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Effect of tetrandrine on neutrophilic recruitment response to brain ischemia /reperfusion

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KEY WORDS brain ischemia; inflammation; intercellular adhesion molecule-1; NF-kappa B; tetrandrine; reverse transcriptase polymerase chain reaction

ABSTRACT

AIM: To investigate the effect of tetrandrine (Tet) on neutrophilic recruitment response to bain ischemia/reperfusion (I/R). **METHODS**: Middle cerebral artery (MCA) ischemia (2 h)/reperfusion model was built on rats ($\hat{\delta}$). Brain water content, neutrophilic recruitment, the expression of intercellular adhesion molecule-1 (ICAM-1) mRNA and activation of NF-κB in cellular nucleus after brain I/R were measured with dry-wet weight, ^{51}Cr-labeled neutrophil, reverse transcriptase polymerase chain reaction, and electrophoretic mobility shift assay, respectively. **RESULTS**: Brain water and neutrophilic recruitment were parallelly increased from 3 h to 24 h after reperfusion ($P < 0.01$). The expression of ICAM-1 mRNA was detected at 1 h after reperfusion ($P < 0.01$), increased to the highest peak at 12 h ($P < 0.01$), and at 24 h decreased to level at 3 h. The activation of NF-κB was detected at 0.5 h after reperfusion ($P < 0.01$), increased to the highest peak at 6 h ($P < 0.01$), and at 24 h decreased to level at 1 h. Tet 10 and 20 mg/kg decreased brain water content, neutrophilic recruitment, the expression of ICAM-1 mRNA, and the activation of NF-κB at 6 h, 12 h, and 24 h after reperfusion. **CONCLUSION**: Tet inhibited neutrophilic recruitment, expression of ICAM-1 mRNA, and activation of NF-κB after brain I/R.

INTRODUCTION

Accumulated data strongly suggest that the neutrophilic recruitment response to brain ischemia (I)/reperfusion(R) is contributed to secondary neuronal injury after brain I/R through release of cytotoxic products and oxygen free radicals from activated leukocytes, hence, anti-inflammatory therapy is used as an interventive therapy for brain I/R injury[1]. However, the process of neutrophilic recruitment from circulatory blood is a multiple step in sequence, which is facilitated by the multiple molecule such as adhesion molecules, cytokines, and chemokines[2]. After brain I(2 h)/R, intercellular adhesion molecule-1 (ICAM-1) protein is up-regulated from 4 h to 72 h after repufersion, and anti-ICAM-1 antibody can attenuate brain I/R injury in animal experience[3,4]. These data implied that ICAM-1 played an important role in neutrophilic recruitment response to brain I/R. However, it is reported that nuclear factor-κB (NF-κB), an inducible multisubunit transcription factor of higher eukaryotes, provides the linkage between early outcellular signals and expression of inducible genes involved in the inflammatory cascade[5]. Binding sites for NF-κB existed in promoter regions of the genes for E-selectin, vascular cell adhesion moleule-1 (VCAM-1), and ICAM-1[5]. Therefore, it is suggested that the expression of ICAM-1 after brain I/R may be triggered by activated NF-κB.

The cerebral edema response to brain I/R is an important role in death after brain I/R because the cerebral edema allows intracranial hypertension and cerebral hernia, and neutrophilic recruitment may aggravate cerebral edema after brain I/R[6].

Tetrandrine (Tet), which is purified from *Fourstamen stephania* root, has been demonstrated to prevent brain against I/R through antagonizing Ca^{2+} overloading and eliminating free radicals and apoptosis[7]. However, Tet has never been proved to have anti-inflammatory effect through inhibiting expression of cellular adhesion molecule and activation of NF-κB.

Therefore, the purpose of the present study is to determine whether Tet attenuates neutrophilic recruitment re-

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sponse to brain I/R, and inhibites expression of ICAM-1 and activition of NF-κB.

MATERIALS AND METHODS

Drugs and reagents Tet (purity 98 %) was made in Jinhua Pharmaceutical Factory (Zhejiang province, China). Gel shift assay system to measure NF-κB was obtained from Promega Co (USA). ^{51}Cr was made in NEN Co (USA). Two pairs of primer to amplify ICAM-1 cDNA and β-actin cDNA were synthesized in Shanghai Sangon Co (China).

