

Original Article

Cerebellar neuronal apoptosis in heroin-addicted rats and its molecular mechanism

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Abstract: Background: The overall objective of this study was to investigate neuronal apoptosis and expression of apoptosis related proteins (c-jun, cytc and Bax) in the cerebellum of rates with heroin addiction. Material/Methods: 40 adult male Sprague-Dawley rats which weighing 200-220 g were randomly divided into 5 groups (n = 8 per group): control group, 10-day heroin-addicted group, 20-day heroin-addicted group, 30-day heroin-addicted group and 40-day heroin-addicted group. Rats in the control group were treated with normal saline. Rats in the addiction groups (20 d, 30 d, 40 d) were all given subcutaneous injection with heroin for 15 days to induce heroin addiction. After injected with heroin for 15 days, rats were treated with naloxone at a dose of 5 mg/kg to induce abstinence for 30 mins to examine the addiction of rats. They were then continued to be treated with heroin for another 10 days, 20 days, 30 days, and 40 days respectively to establish heroin-addicted models. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) was employed to identify apoptotic cells [6]. Immunohistochemistry and Western blot assay were also used in the study to examine the protein expressions of c-jun, cytc and Bax in the cerebellum. Results: Compared with the control group, the proportion of apoptotic neurons increased significantly in the heroin addiction groups (10 d, 20 d, 30 d, 40 d) ($P < 0.05$), also accompanied by markedly increased expressions of c-jun, cytc and Bax ($P < 0.05$) depending on doses of heroin in the cerebellum. Thus, the significant differences were observed in heroin addiction groups (10 d, 20 d, 30 d, 40 d) and control group ($P < 0.05$). Conclusion: Long-term use of heroin may induce neuronal apoptosis in the cerebellum by raising the expressions of pro-apoptotic c-jun, cytc and Bax, which might be one of mechanisms underlying the heroin-induced cerebellum neuronal damage.

Keywords: Heroin addiction, cerebellum, apoptosis, c-jun, cytc, Bax

Introduction

Today, heroin abuse (Drug addiction) still keeps one of the major social problems in the world. Heroin derived from opium which can be further refined to extract morphine, it was extracted by chemical methods when added acetic anhydride, chloroform, and other chemicals in morphine and is the diacetylpyridine derivative of morphine. Long-term inhaling or injecting heroin can cause a deleterious effect on morphology and functionality in numerous reports [1-3]. Thus, Apoptosis is one of the serious

effect of long-term inhaling or injecting heroin induced neuronal damage [4, 5], the neuronal apoptosis and its mechanisms have been investigated in vitro, but few studies are conducted to investigate these in vivo. Recently, some researchers investigated the neuronal apoptosis in the spinal cord, hippocampus or cerebral cortex in vivo in morphine addiction [6-8]. In the present studies, TUNEL (TdT-mediated dUTP nick end labeling), immunohistochemistry and Western blot assay were employed to evaluate the neuronal apoptosis in the cerebellum of rats with heroin addiction,

and further study the potential mechanisms of heroin-induced neuronal apoptosis.

Material and methods

40 adult male Sprague-Dawley (SD) rats weighing 200-220 g were purchased from the Experimental Animal Center of the Experiment Animal Department of First Affiliated Hospital of Xinjiang Medical University [SYXK (Xin) 2013, IACUC, accredited number: 20131224002]. In the study, rats were randomly assigned into 5 groups: control group was treated with normal saline; the 10-day heroin-addicted group; the 20-day group heroin-addicted group; the 30-day heroin-addicted group; and the last group, the 40-day heroin-addicted group (n = 12 per group).

