Effects of penehyclidine hydrochloride on apoptosis of lung tissues in rats with traumatic acute lung injury

WANG Ling-li, ZHAN Li-ying, WU Xiao-jing and XIA Zhong-yuan

【Abstract】Objective: To investigate the effects of penehyclidine hydrochloride on apoptosis of lung tissue cells and its mechanism in acute lung injury following blunt chest trauma in rats.

Methods: Sprague Dawley (SD) rats (n=54) weighing (250±25) g were divided equally and randomly into three groups: normal control group (C group, n=18), trauma model group (T group, n=18) and penehyclidine hydrochloride treatment group (P group, n=18). Each group was further divided into three subgroups according to the time points of 3, 12 and 24 hours after experiment (at each time point, n=6 for each subgroup). Rats of P group were intraperitoneally injected with penehyclidine hydrochloride for 2 mg/kg immediately after blunt chest trauma and rats in its 24 hours subgroup were once again injected with penehyclidine hydrochloride in the same dose 12 hours after injury. Lung tissue samples were collected at every time point and cell apoptosis in lung tissues were measured by TUNEL. Apoptotic index (AI) was calculated, expressions of bax and bcl-2 were detected by immunohistochemical staining of SABC, and lung tissue sections were taken for light and electron microscopic observation.

Results: As compared with C group, at every time point, AI and expressions of bax and bcl-2 in T group were higher (P<0.05), and the ratio of bcl-2/bax markedly decreased (P<0.05), especially in the 24 hours subgroup. The ratio in T group (0.468±0.007) was lower than that in C group (1.382±0.058, t=12.5, P<0.01). Lung tissue injuries were significant under a light microscope, and the number of apoptotic cells increased obviously under a transmission electron microscope. As compared with T group at the same phase, AI and expression of bax decreased in P group (P<0.05 and P<0.01), while the expression of bcl-2 increased significantly (P<0.01), and the ratio of bcl-2/bax markedly increased (P<0.05), especially in the 24 hours subgroup. The ratio in P group (1.012±0.070) was much higher than that in T group (0.468±0.007, t=8.3, P<0.01). The injury of lung tissues was relieved, and apoptosis of cells decreased obviously under a transmission electron microscopic observation.

Conclusions: Apoptosis and expressions of bax and bcl-2 in lung tissues might be involved in the pathogenesis of lung injury induced by blunt chest trauma. Penehyclidine hydrochloride can alleviate lung injuries by inhibiting apoptosis of lung tissue cells, during which effects of penehyclidine hydrochloride on regulating expressions of bax and bcl-2 may play an important role.

Key words: Penetuniformine; Acute lung injury; Apoptosis; BAX protein, human; Genes, bcl-2; Wounds and injuries

METHODS

Animals and reagents

A total of 54 male Sprague Dawley (SD) rats weighing (250±25) g were provided by the Experimental Animal
Center of Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China. Penehyclidine hydrochloride injection was purchased from Chengdu List Pharmaceutical Co, China. Bax and bcl-2 polyclonal antibodies and a strept avidin biotin complex (SABC) kit were procured from Wuhan Boster Bioengineering Co, China. The Roche in situ cell-apoptosis-assay kit was obtained from Shanghai Runwell Technology Co, China.

Animal model, grouping and experimental methods

Animal model and grouping

SD rats (n=54) fasted for 12 hours before experiment but with pure water ad libitum. They were then distributed equally and randomly into three groups: normal control group (C group, n=18), trauma model group (T group, n=18) and penehyclidine hydrochloride treatment group (P group, n=18). Each group was further divided equally into three subgroups according to the time points of 3, 12 and 24 hours after experiment (at every time point, n=6 for each subgroup of each group). The rats were anesthetized using isoflurane. As Raghavendran et al. had described in their study, we induced lung contusions in those anesthetized rats by dropping a 0.3 kg weight from a height of 95 cm through a vertical stainless steel tube onto a platform resting on the chests of SD rats. Rats of P group were intraperitoneally injected with penehyclidine hydrochloride for 2 mg/kg immediately after blunt chest trauma and rats in its 24 hours subgroup were once again injected with penehyclidine hydrochloride in the same dose 12 hours after injury. Lung tissues were collected at each respective time point. These rodents were intraperitoneally anesthetized with 2% pentobarbital (45 mg/kg). Then exsanguination and thoracotomy were performed to harvest lung tissue specimens. These right upper lung tissue samples were fixed in 4% paraformaldehyde, then embedded in paraffin and prepared in slices of 5 µm thickness. Expressions of bax and bcl-2, and the levels of cell apoptosis were determined through standard assay techniques.