Animals Wistar rats (♂, 150 g - 190 g, Grade Ⅱ, Certificate № 980317), supplied by Laboratory Animal Center (Third Military Medical University), were divided into 5 groups. At the beginning of reperfusion (0 h), Tet 5, 10, and 20 mg/kg was injected ip, respectively and the equal volume of saline was injected ip in vehicle rats. In sham-operated group, the fishing line was only inserted into common carotid for 10 mm but middle cerebral artery (MCA) was not blocked, and other treatments were similar to vehicle group.

Brain I/R In order to block MCA, a fish line (Φ:0.24 - 0.28 mm) was pushed for 20 mm from common carotid, and 2 h later, it was pulled out to recover perfusion as described by Negasawa and Kogure[8].

Brain water content Brain water content in rat was measured with dry-wet weight.

Neutrophilic recruitment Neutrophilic recruitment after brain I/R was measured with ^{51}Cr-labeled neutrophil (kBq/g)[9].

Expression of ICAM-1 mRNA Reverse transcriptase polymerase chain reaction (RT-PCR) was used to determine the level of ICAM-1 mRNA in regions of brain I/R. The integral intensity of ICAM-1 and β-actin bands were determined with Kodak Digital Science 1D software, and quantity of ICAM-1 mRNA was expressed as ratio of integral intensity of ICAM-1 to integral intensity of β-actin[3,10]. The sequence of primer used in RT-PCR analysis and predicted base pair (bp) size of fragment were shown in Tab 1.

Activation of NF-κB Cell nuclei were separated from cytoplasm[11]. Electrophoretic mobility shift assay (EMSA) was used to determine the level of activated NF-κB in cell nuclei according to Promega's directions and quantity of activated NF-κB was expressed as integral intensity of NF-κB, which were determined with Kodak Digital Science 1D software.

Statistical analysis Results were expressed as $\bar{x} \pm s$ and analyzed using ANOVA followed by Student-Newman-Keul's test.

Tab 1. The sequence of primer and predicted base pair (bp) size of fragments in RT-PCR.

mRNA	bp	Primer sequence
ICAM-1	598	5′-AGG TGT GAT ATC CGG TAG AC-3′
		5′-CCT TCT AAG TGG TTG GAA CA-3′
β-Actin	318	5′-TGT ACG TAG CCA TCC AGG CT-3′
		5′-TTC TCC AGG GAG GAA GAG GA-3′

RESULTS

Brain water content The brain water content of sham-operated rats was not changed. There was no significant difference between vehicle group at 1 h after reperfusion and sham-operated group ($P > 0.05$, Tab 2). At 3 h, 6 h, 12 h, and 24 h after reperfusion, brain water content was increased in I/R group, respectively as compared with sham-operated group ($P < 0.01$). There was no significant difference between Tet 5 mg/kg-treated group and vehicle group ($P > 0.05$). When the dose of Tet was elevated to 10 and 20 mg/kg, the brain water content was decreased ($P < 0.01$, Tab 2).

The neutrophilic recruitment The neutrophilic recuitment of sham-operated group was not changed. There was no significant difference between vehicle group at 1 h after reperfusion and sham-operated group ($P > 0.05$), but at 3 h, 6 h, 12 h, and 24 h after reperfusion, neutrophilic recruitment in vehicle group was increased ($P < 0.01$, Tab 3). Tet 5 mg/kg had no effects on neutrophilic recruitment at 6 h, 12 h, and 24 h after reperfusion ($P > 0.05$), but Tet 10 and 20 mg/kg reduced neutrophilic recruitment by 36 % and 66 %, 45 % and 70 %, 47 % and 62 % ($P < 0.01$), respectively (Tab 3).

Expression of ICAM-1 mRNA Expression of ICAM-1 mRNA in vehicle group was detected at 1 h after reperfusion but not in sham-operated group (Tab 4, Fig 1). The level of ICAM-1 mRNA in vehicle group at 3 h, 6 h, and 12 h after reperfusion was as 2.1, 2.8, and 4.9 times as that at 1 h, respectively ($P < 0.01$), and at 24 h it was decreased to the level at 3 h ($P > 0.05$, Tab 4, Fig 1). There was no significant difference between Tet 5 mg/kg-treated group and vehicle group at 6 h, 12 h, and 24 h after reperfusion ($P > 0.05$, Tab 4, Fig 1).