Rats in the addiction groups were given subcutaneous injection with heroin (Sigma-Aldrich, St. Louis, MO, USA) twice a day (at the time of 10:00 am and 20:00 pm) with an escalating dose. The regimen of chronic (for instance, 10-day model) escalating dose heroin administration included a dose increase every second day: the first two days dose administered was 7.5 mg/kg/day and was increased to 15 mg/kg/day on the 3rd and 4th day. On day 5 and day 6 the dose was 30 mg/kg/day; and 45 mg/kg/day on the day of 7th and 8th; the dose went up to 60 mg/kg/day on day 9 and 10. This pattern of exposure using heroin has been found to induce physiological dependence in rats [9]. In control group, rats received an equal volume of normal saline. Randomly selected five heroin-addicted rats were then treated with naloxone (Lot number: H20055758; Beijing Sihuan Pharmaceutical Factory) at 5 mg/kg to induce abstinence for 30mins. The recognizable abstinence symptoms were observed including standing (1, 1-5 times; 2, 6-10 times; 3, > 11 times), wet-dog shaking, stretching, teeth chatter, jumping, cunnilingus (1, 1-3 times; 2, 4-6 times; 3, > 7 times). The abstinence symptoms were scored. Once the heroin addicted model have built, rats in experimental groups were injected with the heroin (dose = 60 mg/kg/day) rats for another 10 days, 20 days, 30 days, 40 days, respectively.

Sample collection

Rats were randomly selected from each group were examined under the same procedures.

We removed muscle and fascia from skull and exposed the foramina magnum. The skull was opened along the sagittal plane via the foramina magnum. The dura mater was carefully removed, and then the whole brain was assessed. The half of cerebellum tissue was separated and stored in liquid nitrogen for later western blot assay assessment. The remaining tissue were kept in 10% paraformaldehyde. The cerebellum was collected followed by dehydration and transparentization. After embedding in paraffin, sections were assessed for histopathological examination and immunohistochemistry. The coronal sections (4 µm) were cut and adherent to slides.

Detection of neuronal apoptosis

TUNEL detection kit (Roche, France) were employed for the detection of neuronal apoptosis. Briefly, paraffin embedded sections were deparaffinized and dehydrated. After washing in PBS, all sections were rinsed with 3% hydrogen peroxide for 10 mins followed by washing in PBS. Then they were treated with 20 µg/mL proteinase K for 20 mins at 37°C. After washing in PBS thrice (5 mins for each), sections were incubated with 50 µl TdT + 450 µl dUTP at 37°C for an hour. Following with washing in PBS thrice (5 mins for each), sections were treated with pod conjugated antibody at 37°C for 20 mins. Then after washing in PBS thrice (5 mins for each), sections were again treated with 0.04% DAB at room temperature for visualization for 2-10 mins, dissolved with water. After washing in water, counter-staining was done with hematoxylin followed by mounting with neutral resin. In the negative control, just added in dUTP. The positive control sections were pre-treated with 100 µl DNase I for 10 mins followed by TUNEL staining. Cells with pale brown in the nucleus were seen as positive for TUNEL. A total number of 100 cells were calculated at a high magnification, and the percentage of TUNEL positive cells was calculated.

Immunohistochemistry for c-jun, cytc and Bax

Immunohistochemistry for c-jun, cytc and Bax was done with detection kit (Abcam company). Paraffin-embedded sections were deparaffinized and dehydrated. After washing in PBS thrice (3 mins for each), then were treated with 3% H₂O₂ at room temperature for 10 mins to inactivate endogenous peroxidase. After wash-