Assay of bax and bcl-2 expressions

Expressions of bax and bcl-2 were determined using an SABC kit. The staining was determined positive when cytoplasm turned brownish yellow. Five different visual fields under a light microscope (×400) of each respective section were chosen and the mean optical densities (ODs) of bax positive and bcl-2 positive cells from each section were analyzed with a color image pattern analysis system supplied by the Department of Pathology, Wuhan University, China.

HE staining, light and electron microscopy

After routine HE staining, pathological changes of the lungs were observed under a light microscope (×100). From each 12 hours subgroup, two lung tissue samples were randomly selected, and then fixed in glutaraldehyde and osmium tetroxide followed by dehydration, and finally embedded in epoxy. The specimens were further prepared and made into ultrathin sections and stained with uranyl acetate and lead nitrate. Using a Hitachi H2600 perspective electron microscope supplied by the Medical Structure Center of Wuhan University, China, cellular and sub-cellular changes were examined.

Statistical analysis

Data were expressed as mean ± standard deviation (SD) and analyzed with One-Way ANOVA and Newman-Keuls method using the SPSS 13.0 software. Differences within and between groups were considered statistically significant at *P*<0.05.

RESULTS

As compared with C group, at every time point, AI values of T group were markedly higher, and increased gradually over the whole time course. AI values of P group were lower than those of T group, but higher than those of C group (*P*<0.05, Table 1 and Figure 1).

Compared with C group, expressions of bax and bcl-2 in T group markedly elevated. As time went by, the expression of bax increased gradually, but the expression of bcl-2 decreased gradually. Thus, the ratio of bcl-2/bax markedly decreased during the entire experiment over 24 hours. In P group, the expression of bax was significantly lower, while the expression of bcl-2 was higher and the ratio of bcl-2/bax elevated, compared with T group (*P*<0.01, Tables 2, 3, 4 and Figure 2).
Table 1. Changes of AI values (%) in three groups (x ± s)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Time after experiment (h)</th>
<th>3</th>
<th>12</th>
<th>24</th>
</tr>
</thead>
<tbody>
<tr>
<td>C group</td>
<td>2.972±0.005*</td>
<td>2.935±0.013*</td>
<td>2.964±0.003*</td>
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<tr>
<td>T group</td>
<td>8.594±0.006*</td>
<td>17.057±0.027*</td>
<td>23.402±0.023*</td>
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<tr>
<td>P group</td>
<td>5.670±0.004*</td>
<td>11.541±0.015*</td>
<td>16.208±0.015*</td>
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</table>

*P<0.05, compared with T group at the same time point; *P<0.05, compared with the 12 hours subgroup of T group.

Table 2. Changes of OD values of bax in three groups (x ± s)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Time after experiment (h)</th>
<th>3</th>
<th>12</th>
<th>24</th>
</tr>
</thead>
<tbody>
<tr>
<td>C group</td>
<td>0.065±0.003</td>
<td>0.081±0.007</td>
<td>0.072±0.001</td>
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</tr>
<tr>
<td>T group</td>
<td>0.304±0.006*</td>
<td>0.275±0.006*</td>
<td>0.231±0.004*</td>
<td></td>
</tr>
<tr>
<td>P group</td>
<td>0.407±0.006*</td>
<td>0.361±0.015*</td>
<td>0.359±0.004*</td>
<td></td>
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</table>

*P<0.01, compared with C group at the same time point; *P<0.01, compared with T group at the same time point; *P<0.01, compared with the 12 hours subgroup of T group.

