed 3 times, mixed with DCFH-DA (10 μmol/L) solution, incubated at 37°C for 20 min, inverted and mixed every 3–5 min, and centrifuged. Next, the supernatant was removed and the cells were resuspend in PBS, which was repeated 3 times. The non-specific esterase of DCFH-DA cells removes the acetyl group, and the ROS is further oxidized to produce the fluorescent compound 2,7-dichlorofluorescein (DCF). The fluorescence signal intensity of DCF was measured by the flow cytometer, and the fluorescence intensity was measured at the excitation wavelength of 488 nm and the emission wavelength of 525 nm. The pure DCFH-DA solution was used for zero adjustment, and the value of the blank treatment group was 100%. The above experimental steps were repeated 3 times.

Western blot analysis

The hippocampal tissue specimen of the experimental animal was put it in the cold lysis buffer (Bebo, Shanghai, China), homogenized at low temperature and centrifuged, following which the supernatant was taken. The expression of p-JNK, JNK, P53, PUMA, Bcl-2 and Bax proteins in the hippocampal tissue of rats was detected by western blot assay 40 days after high-voltage stimulation. The collected HT22 cells were inoculated in a 6-well plate, cultured for 24 h until the cells grew to 85% confluence, and then the cells were divided into control group, electrical stimulation (ES) group, NAC group, ES + NAC group. After 24 h of treatment, the cells were lysed and centrifuged. Then the supernatant was taken for subsequent experiments. Both the animal hippocampal tissue and HT22 cells were used to determine the protein content by the BCA (Solebold, Shanghai, China) method. After protein denaturation, 12% polyacrylamide gel (SDS-PAGE) (Bio-rad, Shanghai, China) was used for electrophoresis to separate the protein in the gel to the nitrocellulose PVDF membrane. The bovine protein BSA was used to block the membrane at room temperature for 1 h, and the corresponding primary antibodies were added — JNK (9252; Cell Signaling Technology, Beverly, MA, USA), p-JNK (Thr183/Tyr185) (4668; Cell Signaling Technology, Danvers, MA, USA), P53 (ab202026, Abram, Cambridge, UK), PUMA (ab9643, Abram, Cambridge,

UK), Bax (ab3250, Abram, Cambridge, UK) and Bcl-2 (ab59348, Abram, Cambridge, UK) specific antibodies (1:500) and β-actin (10004156, Proteintech, Wuhan, China) (1:1000) — and incubated overnight at 4°C. The membrane was washed with TBST 3 times, and horseradish peroxidase HRP-labelled goat anti-rabbit secondary antibody (A23920, Abbkine, Beijing, China) (1:1000) was added. After 1-hour incubation at room temperature, the membrane was washed 3 times with TBST, and the colour was developed by ECL luminescence. The results were observed after quantification. β-actin was used as an internal reference, and finally the target band was analysed by scanning density, and the ratio of the grey value of each target protein/β-actin grey value was used to reflect the relative protein expression level of each group. This was repeated 3 times to get the average value. Quantity One gel analysis system analyses the protein content of protein bands.

Statistical analysis

All data were presented as mean ± SD, and statistical analysis were performed using SPSS software version 16.0 (SPSS, Inc., Chicago, IL, USA). The two-tailed Student's t-test was used for two-group comparisons, the ANOVA analysis was followed Dunnett's or Tukey's test for three parameter groups. A p value of < 0.05 was considered to be statistically significant. All experiments were independently repeated at least three times.

Ethics approval and consent to participate

This study was conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals of Hebei Medical University, and the experimental protocols used in this research were approved the First Hospital of Hebei Medical University.

RESULTS

The oxidative stress is involved in the process of high-voltage electric burn

To understand whether the oxidative stress takes a part in the process of high-voltage electric burn, the oxidative stress biomarkers in serum including the content of malondialdehyde (MDA), the activities of superoxide dismutase (SOD), 8-oxo-deoxyguanosine (8-OHdG) level and catalase (CAT) activity were detected by Enzyme-linked immunoassay (ELISA) assay and Colorimetric Assay Kits. The results showed that the serum MDA content (Fig. 1A) and the 8-OHdG levels