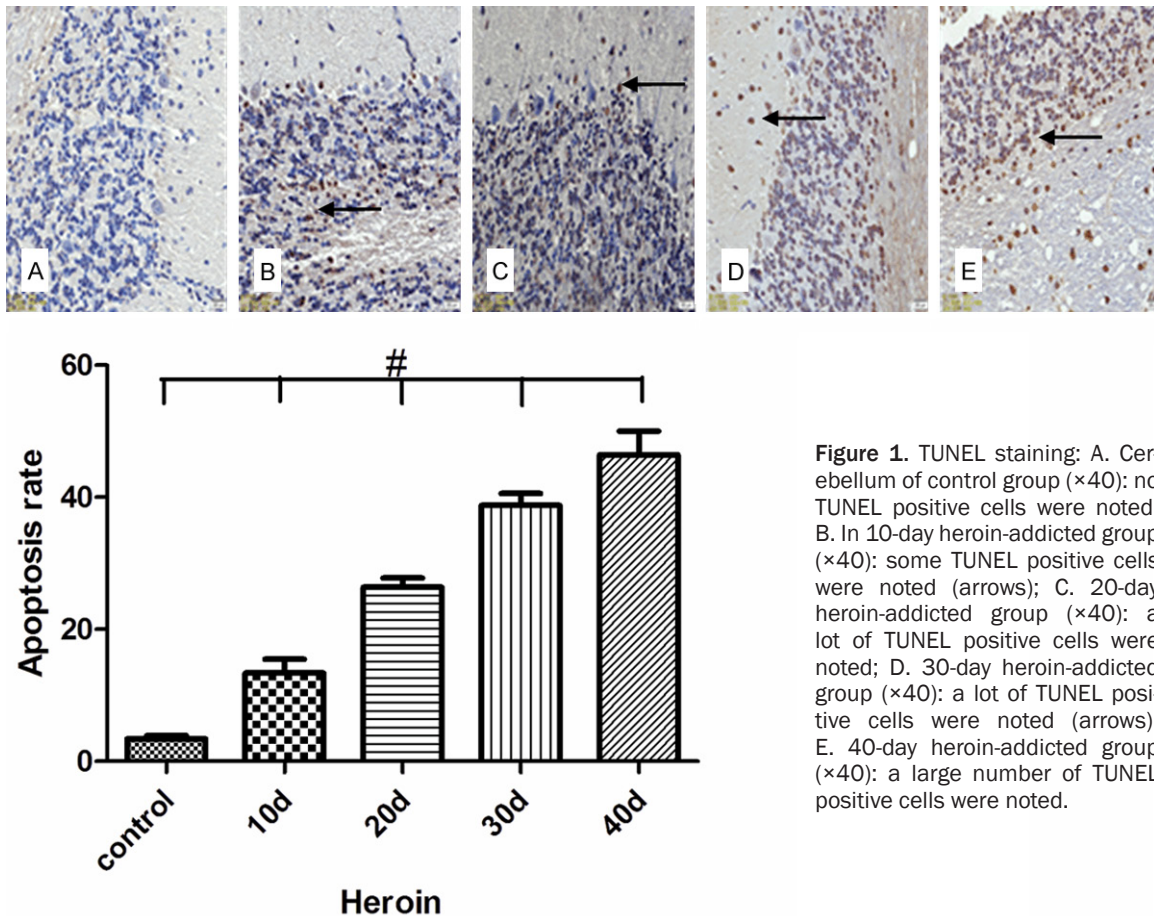


Figure 1. TUNEL staining: A. Cerebellum of control group ($\times 40$): no TUNEL positive cells were noted; B. In 10-day heroin-addicted group ($\times 40$): some TUNEL positive cells were noted (arrows); C. 20-day heroin-addicted group ($\times 40$): a lot of TUNEL positive cells were noted; D. 30-day heroin-addicted group ($\times 40$): a lot of TUNEL positive cells were noted (arrows); E. 40-day heroin-addicted group ($\times 40$): a large number of TUNEL positive cells were noted.

ing in PBS thrice (5 mins for each), antigen retrieval was performed at 98°C (12 mins for each). Sections were kept cool as the room temperature. After washing in PBS (5 mins for each), they were incubated with normal goat serum at room temperature for another 30 mins. Then, these sections were separately treated with primary antibodies (c-jun: 1:50, cytc: 1:100 and Bax: 1:200) at 4°C refrigerator overnight. Followed by washing in PBS thrice (5 mins for each), sections were incubated with HRP conjugated streptavidin at 37°C for 40 mins. Followed by washing in PBS thrice (5 mins for each), observation was done with DAB, terminated with water. After washing in water, counter-staining was done with hematoxylin followed by mounting with neutral resin. In blank control, PBS was employed. In alternative control, normal serum was used instead of primary antibody. The known positive control served as a positive control. These two sections were randomly selected from each group, and three fields were randomly selected at a high magnification. The proportion of positive cells was calculated.

