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Effects of tetrandrine on functions and ultrastructure of alveolar macrophages in smoke inhalation-injured rabbits

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ABSTRACT A rabbit model with severe smoke inhalation injury was used to explore the effects of tetrandrine (Tet) on the release of inflammatory mediators and the level of $[Ca^{2+}]_i$ in alveolar macrophages (AM) as well as on the ultrastructural changes. It was found that Tet reduced the number of white blood cells (WBC) in bronchoalveolar lavage fluid, inhibited the release of LTB_4 and O_2^- from AM, and increased the $[Ca^{2+}]_i$ in AM. The changes in size, pseudopod, and lysosome evacuation in AM treated with Tet were smaller than those in the AM from the untreated. These results suggest that Tet may alleviate the pulmonary injury through inhibiting the activation of AM.

KEY WORDS tetrandrine; smoke inhalation injury; leukotrienes B; superoxide; calcium; macrophages

Alveolar macrophages (AM), the chief constituent of pulmonary reticuloendothelial system, play an important role in host defence, they might also mediate the lung injury by releasing various biologically active substances[1]. Both animal experiments and clinical research had proved that respiratory failure induced by the obstruction of the air passage and pulmonary edema was the main cause of death from the smoke inhalation[2].

Tetrandrine (Tet) is an effective component of *Stephania tetrandra*[3]. Tet could inhibit the release of superoxide anion (O_2^-) and lysosome enzymes from neutrophils[4]. In this paper, the action of Tet on the release of leukotriene B_4(LTB_4) and O_2^-, the level of cytosolic free calcium ($[Ca^{2+}]_i$), and the ultrastructure in AM from smoke inhalation injured rabbits were studied.

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MATERIALS AND METHODS

Materials Tet was produced by Jinhua Pharmaceuticals, Zhejiang. Leukotriene B_4, prostaglandin B_2 (PGB_2), arachidonic acid (AA), calcimycin (A_{23187}), cytochrome C (Cyt-C, Type III) were produced by Sigma Chemicals, USA. Fura-2/AM and fura-2 were produced by Institute of Materia Medica, Chinese Academy of Medical Sciences. Superoxide dismutase (SOD) was produced by Shanghai Institute of Biochemistry, Chinese Academy of Sciences. Percoll solution (Bioengineering Co). The intensity of fluorescence was measured with an F-3010 fluorescence spectrophotometer (Hitachi, Japan). LTB_4 was analyzed with RP-HPLC (Gilson, France). O_2^- was measured with ultraviolet spectrophotometer (Beckman, USA). Ultrapure water was made by an MilliQ System (Millipore Co, USA). The ultrastructure of AM was examined with an JEM-2000 transmission electron microscope (TEM, Japan).

Rabbit model and collection of samples Sixty-six ♂ New Zealand rabbits (2.3±*s* 0.3 kg) were divided into 3 groups: normal control (NC, $n=6$), injured control (IC, $n=30$), and Tet treated (Tet, $n=30$). The smoke inhalation injury was produced according to the method previously described[5]. Rabbits were put into a cabinet full of sawdust smoke with a temperature of 38±2℃ for 4 min, followed by 3 more times of exposure to the smoke with 3-min intervals. The smoke was produced by a mixture of 150 g dry pine sawdust and 30 ml kerosene. The rabbits were killed before injury and 6, 24, 48, 72 h, and 7 d after injury. The lung was lavaged 4 times with 80 ml normal saline (20 ml each time). The WBC in bronchoalveolar lavage fluid (BALF) were counted and assorted. AM were separated with Percoll solution (AM>90%, activity>95%) and resuspended in Hank's balance solution.

For the Tet group, Tet (7.5 mg·2 ml^{-1}·kg^{-1}) was slowly injected through the marginal vein of the ear 10 min after injury. The iv was repeated twice at

intervals of 12 h. Rabbits in the injured control group were injected iv with 5% glucose in normal saline (wt/vol, 2 ml·kg^{-1}).

Methods to determine the parameters

1 WBC in BALF A routine hospital method was used.

2 LTB_4 from AM The AM number in the solution was adjusted to 2.5×10^6 cells·ml^{-1}. Calcimycin (1 μmol·L^{-1}) was used as a stimulant. The amount of LTB_4 released by AM was measured with reverse-phase HPLC[6].

3 Superoxide anion from AM The concentration of AM was adjusted to 5×10^5 cells·ml^{-1}. Cell suspension (2 ml) was incubated at 37℃ for 15 min, then mixed with cytochrome C 1 mg, calcimycin 1 μmol·L^{-1}, and $CaCl_2$-$MgCl_2$ 0.5 mmol·L^{-1}. The mixture was further incubated at 37℃ for 15 min. The reaction was terminated by an ice-cold water bath. The cell suspension was centrifuged at 4℃ at $1590\times g$ for 5 min. The amount of O_2^- in the suspension was measured with an uv spectrophotometer at 550 nm[7].