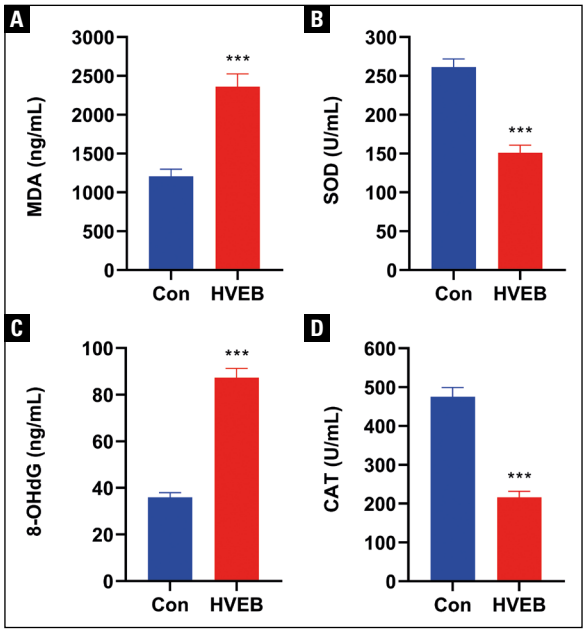

Figure 1. Alterations of oxidative stress biomarkers in serum of patients with the high-voltage electrical burns. The levels of MDA (**A**), SOD activity (**B**), 8-OHdG (**C**) and CAT activity (**D**) were detected by ELISA assay and Colorimetric Assay Kit. ***$p < 0.001$ vs. the control group ($n = 30$).

(Fig. 1C) were significantly increased ($p < 0.001$), while the SOD (Fig. 1B) and CAT (Fig. 1D) activity were significantly decreased ($p < 0.001$) in HVEB patient group compared with the control group. These results indicated that the oxidative stress injury is involved in the process of high-voltage electric burn in patients. The patients with high-voltage burn usually have different neuropsychological symptoms during acute phase, including anxiety (58%), sleep difficulties (60%), depression (49%), and memory/attention difficulties (62%) and so on.

Changes of behavioural tests in rats with high-voltage electric burn

To develop a PTSD animal model triggered by high-voltage electric burn, the rats were treated with high-voltage electric stimulation and the behavioural tests were performed to screen the rats with PTSD, including Morris water maze test, open field test and elevated plus-maze apparatus (Fig. 2A). The single-prolonged stress (SPS) model was used as the positive control of the PTSD. The representative images of swimming trajectory to find the platform for rats of each group in MWM test were shown in (Fig. 2B). The results of MWM test showed that the time spent in the target quadrant was decreased, the distance