Table 3. Changes of OD values of bcl-2 in three groups (x ± s)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Time after experiment (h)</th>
<th>3</th>
<th>12</th>
<th>24</th>
</tr>
</thead>
<tbody>
<tr>
<td>C group</td>
<td>0.049±0.005</td>
<td>0.056±0.003</td>
<td>0.052±0.002</td>
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</tr>
<tr>
<td>T group</td>
<td>0.426±0.008*</td>
<td>0.455±0.009*</td>
<td>0.493±0.008*</td>
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<tr>
<td>P group</td>
<td>0.317±0.008*</td>
<td>0.324±0.010*</td>
<td>0.356±0.023*</td>
<td></td>
</tr>
</tbody>
</table>

*P<0.01, compared with C group at the same time point; *P<0.01, compared with T group at the same time point; *P<0.01, compared with the 12 hours subgroup of T group.

Table 4. The ratio of bcl-2/bax in three groups (x ± s)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Time after experiment (h)</th>
<th>3</th>
<th>12</th>
<th>24</th>
</tr>
</thead>
<tbody>
<tr>
<td>C group</td>
<td>1.368±0.023</td>
<td>1.378±0.046</td>
<td>1.382±0.058</td>
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<tr>
<td>T group</td>
<td>0.713±0.018*</td>
<td>0.603±0.022*</td>
<td>0.468±0.007*</td>
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<tr>
<td>P group</td>
<td>1.284±0.033*</td>
<td>1.114±0.013*</td>
<td>1.012±0.070*</td>
<td></td>
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</table>

*P<0.05, *P<0.01, compared with C group at the same time point; *P<0.01, compared with T group at the same time point; *P<0.01, compared with the 12 hours subgroup of T group.

Figure 1. Apoptosis of lung tissue cells in each group (TUNEL×400). A: Almost no cells are positive for TUNEL in C group. B: Many cells are positive for TUNEL in T group. The nucleuses are brownish yellow or dark brown. C: Few cells are positive for TUNEL in P group.

Figure 2. Expressions of bax and bcl-2 in lung tissue cells of T group and P group (SABC×400). After staining, the cytoplasm of positive cells is brown, which can be found in bronchial epithelial cells, alveolar epithelial cells and inflammatory cells. A: There are many bax positive cells in T group. B: There are few bax positive cells in P group. C: There are few bcl-2 positive cells in T group. D: There are many bcl-2 positive cells in P group.

Figure 3. Pathological changes of lung tissues (HE×100). A: Pathological changes of normal lung tissues (C group). B: Pathological changes of lung tissues after a chest impact (T group). The lung tissues are damaged significantly, exhibiting interstitial edema, hemorrhage, thickening of the alveolar wall, and infiltration of inflammatory cells into the interstitial and alveolar spaces. C: Histological changes of lung tissues after penicillin hydrochloride treatment (P group). There are minimal edema, hemorrhage and inflammatory cells.
Lung tissues from C group revealed no abnormal histopathological findings. But those from T group showed that there were red spots of varying sizes scattered on the surface of bilateral lungs. Upon closer examination, the red spots revealed consolidation changes. Under a light microscope, the lung tissues were found to be damaged significantly, presenting with interstitial edema, hemorrhage, thickening of the alveolar wall, and infiltration of inflammatory cells into the interstitial and alveolar spaces. Under a transmission electron microscope, microvilli of alveolar type II cells appeared to be detached, with lamellar body cavitations, swelling of the mitochondria and nuclear condensation. Such histopathological changes were also found in P group, but to a much milder extent.

**DISCUSSION**

Apoptosis is a programmed cell death regulated by genes. In recent years, there have been growing concerns about effects of apoptosis on the pathogenesis of ALI. Cytokines may take a part in the progression of ALI by inhibiting apoptosis of inflammatory cells, thus extending the inflammatory reaction time. Such prolonged inflammatory reaction promotes the apoptosis of pulmonary vascular endothelial cells and alveolar epithelial cells, which thereby increases the damage extent of the capillaries and alveoli, hence causing an increased permeability of the blood vessels and alveolar walls. The resulting exudation leads to a decreased diffusing capacity of alveoli. Dong et al have found that apoptosis of vascular endothelial cells and alveolar epithelial cells was significantly increased after chest impact, causing permeability of the blood-gas barrier to increase, further leading to a pulmonary edema, which confirms that apoptosis is an important factor in the pathogenesis of pulmonary edema secondary to injury. Our study revealed that AI of trauma model group was higher than that of normal control group, and the lung tissues of T group were significantly damaged under a light microscope, presenting with interstitial edema, hemorrhage, thickening of the alveolar wall and infiltration of inflammatory cells into the interstitial and alveolar spaces. Under a transmission electron microscope, microvilli of alveolar type II cells were found to be detached, together with cavitations of lamellar bodies, swelling of the mitochondria and nuclear condensation. All these findings were compatible with ALI and apoptosis after chest impact.