4 $[Ca^{2+}]_i$ in AM A modified Fura-2 method[8] was used. The AM suspension was washed twice with calcium-free Tyrode solution and the concentration was adjusted to 5×10^5 cell·ml^{-1}. Cell suspension (2 ml) was mixed with Fura-2/AM and bovine serum albumin to a final concentration of 2.5 μmol·L^{-1} and 0.1% (wt/vol), respectively. It was incubated at 37℃ for 30 min, and centrifuged at $170\times g$ for 10 min. The pellet was washed once with calcium-free solution and resuspended in 2 ml of this solution. The suspension was incubated for 10 min at 37℃ and the intensity of fluorescence was measured. Triton X-100 (0.1%, 20 μl) was added to destroy the cell membrane and then the maximal fluorescence strength (F_{max}) was determined. EDTA-Na_2 (9 mmol·L^{-1}) was added (pH was adjusted to 8.7 with Tris) to chelate all the calcium ions in order to measure the minimal intensity of fluorescence (F_{min}). $[Ca^{2+}]_i$ values were calculated with the formula: $[Ca^{2+}]_i=K_d(F-F_{min})/(F_{max}-F)$, where $K_d=224$ nmol·L^{-1}.

AM ultrastructure The rabbits from NC group ($n=4$), IC group ($n=6$), and Tet group ($n=6$) were killed at 24 h after injury. The lung lavaged with normal saline. The BALF was filtered with a nylon net, then centrifuged at $1100\times g$ for 10 min. The pellet was fixed with 3% glutaraldehyde and processed according to the routine method for TEM.

Statistical methods The results were expressed as $\bar{x}\pm s$. ANOVA was employed to determine the variance in different groups.

RESULTS

WBC in BALF The WBC, AM, and neutrophil (Neu) numbers in BALF in the IC group were increased ($P<0.05$, $P<0.01$) within 24 h after injury. The proportion of Neu was increased even more significantly than that of AM. The number of AM approached normal level by 48 h and another peak appeared around d 7 post injury. The number of Neu remained at a high level (Tab 1).

Tab 1. The numbers of white blood cells (WBC), alveolar macrophages (AM), and neutrophils (Neu) in bronchoalveolar lavage fluid (BALF) after smoke inhalation injury and treated with tetrandrine (Tet). Cells·ml^{-1} BALF, $n=6$, $\bar{x}\pm s$. $^aP>0.05$, $^bP<0.05$, $^cP<0.01$ vs normal control; $^dP>0.05$, $^eP<0.05$, $^fP<0.01$ vs the injured control.

	Normal control	6 h	24 h	48 h	72 h	7 d
Injured control						
WBC	2.07±0.35	5.06±1.54^c	4.03±1.40^b	2.41±0.71^a	2.59±0.75^a	6.10±1.85^a
AM	1.76±0.30	2.93±0.61^c	2.87±0.92^b	1.75±0.52^a	1.86±0.43^a	4.25±1.13^c
Neu	0.08±0.03	1.82±1.01^c	0.90±0.34^c	0.37±0.16^c	0.45±0.27^b	0.66±0.23^c
Tetrandrine-treated						
WBC	2.07±0.35	2.84±0.58^{ae}	2.89±0.85^{ae}	1.97±0.42^{ae}	1.81±0.35^{ae}	3.66±0.87^{ae}
AM	1.76±0.30	1.92±0.47^{ae}	2.05±0.55^{ae}	1.46±0.37^{ae}	1.34±0.21^{be}	2.77±0.69^{ae}
Neu	0.08±0.03	0.47±0.22^{ae}	0.44±0.23^{ce}	0.22±0.09^{ce}	0.27±0.18^{ce}	0.28±0.06^{ce}

LTB_4 release by AM In the IC group, the amount of LTB_4 released by AM increased markedly at 6 h post injury ($P<0.05$). It reached a peak at 24 h and returned to normal by 48 h. The amount of LTB_4 in the Tet group also increased, but to a less extent than that in the IC group, particularly at 24 h ($P<0.05$, Fig 1).