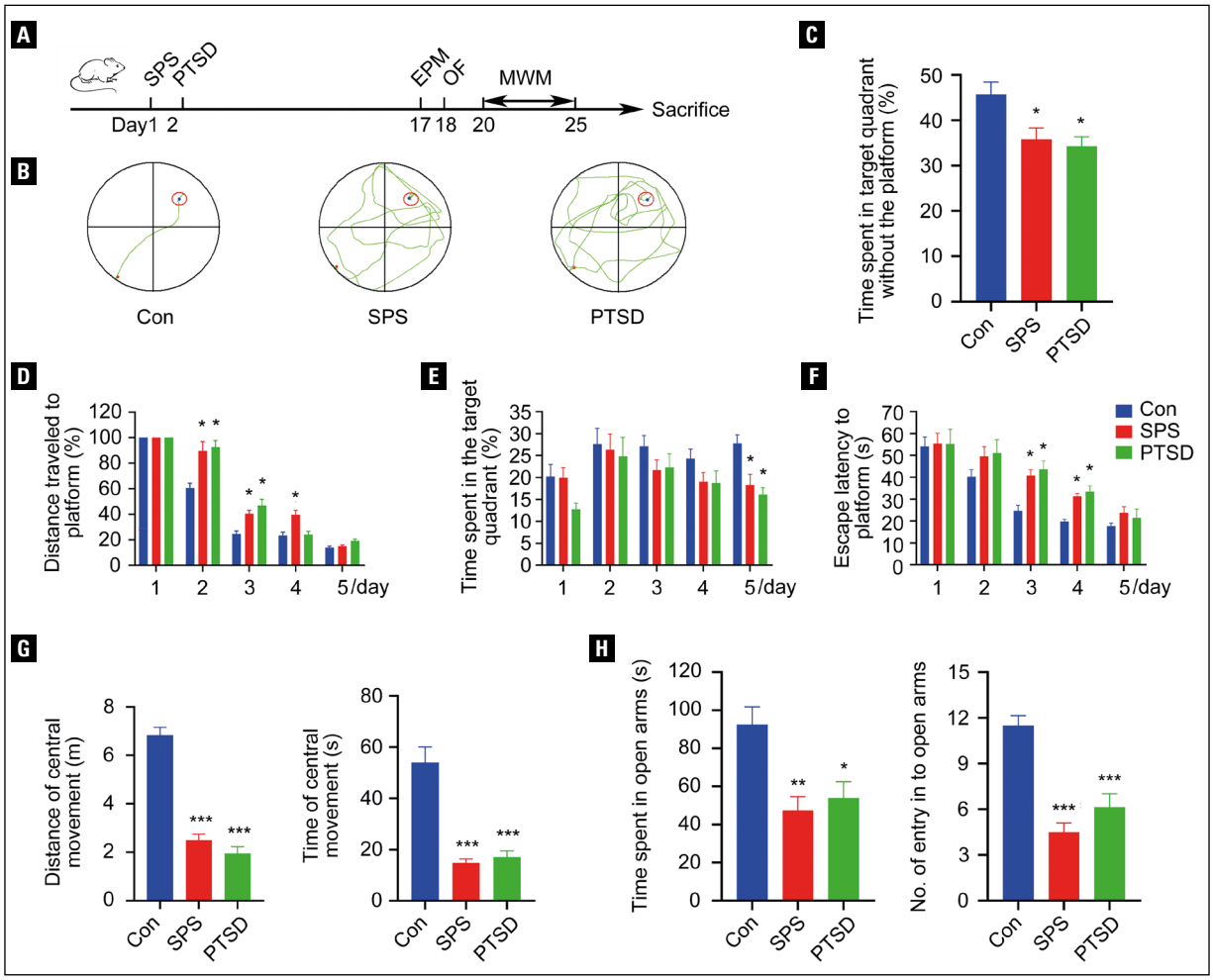

Figure 2. Changes of behavioural performance tests in PTSD rat model induced by high-voltage electric burn. **A.** Timeline of experimental procedures; **B.** Representative images of swimming trajectory to find the platform in Morris water maze (MWM) test for rats in the indicated groups; **C.** Time spent in target quadrant without the platform in MWM test; **D.** Distance travelled to platform in MWM test; **E.** Time spent in the target quadrant in MWM test; **F.** Escape latency to finding a hidden platform during five days of navigation training in MWM test; **G.** Distance and time of central movement in the open-field test (OFT); **H.** Time spent in open arms and number of entry in open arms in elevated plus-maze (EPM) test. Data were presented as mean ± SD (n = 6). *p < 0.05, **p < 0.01, ***p < 0.001 vs. the control group.

travelled to platform and the escape latency were increased in rats of SPS and PTSD groups compared with that of control group ($p < 0.05$) (Fig. 2C–F). In the open field test, the distance in central movement, as well as the time in central region of rats in SPS and PTSD groups were reduced significantly compared with that of control group ($p < 0.001$) (Fig. 2G). The results of time spent in open arms and the number of entry in open arms in elevated plus-maze (EPM) test also showed the similar trend for rats in SPS and PTSD groups (Fig. 2H). These results suggested that high-voltage electric stimulation could induced the PTSD-like symptoms, such as the reduced ability of spatial learning and memory, alertness and anxiety, conditioned fear and sensitized fear and decreased environmental adaptability.

ROS-JNK-P53 pathway is involved in the neuronal apoptosis in rats with PTSD induced by HVEB

To investigate the mechanisms of oxidative stress in the pathogenesis of PTSD triggered by High-voltage electrical burn, we tested the oxidative stress biomarkers, apoptosis related proteins and its upstream signalling molecular in the hippocampus of rat model with PTSD induced by HVEB. The results of ELISA showed that the MDA contents were increased ($p < 0.05$) (Fig. 3A) and the SOD activity was decreased ($p < 0.001$) (Fig. 3B) in the serum of rats with PTSD compared with the control group. DHE staining results showed that the ROS generation in the hippocampal CA3 area of rats with PTSD was increased compared with the control group (Fig. 3C). The expression levels of p-JNK, P53, PUMA and Bax proteins