The levels of ICAM-1 mRNA in Tet 10 and 20 mg/kg treated group, at 6 h, 12 h, and 24 h after reperfusion, were decreased by 40 % and 74 %, 39 % and 58 %, 32 % and 57 %, respectively, as compared with those of vehicle group ($P<0.01$, Tab 4, Fig 1).

Activation of NF-κB Activation of NF-κB was detected in vehicle group at 0.5 h after reperfusion but not in sham-operated group (Tab 5, Fig 2). There was no significant difference between the level of activated NF-κB at 1 h and that at 0.5 h after reperfusion ($P>0.05$), and at 3 h, 6 h, and 12 h it was as 2.2, 2.6, and 1.6 times as that at 1 h, respectively ($P<0.01$), and at 24 h it was decreased to the level at 1 h ($P>0.05$) (Tab 5, Fig 2). There was no significant difference between Tet 5 mg/kg-treated group and vehicle group at 6 h, 12 h, and 24 h after reperfusion ($P>0.05$, Tab 5, Fig 2). The levels of activated NF-κB in Tet 10 and 20 mg/kg-treated group were decreased by 38 % and 70 %, 48 % and 65 %, 42 % and 75 % at 6 h, 12 h, and 24 h after reperfusion, respectively, as compared with vehicle group ($P<0.01$, Tab 5, Fig 2).

DISCUSSION

The present experience showed that neutrophilic recruitment and cerebral edema were parallelly increased after brain I/R, which was consistent with previous reports[3]. In addition, they were parallelly decreased by Tet. Therefore it is indicated that cerebral edema is

Tab 2. Effect of tetrandrine on brain water content after ischemia (2 h)/reperfusion. $n=6$ **rats.** $\bar{x}\pm s$. $^{a}P>0.05$, $^{c}P<0.01$ *vs* **sham-operated group at the same time points.** $^{d}P>0.05$, $^{f}P<0.01$ *vs* **vehicle group at the same time points.**

Groups	Brain water content/% 1 h	3 h	6 h	12 h	24 h
Sham-operated	75.1±1.6	75.4±2.0	74.6±1.7	75.0±1.6	75.0±0.9
Vehicle	70.0±1.0[a]	79.6±1.0[c]	82.1±0.9[c]	86.1±1.0[c]	88.4±1.3[c]
Tet 5 mg/kg	–	–	82.4±0.6[cd]	86.8±1.5[cd]	88.9±1.1[cd]
Tet 10 mg/kg	–	–	79.6±1.5[cf]	83.5±1.4[cf]	85.4±1.2[cf]
Tet 20 mg/kg	–	–	77.0±1.0[cf]	79.9±1.0[cf]	82.3±1.6[cf]

Tab 3. Influence of tetrandrine on neutrophilic recruitment of ischemia (2 h)/reperfusion. $n=6$ **rats.** $\bar{x}\pm s$. $^{a}P>0.05$, $^{c}P<0.01$ *vs* **sham-operated group at the same time points.** $^{d}P>0.05$, $^{f}P<0.01$ *vs* **vehicle group at the same time points.**

Groups	Neutrophilic recruitment/kBq·g^{-1} 1 h	3 h	6 h	12 h	24 h
Sham-operated	0.16±0.07	0.17±0.08	0.15±0.10	0.14±0.11	0.16±0.09
Vehicle	0.15±0.05[a]	0.43±0.07[c]	0.87±0.13[c]	1.10±0.25[c]	1.70±0.27[c]
Tet 5 mg/kg	–	–	0.85±0.19[cd]	1.1±0.16[cd]	1.7±0.23[cd]
Tet 10 mg/kg	–	–	0.56±0.09[cf]	0.60±0.11[cf]	0.90±0.17[cf]
Tet 20 mg/kg	–	–	0.30±0.06[cf]	0.33±0.09[cf]	0.64±0.10[cf]

Tab 4. Effect of tetrandrine on ICAM-1 mRNA after ischemia (2 h)/referpusion. $n=6$ **rats.** $\bar{x}\pm s$. $^{c}P<0.01$ *vs* **vehicle at 1 h reperfusion time point.** $^{d}P>0.05$ *vs* **vehicle at 3 h referpusion time point.** $^{g}P>0.05$, $^{i}P<0.01$ *vs* **vehicle group at the same time point.**