Western blot assay of c-jun, cytc and Bax

The cerebellum was taken out of the liquid nitrogen and homogenized with a buffer (1:10) followed by centrifugation at $800\times g$ at 4°C for 10 mins. The supernatant was obtained and protein quantification was detected with BCA method. The proteins were stored at -80°C . And, $20\text{ }\mu\text{g}$ of total proteins were subjected to 12% SDS-PAGE and then transferred onto PVDF membranes which were then blocked in 5% skim milk at room temperature for 1 h. Following washing in TBST (Tris-HCl and Tween buffer) (5 min for each), the membranes were treated with rabbit anti-c-jun, cytc and Bax polyclonal antibody (1:1000, 1:5000, 1:2000; Abcam company) at 4°C refrigerator overnight. After washing in TBST (5 min for each), these membranes were incubated with secondary antibody solution (Invitrogen, USA) at 37°C for an hour, the membranes were underwent visualization with chromogenic substrate. Representative photographs were captured and the bands on membranes were analyzed with Gel Doc 2000 system (BioRad, USA).

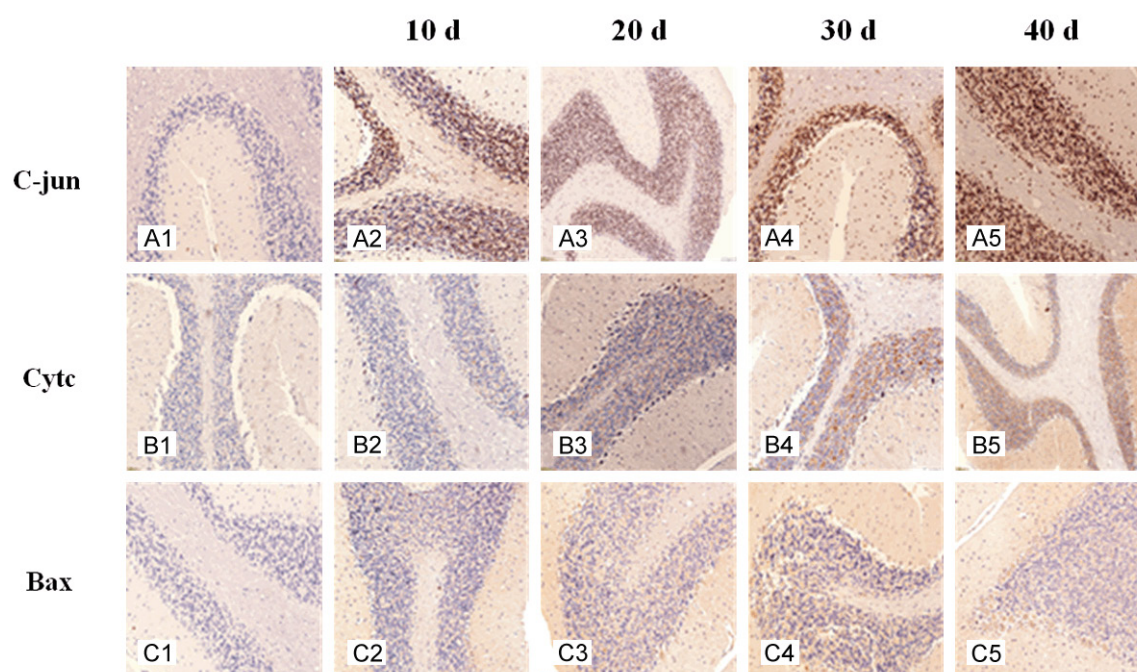


Figure 2. Protein expressions of c-jun, cytc and Bax in cerebellum neurons of rats with heroin addiction. (Immunohistochemistry, Original magnification: $\times 200$). A1~A5: c-jun protein expression in control group, heroin addiction groups 10 d, 20 d, 30 d and 40 d; B1~B5: cytc protein expression in control group, heroin addiction groups 10 d, 20 d, 30 d and 40 d; C1~C5: Bax protein expression in control group, heroin addiction groups 10 d, 20 d, 30 d and 40 d.