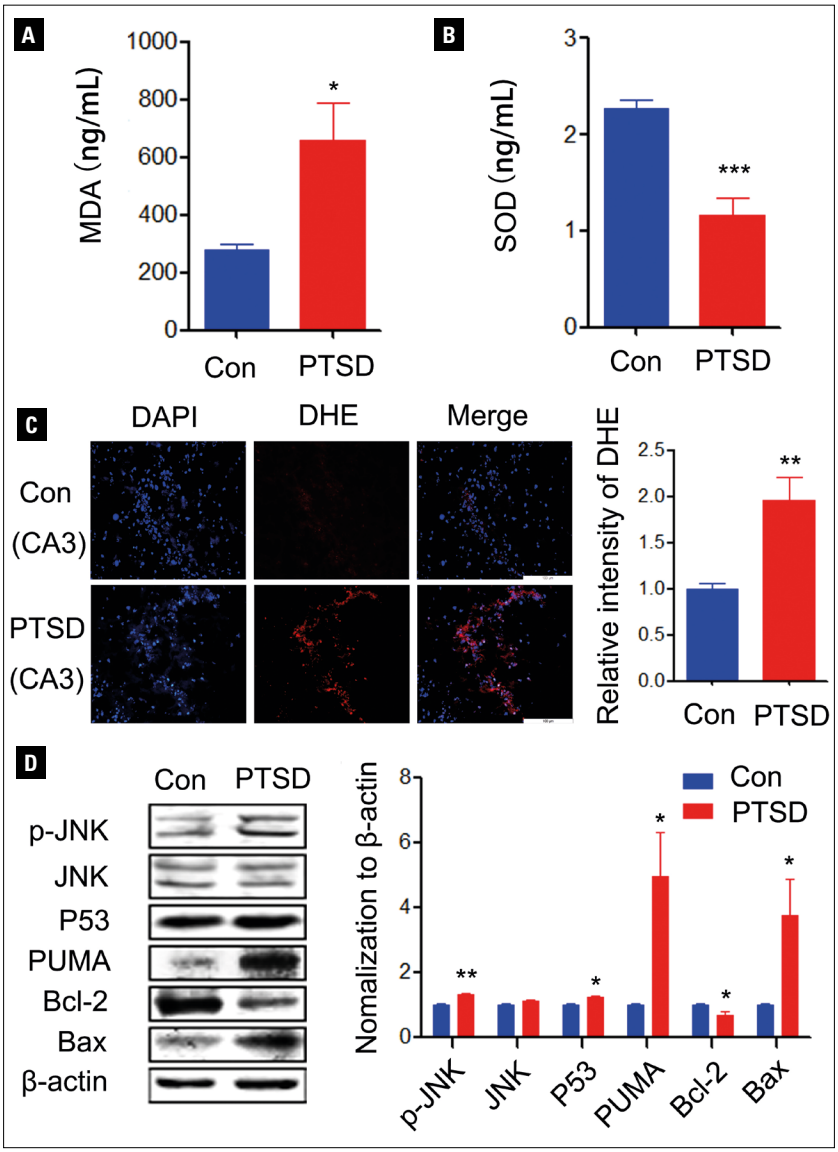

Figure 3. Oxidative stress mediated hippocampal neuronal apoptosis through ROS-JNK-P53 pathway in rats with PTSD induced by high-voltage electrical burns. (A-C) The serum levels of MDA content (**A**), and SOD activity (**B**) were detected by ELISA assay, and DHE fluorescence staining (**C**) was applied to detect ROS generation in the hippocampal CA3 region of rats in PTSD or control group. Scale bars = 100 μm. (**D**) The expression of p-JNK, JNK, P53, PUMA, Bcl-2 and Bax protein were detected by western blot analysis in the hippocampus of PTSD or control rats. The β-actin was used as a loading control. *$p < 0.05$, **$p < 0.01$. ***$p < 0.001$ vs the control group ($n = 6$).

were higher, but the Bcl-2 protein was lower in the hippocampus of rats with PTSD than that of control group ($p < 0.05$) (Fig. 3D). These results suggested that oxidative stress mediated hippocampal neuronal apoptosis may through ROS-JNK-P53 pathway in rats with PTSD induced by high-voltage electrical burns.

Oxidative stress participated in the neuronal apoptosis induced by electrical stimulation

To verify if the oxidative stress participated in the neuronal apoptosis induced by electrical stimulation, we developed an *in vitro* electrical stimulation (ES) cell model using HT-22 cell lines. The HT22 cells were treated with different voltage of electrical stimulation (ES) (0 to 100 V) for 5 s and continuously cultured for 24 h, CCK-8 regent was used to determine the cell viability for screening the stimulation conditions preliminarily. The results showed that after 24 hours of treatment of HT22 cells with different voltage of electrical stimulation for 5s, the cell viability was gradually decline and caused significant decrease at 75 V and 100 V compared to control group, and the cell survival rate was significantly reduced in a voltage-dependent

manner ($p < 0.001$) (Fig. 4A). So we selected the 50 V voltage as the best stimulation condition to prepare the ES cell model. In order to explore whether the antioxidant NAC has a protective effect on the injury induced by electrical stimulation in HT22 cells [33], different concentrations of NAC were used to incubate the HT22 cells for 24 h, and the cell survival rate was detected by CCK-8 assay. The results showed that the cell viability was decreased significantly at the concentration of 2 mmol/L NAC ($p < 0.001$) (Fig. 4B). Finally, we selected the concentration of 1 mmol/L NAC for the subsequent experiments. Next, the HT22 cells were stimulated with 50 V voltage electrical current for 5 s and continuously incubated with or without 1 mmol/L NAC for 24 h. We found that the viability of HT22 cells were decreased ($p < 0.001$) (Fig. 4C–F), but the ROS generation ($p < 0.001$) (Fig. 4D–G) and the cell apoptosis rate ($p < 0.001$) (Fig. 4E, H) were increased significantly by the electrical stimulation, and the antioxidant NAC could rescue the ROS generation and improved the cell apoptosis rate in HT22 cells. These results suggested that oxidative stress participated in the neuronal apoptosis induced by electrical stimulation *in vitro*.