Groups	Level of ICAM-1 mRNA 1 h	3 h	6 h	12 h	24 h
Sham-operated	0	0	0	0	0
Vehicle	0.35±0.05	0.75±0.11[c]	0.99±0.14[c]	1.73±0.26[c]	0.82±0.15[cd]
Tet 5 mg/kg	–	–	1.00±0.19[g]	1.67±0.26[g]	0.83±0.13[g]
Tet 10 mg/kg	–	–	0.59±0.10[i]	1.06±0.14[i]	0.56±0.09[i]
Tet 20 mg/kg	–	–	0.26±0.05[i]	0.73±0.11[i]	0.35±0.07[i]

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Tab 5. Effect of Tet on activation of NF-κB after ischemia (2 h)/reperfusion. $n = 6$ rats. $\bar{x} \pm s$. $^aP > 0.05$, $^cP < 0.01$ *vs* vehicle at 1 h after reperfusion. $^dP > 0.05$, $^fP < 0.01$ *vs* vehicle group at the same time point.

Groups	10^{-3} × Level of activated NF-κB					
	0.5 h	1 h	3 h	6 h	12 h	24 h
Sham-operated	0	0	0	0	0	0
Vehicle	1.51 ± 0.23^a	1.34 ± 0.21	2.8 ± 0.4^c	3.4 ± 0.7^c	2.1 ± 0.4^c	1.23 ± 0.11^a
Tet 5 mg/kg	–	–	–	3.4 ± 0.6^d	2.1 ± 0.3^d	1.13 ± 0.23^d
Tet 10 mg/kg	–	–	–	2.1 ± 0.4^f	1.10 ± 0.24^f	0.71 ± 0.11^f
Tet 20 mg/kg	–	–	–	1.01 ± 0.23^f	0.73 ± 0.11^f	0.31 ± 0.09^f

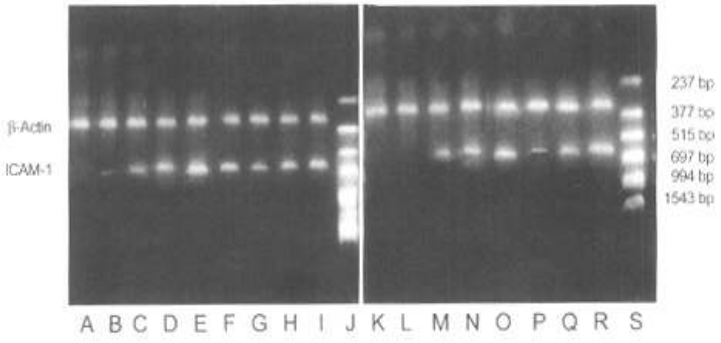

Fig 1. RT-PCR of ICAM-1 mRNA after ischemia/reperfusion. J and S: Markers. A, K, and L: Sham-operated groups; B, C, D, E, and F: Vehicle group at 1 h, 3 h, 6 h, 12 h, and 24 h after reperfusion, respectively; M, N, and O: Tet 20, 10, and 5 mg/kg at 6 h after reperfusion, respectively; G, H, and I: Tet 20, 10, and 5 mg/kg at 12 h after reperfusion, respectively; P, Q, and R: Tet 20, 10, and 5 mg/kg at 24 h after reperfusion, respectively.

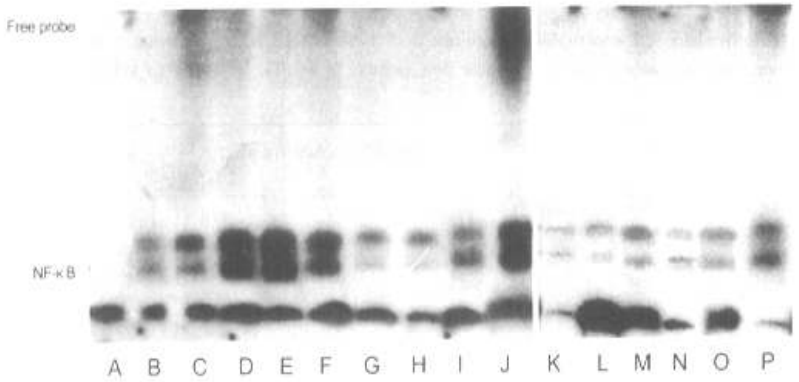

Fig 2. Electrophoretic mobility shift assay of NF-κB after ischemia/reperfusion. A: Sham-operated group; B, C, D, E, F, and G: Vehicle group at 0.5 h, 1 h, 3 h, 6 h, 12 h, and 24 h after reperfusion, respectively; H, I, and J: Tet 20, 10, and 5 mg/kg at 6 h after reperfusion, respectively; K, L, and M: Tet 20, 10, and 5 mg/kg at 12 h after reperfusion, respectively; N, O, and P: Tet 20, 10, and 5 mg/kg at 24 h after reperfusion, respectively.