Table 1. Percentage of cerebellum neurons cell positive of c-jun, cytc and Bax in rats with heroin addiction (n = 8, $\bar{x} \pm s$, %)

group	c-jun	cytc	Bax
Control	3.067 \pm 1.901	4.167 \pm 2.754	3.3 \pm 2.364
10 d	17.53 \pm 6.577*	9.833 \pm 2.255#	6.3 \pm 0.8544*
20 d	25.13 \pm 4.801#	17.67 \pm 2.517#	14.33 \pm 2.082*
30 d	45.33 \pm 7.506#	27.67 \pm 2.517#	21.33 \pm 3.512#
40 d	70 \pm 13.23#	44.33 \pm 10.07#	31.67 \pm 3.512#

Compared with control group, *P < 0.05; #P < 0.01.

Neuronal apoptosis in cerebellum of rats with heroin addiction

The apoptotic cells were different from surrounding cells. Compared with control group, there is fewer apoptotic cell. However, in 10-day heroin-addicted group, 20-day heroin-addicted group, 30-day heroin-addicted group and 40 d

heroin-addicted group, a large amount of apoptotic cells were observed in the nucleus of cerebellum neurons (**Figure 1**). The rates of apoptotic cells were 3.4 \pm 1.14%, 13.44 \pm 4.67%, 26.4 \pm 3.01%, 38.80 \pm 3.96% and 46.4 \pm 8.05% in control group, in 10-day heroin-addicted group, 20-day heroin-addicted group, 30-day heroin-addicted group and 40 d heroin-addicted group, respectively. The apoptotic rate in heroin addiction group (10 d, 20 d, 30 d and 40 d) was significantly higher than that in control group (P < 0.01), however, there was also a significant difference among heroin addiction groups (10 d, 20 d, 30 d and 40 d), there is an increasing trends with apoptotic cells rate corresponds to the escalating dose of subcutane-

Statistical analysis

Quantitative data were reported as mean \pm standard deviation ($\bar{x} \pm s$). Statistical analyses were performed using one-way analysis of variance (ANOVA) or t-test. And P < 0.05 was considered statistically significant.

Results

Established model of heroin addiction

In control group, the abstinence score was 3.67 \pm 1.63, but markedly lower than that in heroin group (13.83 \pm 1.472; P < 0.01). This suggests that heroin addiction was induced in these rats.