The electrical stimulation induced neuronal apoptosis through ROS/JNK/P53 signalling pathway

The results of *in vivo* experiment have indicated that oxidative stress mediated hippocampal neuronal apoptosis may through ROS-JNK-P53 pathway in rats with PTSD induced by high-voltage electrical burns. To validate this conclusion, we used western blot assay to detect the expression of the JNK/P53 signalling molecular and the apoptosis related proteins in electrical stimulation-induced HT22 cells. The results showed that the expression of p-JNK, P53, PUMA, and Bax proteins were increased significantly ($p < 0.01$) in HT22 cells treated with electrical stimulation compared with the control group, and the antioxidant NAC could block these changes in the JNK/P53 signalling proteins and cell apoptosis related proteins (Fig. 5A, B). The above results indicate that JNK and P53 are involved in the apoptosis induced by electrical stimulation, and NAC reversed the activation of JNK and P53, and inhibited the up-regulation of PUMA, Bax and the down-regulation of Bcl-2. In addition, the JNK inhibitor SP600125 could significantly inhibited the percentage of HT22 cell apoptosis induced by electrical stimulation ($p < 0.001$) (Fig. 5C, D). These results suggested that the ROS-JNK-P53 pathway plays a crucial role in the neuronal apoptosis induced by electrical stimulation.

DISCUSSION

Post-traumatic stress disorder (PTSD) caused by high-voltage electrical burn has a high disability and fatality rate in clinic [5, 12], its pathogenesis is still unknown, and there has been a lack of effective clinical treatment measures. Oxidative stress induced by burns causes cell apoptosis is a research hotspot in the field of burn medicine. Current studies have found that PTSD caused by high-voltage electric burn is closely related to oxidative stress [4]. This study reported that oxidative stress mediates hippocampal neuronal apoptosis through ROS/JNK/P53 pathway in PTSD rats induced by High-voltage electrical burn, which will provide an experimental basis for the prevention and treatment of PTSD induced by high--voltage electrical burn.

Oxidative stress triggered by burns increases the production of oxygen free radicals in the body and/ /or decreases the body's antioxidant capacity, which causes lipid peroxidation, protein oxidation, DNA damage and so on, and result in multiple organ dysfunction or even failure in burn patients, eventually leads to the death of burn patients [3, 13, 34]. But how the oxidative stress participate the pathogenesis of PTSD caused by high-voltage electric burn and it underlying mechanisms is still not unclear. In this study, we confirmed that the oxidative stress injury is involved in the disease process of high-voltage electric burn patients. At the same time, we developed a PTSD rat model triggered high-voltage electric burn. The results of water maze, open field tests and elevated plus-maze apparatus tests showed that the high-voltage electric burn PTSD model rats exhibited PTSD--like symptoms, such as the reduced ability of spatial learning and memory, increased alertness and anxiety, and decreased environmental adaptability. We found that the levels of MDA contents in the PTSD model rats were increased significantly after high-voltage electric burns, and the SOD activities was decreased significantly. The related proteins in the ROS/JNK/ /P53 pathway in the hippocampus were confirmed that oxidative stress induced by high-voltage electric burn mediated the upregulation of p-JNK, P53, PUMA proteins and the downregulation of Bcl-2 protein. Previous studies have found that PUMA is a master