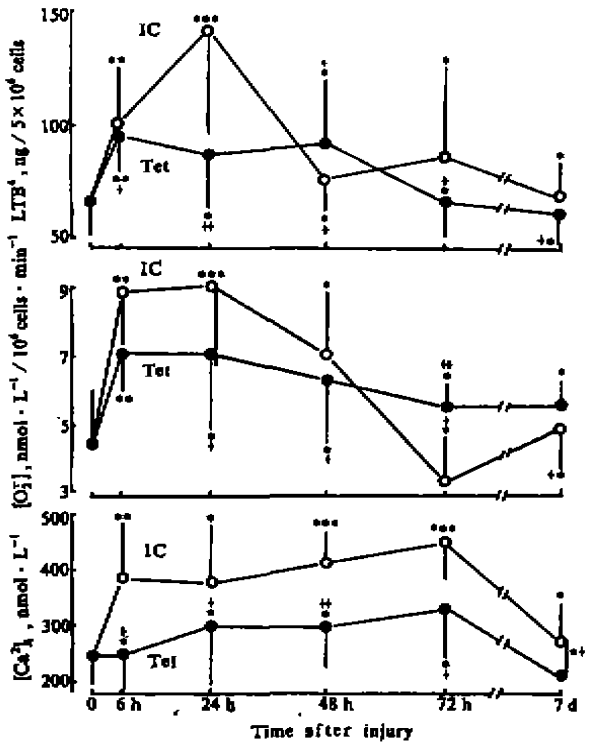

Fig 1. LTB_4 ($n=6$) and O_2^- ($n=5$) released by alveolar macrophages, and $[Ca^{2+}]_i$ ($n=5$) in alveolar macrophages from smoke inhalation injured rabbits treated with tetrandrine (Tet). $^*P>0.05$, $^{**}P<0.05$, $^{***}P<0.01$ *vs* normal control, 0 h; $^+P>0.05$, $^{++}P<0.05$, $^{+++}P<0.01$ *vs* injured control (IC).

Generation of superoxide anion by AM In both IC and Tet groups, the amount of O_2^- generated by AM increased within 48 h after injury. At 72 h the O_2^- generation in IC group was lower than the normal level and returned to normal level by d 7. The O_2^- generation in Tet group approached normal level by 72 h (Fig 1).

$[Ca^{2+}]_i$ concentration in AM In the IC group, the $[Ca^{2+}]_i$ level in AM increased post injury (at 6, 48, and 72 h, $P<0.05$, $P<0.01$). It reached a peak at 72 h and returned to normal by d 7 (Fig 1). Compared with the IC group, the increase of $[Ca^{2+}]_i$ in the Tet group was inhibited especially at 48 h ($P<0.05$, Fig 1).

Ultrastructure of AM In the IC group at 24 h after injury, the pseudopods of AM increased in size and number. There was also an increase in endoplasma reticula, lysosomes, phagolysosomes, and endocytoplasmic vesicles. In some of the AM, vacuolar degeneration and necrosis of endoplasmic reticula and mitochondria were seen (Fig 2A and B, Plate 3). In the Tet group, the AM showed some pathological changes, but the degrees of activation and vacuolar degeneration were much less than those in the IC group (Fig 2C and D, Plate 3).

DISCUSSION

In this study, we found there was an increase in AM and neutrophil (Neu) numbers in the alveoli after smoke inhalation.. The synthesis and release of O_2^- and LTB_4 by these AM were also increased within 24 h post injury. There was a significant positive correlation between the number of AM in BALF and the amount of LTB_4 released by AM ($r=0.86$, $P<0.05$), which indicates that AM did take part in the development of lung injury.

Recent research had proven that calcium ions were the key factor in the activation of inflammatory cells. WBC have many functions, including chemotaxis, migration, phagocytosis, release of lysosome enzymes and inflammatory mediators, etc., which are considered to be controlled by calcium ions in cyto-

plasm[9]. In this study, the increase of free calcium in the cytoplasm of AM was observed during the first 72 h after smoke inhalation. TEM examination showed that, the size of the cell became expanded, the pseudopods increased, and the endoplasmic reticulum enriched, all these implied the activation of AM.

Tet, as an anti-inflammatory agent and antiallergic agent, could inhibit the elevation of free calcium level in the cytoplasm of neutrophils induced by LTB_4, platelet activating factors, and A_{23187}[10]. In this study, we found that Tet could reduce the numbers of both AM and Neu in BALF, inhibit the release of O_2^- and LTB_4, as well as the elevation of free calcium level in the cytoplasm of AM which is in good agreement with above results. These results suggests that Tet could control the activity of AM and alleviate the inflammatory changes in trachea and lungs. These actions might be attributed to its effect of inhibiting the increase of $[Ca^{2+}]_i$ in AM by prohibiting the inward flow of extracellular calcium ions and/or the release of calcium ions from intracellular stores. The mechanism for actions of Tet on the calcium channels of AM needs to be further explored.

It should be mentioned that at 7 d, although Tet significantly inhibited the increase of WBC, AM, and Neu numbers, the values in both the injured control and the Tet-treated groups were much higher than those of normal control. The reason is not clear at present. It might be due to the secondary infection of the lungs. Further investigation will be carried out.

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粉防己碱对烟雾吸入伤兔肺泡巨噬细胞功能及超微结构的影响

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A 摘要 采用兔重度烟雾吸入伤模型，观察粉防己碱(Tet)对肺泡巨噬细胞(AM)释放炎症介质、内钙水平及超微结构的影响. 结果发现，Tet 可减少 BALF 中 WBC 数目，抑制 AM 释放 LTB_4 和 O_2^-，降低内钙水平；同时使 AM 体积增大，伪足增多和溶酶体排空等改变减轻. 提示：Tet 通过抑制 AM 的活化可能减轻肺损伤.

关键词 粉防己碱；烟雾吸入伤；白三烯 B；超氧阴离子自由基；钙；巨噬细胞