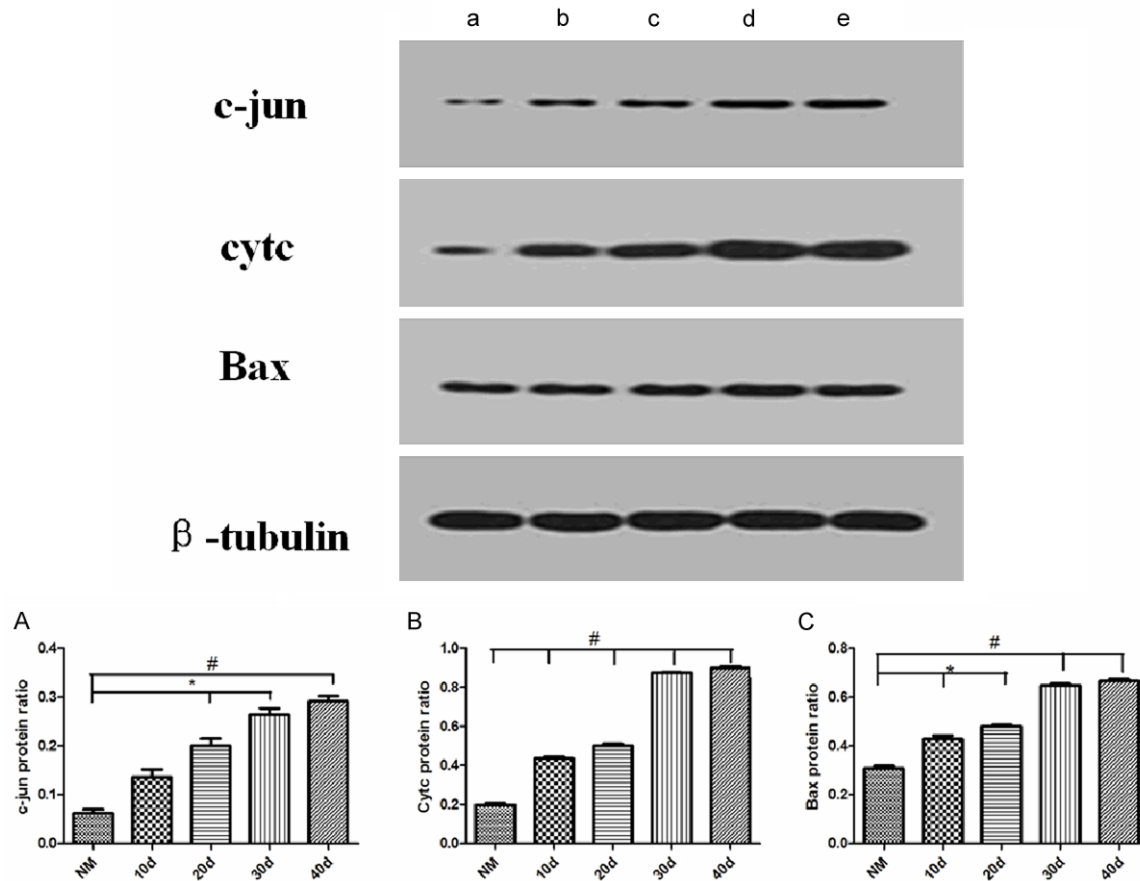


Figure 3. Western blot assay of c-jun, cytc and Bax protein expression in cerebellum neurons cell with heroin addiction. a: Control group; b: 10 d heroin-addicted group; c: Heroin addiction 20 d heroin-addicted group; d: 30 d heroin-addicted group; e: 40 d heroin-addicted group; A: c-jun protein ratio in cerebellum neurons cell with heroin addiction (10 d, 20 d, 30 and 40 d) and control group; B: cytc protein ratio in cerebellum neurons cell with heroin addiction groups (10 d, 20 d, 30 and 40 d) and control group; C: Bax protein ratio in cerebellum neurons cell with heroin addiction groups (10 d, 20 d, 30 d and 40 d) and control group; (* $P < 0.05$; # $P < 0.01$).

ous injection of heroin with days. This suggests that long-term use of heroin may cause in amount of apoptotic neurons in rats.

Protein expression of c-jun, cytc and Bax in neurons of cell with heroin addiction

In the immunohistochemistry, cells with yellow-brown granules in the cytoplasm or nucleus were regarded as positive (Figure 2). Results showed, when compared with control group, the expressions of c-jun, cytc and Bax increased dramatically correspond to the increases dose of heroin in heroin addiction groups (10 d, 20 d, 30 d and 40 d) ($P < 0.05$). However, significant differences in protein expressions of c-jun, cytc and Bax were observed in heroin addiction groups (10 d, 20 d, 30 d and 40 d) ($P < 0.05$) continuing to use heroin drugs (Table 1).

Western blot assay also revealed that the expressions of c-jun, cytc and Bax increased in all heroin addiction groups (10 d, 20 d, 30 d and 40 d) and also in control group ($P < 0.05$, Figure 3).