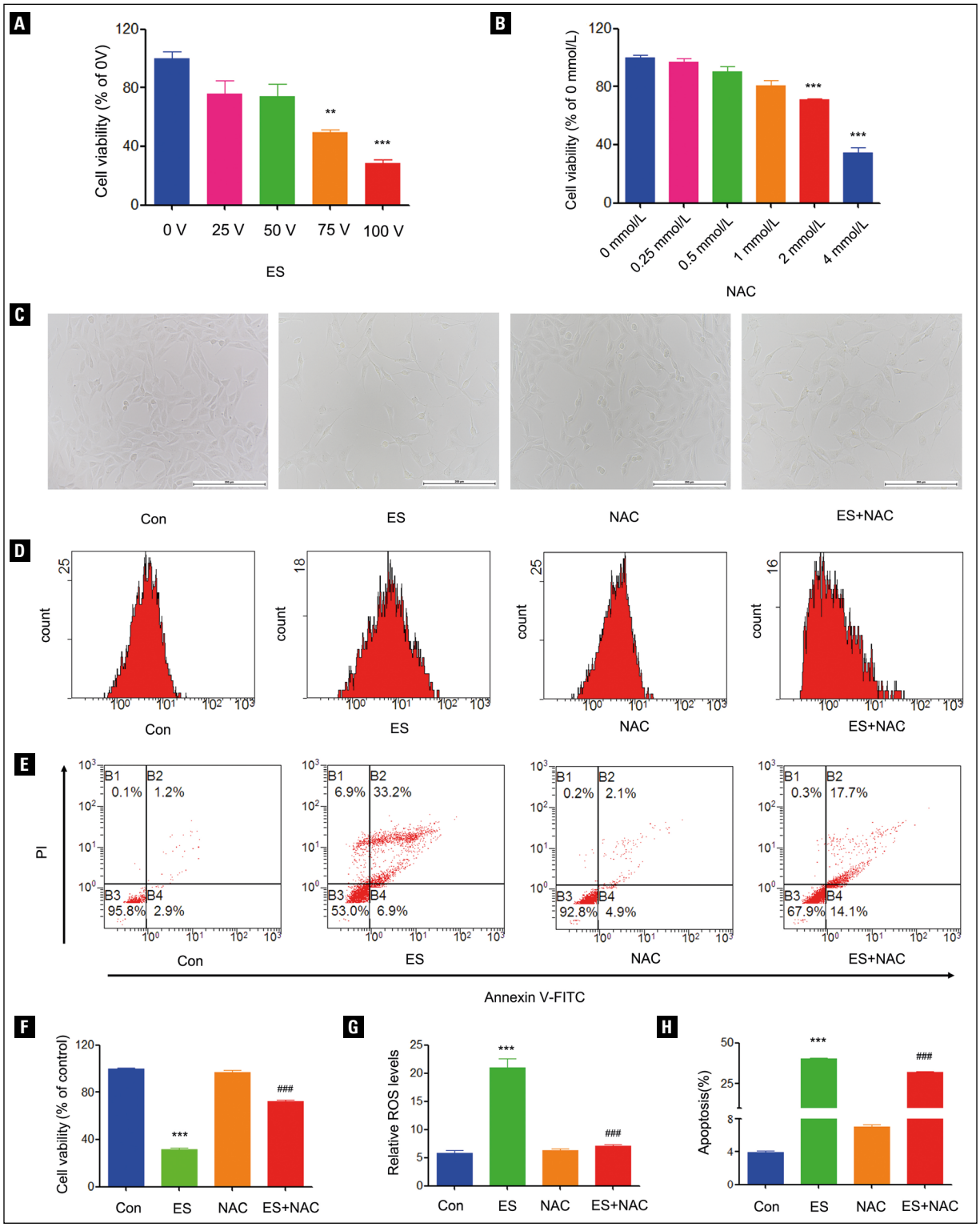

Figure 4. Oxidative stress participated in the neuronal apoptosis induced by electrical stimulation in HT22 cells. **A, B.** HT22 cells were treated with different voltage (0, 25, 50, 75 and 100 V) of electrical stimulation (ES) for 5 s and continuously cultured for 24 h (A) or incubated with different concentrations (0, 0.25, 0.5, 1, 2 and 4 mmol/L) of N-Acetyl-L-cysteine (NAC) for 24 h (B), and then the cell viability was assessed using a CCK-8 assay. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. the control group; **C, F.** The effects of electrical stimulation (50 V for 5 s) on the morphology (C) and the cell viability (F) of HT22 cells in the absence or presence of NAC (1 mmol/L). Scale bar = 200 μm; **D, G.** The protective effects of NAC against electrical stimulation-induced ROS generation were measured by flow cytometry. HT22 cells were treated with electrical stimulation in the absence and presence of NAC, and then were loaded with DCFH-DA to detect the generation of intracellular ROS; **E, H.** The protective effects of NAC against electrical stimulation-induced cell apoptosis were measured by flow cytometry. Apoptotic HT22 cells were identified using Annexin V-FITC/PI dual staining. Data were shown as the mean ± SD (n = 3). $^{***}p < 0.001$ vs. the control group; $^{###}p < 0.001$ vs. the ES group.