Bcl-2 and bax are members of the bcl-2 gene family. The inhibiting effects of bcl-2 on apoptosis may be related to Ca\(^{2+}\) in the endoplasmic reticulum, which inhibits apoptosis through releasing Ca\(^{2+}\) directly or indirectly. Some researchers have proposed an idea that bcl-2 acts as an anti-oxidant and prevents cell death by its anti-oxidizing effects or suppressing the production of oxygen free radicals. It has already been confirmed that bcl-2 can protect against lipid membrane peroxidation, maintain the redox status of cells and increase glutathione cellular levels. As a matter of fact, researches in the field of anti-oxidants have currently become a hot spot. In addition, bcl-2 can inhibit apoptosis by combining with bax to form a heterogeneous dimer. When the expression level of bax increases, the effect of bcl-2 is antagonized, thus promoting apoptosis. Therefore, the effects of bcl-2 and bax on regulating apoptosis depend not only on the level of self-expression, but also on the ratio of bcl-2/bax. Husain found that bcl-2 is related to ALI. Our study revealed that acute lung trauma caused the expression of bax to increase gradually over the experimental time course. The expression of bcl-2 also elevated, but its magnitude was relatively small and decreased gradually with the disease progression, most probably due to the compensatory mechanisms within bodies. The ratio of bcl-2/bax markedly decreased and AI markedly increased, which indicated that changes in the expressions of bcl-2 and bax directly influenced the development of external trauma-induced lung injury.

Traditional atropine derivative drugs, such as anisodamine, have been used in the prevention and treatment of ALI. Several other studies have shown that anisodamine has antioxidant properties that may be effective against free radical-induced cellular injury. Anisodamine also acts as a Ca\(^{2+}\) antagonist and can induce the expression of bcl-2 in lung tissues of subjects suffering from endotoxin shock, thus playing a protective role for the lungs. However, traditional atropine derivatives have a short half-life and many side effects due to their lacking selectivity of M-receptor subtypes, leading to a limitation of their clinical applications. Penehyclidine hydrochloride is a new highly selective drug of anisodamine-type. It has no clinical effects on M\(_2\)-receptors and can effectively avoid the side effects of its previous generations: lacking selectivity of M-receptor subtypes and having a short therapeutic time. Previous studies have shown that
Penehyclidine hydrochloride has the effect of traditional belladonna derivatives, such as relieving microvascular spasm, reducing glandular secretion, relaxing bronchial smooth muscles and increasing respiratory flow. A recent study found that penehyclidine hydrochloride can improve microcirculation, reduce permeability of capillary walls and suppress the release of lysosome, hence to improve cellular protections. From these findings, we can derive that penehyclidine hydrochloride has a greater advantage over traditional belladonna alkaloids in the management of ALI. Several studies have shown that penehyclidine hydrochloride has a significant protective effect against ALI in rats by inhibiting the expressions of inflammatory cytokines such as TNF-α and interleukin. It also prevents lipid peroxidative injury and lipopolysaccharide-induced neutrophil accumulation within the lungs, reducing apoptosis of lung tissue cells. Some studies have shown that penehyclidine hydrochloride preconditioning can provide cerebral protections by promoting the expression of bcl-2 at an earlier stage to maintain a high level and an extended duration, prohibiting the expression of bax, increasing the ratio of bcl-2/bax and logically inhibiting apoptosis. Within our study, in P group, the expression of bax decreased remarkably while the expression of bcl-2 increased significantly, the ratio of bcl-2/bax increased markedly and AI decreased, implying that the injury of lung tissues was minimized. Therefore, we believe that protective effects of penehyclidine hydrochloride on the lungs may be partly due to the regulation of apoptosis related genes: bax and bcl-2. However, there is still much to be done in order to elucidate the meaning of intracellular signal transduction in the mechanism of apoptosis in lung tissues during ALI secondary to blunt chest trauma and the exact protective effects of penehyclidine hydrochloride during ALI.

REFERENCES


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