Discussion

In Recent years, long-term using of heroin can cause varying degrees of damage to the brain tissue which Ketamine can lead to atrophy of the cerebral cortex [10], cocaine can cause front-cortical and striatal damages in the treated rat by MRI [11], frontal white matter, callosum and hippocampal damaged by DTI in human cocaine addicts [12-14]. Heroin regarded as opioid important derivatives, long-term abuse induces pathological changes in the nervous system. Many cases which heroin abuse

lead to death exist ischemic neurological changes, cerebral edema and congestion. There were noticeable the symmetry side of the brain ischemic injury and necrosis, decreased neuronal density partially among intravenous heroin in people. Some people were affected by Spongiform leukoencephalopathy after in-taking heating heroin, reducing the gray matter of the hippocampus or the Purkinje cell layer [15]. Thus, heroin is equipped with clinically neurotoxin in which involved in gray matter loss in the frontal, cingulate and occipital cortices, neuronal apoptosis, mitochondrial dysfunction, synaptic defects, depression of adult neurogenesis and the effect of spongiform leukoencephalopathy, increased intracellular calcium, decreased mitochondrial membrane potential and decreased ATP levels [16-28].

However, the apoptotic mechanisms causes the diverse toxic effects of heroin are complicated and not yet fully understood by current researchers. Apoptosis controls cell death, equipped with a complex regulation and many molecules involved. The researches indicates that cerebellar granule cells cultured in vitro own the excessive activation of JNK pathway when there is low potassium damaged neuron, further induced neuronal apoptosis along with the activation of the JNK pathway [29]. The activation of JNK mainly mediated by affecting the function of gene expression and mitochondrial mediated apoptosis. JNK phosphorylates and promotes apoptotic protein Bax activity to boost, so as to promote the opening of the mitochondrial permeability transition pore, the mitochondrial CytC is released into the cytoplasm to induce apoptosis by caspase-9/caspase-3 factors. Intrinsic apoptotic pathway is primarily mediated by mitochondrial which neuronal mitochondrial outer membrane increased permeability after cerebral ischemia, it may promote apoptosis protein cytochrome C to release and initiating protease cascade reaction, ending inducing cell apoptosis [30, 31]. Ying Wang et al [32, 33] found that rats exposed to heroin, the prefrontal cortex, hippocampus and ventral nucleus of Caspase-3 and Bax factors expression were greatly increased, Bcl-2 factor was down-regulated which indicates that heroin can exert its neurotoxic effects through inducing neurons apoptosis in related cerebral regions and mitochondrial apoptosis pathway may be involved in the molecular mechanism of

injury in mice heroin neurobehavioral development. Bax as Bcl-2 family proapoptotic factor promotes apoptosis through destroying the integrity of mitochondrial membrane while Bax was gathered in the outer membrane of mitochondria and interacted with the mitochondrial outer membrane voltage-dependent anion channel, prompting the mitochondrial permeability transition pore opening [34].

Previous studies on the neurodegeneration of PC12 cells, a dopaminergic cell line, indicated that drugs of abuse can induce apoptotic features and the opioid drugs (heroin and morphine) are more toxic than stimulant drugs (d-amphetamine and cocaine) in this regard [35]. In the fact that cell lines in different brain regions are various, thus, certain brain regions may have more vulnerability to be affected than the other parts when exposed to heroin. Heroin is usually injected, sniffed, snorted, or smoked and the degree of impairment caused by heroin depends on the time (duration) and frequency, dosage and route of administration. Chronic exposure to opioid drugs in the central nervous system would interfere with neurobehavioral deficits through activation of apoptotic pathways by raising the brain expression of proapoptotic factors belonging to apoptotic pathways [36, 37].

In the study, we found a significantly increased expression of c-jun, cytc and Bax depending on escalated doses of heroin and longer term of drug abuse in the cerebellum. Thus, we conclude heroin may cause cerebellar neuronal apoptosis via altering expressions of c-jun, cytc and bax. It is helpful to provide evidence for the mechanism and forensic pathophysiology underlying the neuronal damage due to long term abuse of heroin.

Disclosure of conflict of interest

None.

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