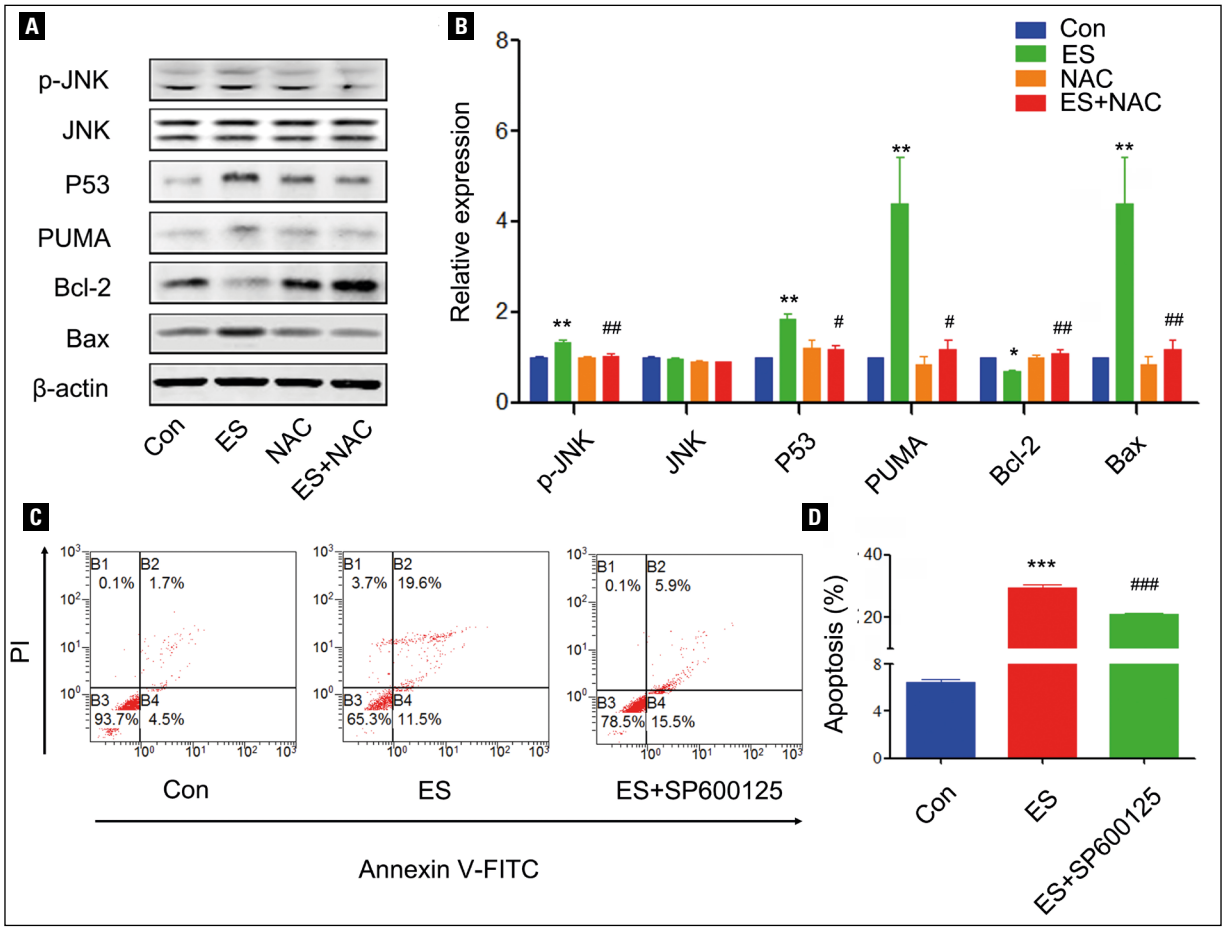

Figure 5. The JNK/P53 signalling pathway was involved in the electrical stimulation-induced apoptosis in HT22 cells. **A, B.** HT22 cells were treated with electrical stimulation (50 V for 5 s) and co-treated with NAC (1 mmol/L) for 24 h. Western blot assay was adopted for p-JNK, JNK, P53, PUMA, Bcl-2 and Bax protein examination; **C, D.** The effects of SP600125 (JNK inhibitor) on the electrical stimulation-induced cell apoptosis were measured by flow cytometry. HT22 cells were cultured in DMEM containing 10% FBS, and were pretreated with 10 μL SP600125 (JNK inhibitor) for 30 min. Then, cells were subjected to electrical stimulation for 5 s and continuously cultured for 24 h. Apoptotic cells were identified by FACS using Annexin V-FITC/PI dual-staining. Representative dot-plot diagrams of Annexin V-FITC/PI flow cytometry **C.** The percentage of apoptotic cells were compared with the total cells **D.** Data were shown as the mean ± SD (n = 3). *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ vs. the control group; #$p < 0.05$, ##$p < 0.01$ and ###$p < 0.001$ vs. the ES group.

regulator of Bax activation and neuronal apoptosis induced by oxidative stress. In this study, the expression of Bax protein has a very obvious change, which is considered to have a certain relationship with PUMA's dominant role in regulating Bax activation [44]. The above experiments confirmed that oxidative stress is closely related to the onset of PTSD in high-voltage electric burn to a certain extent.