closely relevant to neutrophilic recruitment after brain I/R. Neutrophilic recruitment may aggravate cerebral edema via releasing oxygen free radicals from neutrophil to injury blood-brain barrier and releasing inflammatory agents to increase permeability of microvessels of brain[12]. Also, it is resulted from these facts that Tet attenuated, but not completely, neutrophilic recuitment and cerebral edema response to brain I/R. The inhibitory effects of Tet on neutrophilic recruitment and cerebral edema may be relevant to its effect of eliminating oxygen free radicals and inflammatory agents.

In the present study, level of ICAM-1 mRNA was

increased by several folds after brain I/R, which was consistent with former report[3], and level of activated NF-κB was parallelly increased. In addition, NF-κB response to brain I/R was activated before ICAM-1 mRNA was expressed, and they were parallelly decreased by Tet. So there is a very important relationship between expression of ICAM-1 mRNA and activation of NF-κB after brain I/R, and Tet is an inhibitor of NF-κB.

NF-κB plays a crucial role in the inflammatory cascade response to brain I/R. On activation by a wide range of extracellular agents after brain I/R, such as IL-1β, TNF-α, and oxgen free radicals, NF-κB triggers the expression of inflammation genes such as ICAM-1, IL-1β, and TNF-α mRNA[5]. In these extracellular signals, expression of IL-1 and TNF-α are induced by activation of NF-κB, on the other hand, oxygen free radicals are not produced through expression of genes. Therefore, oxygen free radicals may activate NF-κB as a preformed outcellular signal to trigger inflammation, and IL-1 and TNF-α produced by NF-κB may amplify the NF-κB signaling system. Hence, Tet inhibited activation of NF-κB by eliminating oxygen free radicals, and prevented amplification of inflammation by inhibiting IL-1 and TNF-α production. As NF-κB triggers largely inducible genes, which control synaptic transcription, neuronal plasticity, neuronal development, and neurodegenerative disease[5], Tet as an inhibitor of NF-κB activation will possess other important pharmacologic actions.

In conclusion, Tet inhibited expression of neutrophilic recruitment and ICAM-1 mRNA and activation of NF-κB after brain I/R.

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粉防己碱对脑缺血/再灌注导致的中性粒细胞募集反应的作用

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关键词 脑缺血; 炎症; 细胞间粘附分子-1; NF-κB; 粉防己碱; 逆转录聚合酶链反应

目的: 探讨粉防己碱对脑缺血/再灌注(I/R)后中性粒细胞募集反应的作用及分子机制. **方法**: 大鼠(♂)脑中动脉线栓闭塞(2 h)/再通法建立局灶性脑缺血/再灌注模型. 采用干湿重法、^{51}Cr标记中性粒细胞法、逆转录聚合酶链反应和凝胶迁移电泳分别检测脑组织含水量、中性粒细胞募集、细胞间粘附分子-1 (ICAM-1) mRNA 转录的水平以及细胞核内激活的核因子-κB (NF-κB)的水平. **结果**: 脑含水量和中性粒细胞募集数量从再灌注 3 h 到 24 h 平行增加. 在再灌注 1 h 可检测到 ICAM-1 mRNA 的表达, 12 h 达到最高值, 24 h 下降到 3 h 的水平. 在再灌注 0.5 h 可检测到 NF-κB 的激活, 6 h 达到最高值, 24 h 下降到 1 h 的水平. 在再灌注 6 h、12 h 和 24 h, 粉防己碱(10 和 20 mg/kg)可抑制这些变化. **结论**: 粉防己碱可抑制脑缺血/再灌注后中性粒细胞的募集, ICAM-1 mRNA 的转录和 NF-κB 的激活.

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