Previous studies have found that the production of ROS plays a critical role in inducing cell apoptosis [14, 47]. ROS can induce DNA damage, mitochondrial permeability and activation of apoptosis signals, leading to cell death. In this experiment, we used electrical stimulation induced HT22 cells to prepare oxidative damage models. The study showed that the electrical stimulation significantly increased the production of ROS in the oxidative stressed HT22 cell model. As an antioxidant, NAC can effectively protect HT22 cells from apoptosis induced by ROS. The reason for the anti-ROS activity of NAC is to scavenge free radicals through the redox potential of thiols or increase the level of glutathione in cells [35]. Therefore, protecting hippocampal neurons from apoptosis induced by oxidative stress may have a very critical therapeutic value in the treatment of PTSD.

When the cells received ischaemia and hypoxia stimulation persistently, the JNK signalling pathway is activated and participates in cell apoptosis. JNK is one of the members of the mitogen-activated protein kinase (MAPK) pathway. Phosphorylated JNK can induce cell apoptosis, which is very important in the exogenous and endogenous apoptotic pathways

[52]. This experiment showed that high-voltage electrical stimulation induced an increase in p-JNK in the hippocampus of PTSD rats through oxidative stress, which is consistent with the increase in ROS levels. At the same time, cell experiments proved that the electrical stimulation significantly induced the death of HT22 cells in a voltage-dependent manner, which is consistent with previous studies. This is consistent with previous research. The electrical stimulation can induce JNK phosphorylation (p-JNK), while the JNK inhibitor SP600125 can inhibit cell apoptosis, and the NAC can inhibit JNK activation [45]. The above studies have shown that the increase in p-JNK is inseparable from the increase in ROS generation. JNK is a key mediator of inducing cell apoptosis. In addition, studies have reported that the JNK signalling pathway can regulate the P53 signalling pathway and induce cell apoptosis. As a "genome guardian", P53 protein has anti-proliferation and pro-apoptosis functions. It is an important transcription factor and tumour suppressor in the body. In different types of cell stress, P53 undergoes different post-translational modifications and transactivation target genes play a critical role in different cellular pathways, such as apoptosis, autophagy and DNA repair [2]. Current studies have shown that when the body is in a state of sustained high levels of oxidative stress, DNA damage induced by ROS can activate P53 [40] and P53 can activate pro-apoptotic genes Bax, PUMA, etc. through the endogenous (mitochondrial) pathway [24, 51]. or by inhibiting the anti-apoptotic gene Bcl-2 to promote endogenous cell apoptosis [19, 20]. P53 can also directly activate Bax in the cytoplasm through non-transcriptional pathways, and induce endogenous cell apoptosis.

As we all know, when the body encounters a serious life event, it induces the body to produce a high level of oxidative stress, enter the resistance phase and exhaustion phase, and develop into PTSD [29, 46]. The continuous reduction of hippocampal volume in patients with PTSD may be related to the activation of P53 protein induced by high levels of oxidative stress and trigger neuronal apoptosis. However, it is still unclear how hippocampal neurons are damaged by high-voltage electric burn. In this study, we detected the expression of p-JNK, P53 and other related apoptotic proteins in the hippocampus of PTSD model rats through western blot assay, and the results proved that high-voltage electric burn activate the ROS/JNK/P53 pathway. By activating the P53 protein, it triggers the expression of the pro-apoptotic protein PUMA, down-regulates Bcl-2, and promotes neuronal apoptosis. In addition, the *in vitro* experiment of this study also proved that electrical stimulation induced an increase in intracellular ROS production in HT22 cells, and increased the expression of p-JNK and P53 proteins, and caused apoptosis through the endogenous (mitochondrial) pathway, which was manifested in the decreased expression of anti-apoptotic molecule Bcl-2 protein and the increased expression of Bax and PUMA protein. The antioxidant NAC can prevent electrical stimulation induced cell death through JNK inactivation, and inhibit the accumulation of P53 by inhibiting the production of ROS. These results indicated that the development of PTSD caused by high-voltage electrical burn and oxidative stress in the cells model, the ROS-JNK-P53 pathway was involved in the regulation of cell apoptosis.

CONCLUSIONS

In summary, this study concluded that oxidative stress mediates hippocampal neuronal apoptosis through ROS/JNK/P53 pathway in rats with PTSD triggered by high-voltage electrical burn, which will provide an experimental basis for further exploring the pathogenesis of PTSD caused by high-voltage electric burn and screening the potential therapeutic targets.

ARTICLE INFORMATION AND DECLARATIONS

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