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Focal Cerebral Ischemia Induces Alzheimer's Disease-like Pathological Change in Rats*

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Summary: The changes in the tau protein phosphorylation and expression of bcl-2, and bax in rat parietal cortex neurons after focal cerebral ischemia-reperfusion (I/R) were explored, and the relationship between the tau protein phosphorylation and the expression of bax or apoptosis was clarified in order to elucidate the relationship between cerebral infarction and Alzheimer's disease. The rat focal cerebral I/R model was induced by occlusion of the right middle cerebral artery using the intraluminal suture method. The level of tau protein phosphorylation at Ser396, Ser404, Tyr231, Ser199/202 sites and the expression of bcl-2, bax and total tau 5 in rat parietal cortex during focal cerebral ischemia/reperfusion were detected by Western blot. The relationship between the tau protein phosphorylation and the expression of bax, or apoptosis was examined by TUNEL method and double-labeling immunofluorenscence method. The results showed that the level of tau hyperphosphorylation at Ser199 / 202, Ser396, Ser404, Tyr231 sites and the expression levels of bcl-2, and bax were significantly higher in I/R group than in the sham group, but the ratio of bcl-2/bax was decreased. Neuronal apoptosis, bax expression and the tau protein hyperphosphorylation were co-localized. It is suggested that Alzheimer's disease-like pathological changes occur after cerebral I/R. The highly abnormal phosphorylation of tau protein plays a key role in cerebral I/R-induced apoptosis. The cerebral infarction may contribute to Alzheimer's disease occurrence and development.
Key words: cerebral ischemia; Alzheimer's disease; tau protein; phosphorylation; apoptosis; bcl-2; bax

Epidemiological studies show that the prevalence of dementia in ischemic stroke patients is remarkably higher than in control subjects[1, 2]. Many of these dementias develop progressively, and cerebral damage is not the direct cause of the subsequent dementia in over half of these cases[3]. Alzheimer's disease (AD) is the most common cause of senile dementia, and other studies claimed that the vascular risk factors of stroke could increase the morbidity of AD[4]. But the relationship between stroke and AD is unclear by now.

Tau protein is a microtubule-associated protein that is expressed abundantly in the nervous system, and plays a key role in stabilizing microtubules, promoting microtubule assembly, keeping the shapes of neurons and promoting axonal transport. Abnormal functional changes of tau protein may be an essential part of neural dysfunction and neural necrosis. The balance between phosphorylation and dephosphorylation of tau protein is a key modulating factor of microtubule stabilizing. Hyperphosphorylated tau will form paired helical filament (PHF), which leads to the characteristic pathological change of AD-neurofilament tangles (NFTs)[5]. NFTs-like changes and loss of neurons are the typical pathological features of AD. Previous studies also found that tau hyperphosphorylation and NFTs formation were associated with neurocyte apoptosis in AD patients[6]. The expression of apoptosis related genes, bcl-2 and bax, is correlated with hyperphosphorylated tau protein positive cells[7, 8]. Bcl-2 and bax expression changes after cerebral ischemia[9, 10], and cerebral ischemia induces the aberrant hyperphosphorylation of tau protein as well. So we presume that the post-ischemic neurocyte apoptosis may have correlation with the aberrant hyperphosphorylation of tau protein, and they may share the same process with AD by regulating the apoptosis related genes, bcl-2 and bax. Cerebral ischemia may induce AD's progression through hyperphosphorylation of tau protein and neurocyte apoptosis.

In the present study, we further illustrated the relationship between stroke and AD in a rodent model for transient cerebral ischemia, by examining specific phosphoryla-

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tion sites of tau protein and the degree of phosphorylation, the dissolubility of phosphorylated tau protein, the correlation among tau phosphorylation, post-ischemic neuronal apoptosis and expression of bax after cerebral ischemia.

1 MATERIALS AND METHODS

1.1 Main Reagents and Equipment

Tau 5, tau (pSer404), tau (pSer199/202), tau (pTyr231) antibodies were purchased from Biosource International Inc. (USA). bcl-2, bax, tau (pSer396), rabbit anti-rat GAPDH monoclonal antibodies were from Cell Signal Technology (USA). Rhodamine labeled rabbit anti-rat IgG and rabbit anti-mouse fluorescence secondary antibody, BCA protein assay kit and Western blot chemiluminescent kit were purchased from Pierce Biotechnology (USA). TUNEL apoptosis assay kit was purchased from Beyotime Institute of Biotechnology (China). All chemicals were from Sigma Aldrich (USA). Olympus laser scanning confocal microscope electrophoresis apparatus (Japan) and electrophoretic transfer cell were purchased from Biometro (Germany). Freezing microtome was from Thermo Electron Corporation (USA).

1.2 Grouping of Animals

Fifty-seven clean male Sprague-Dawley (SD) rats weighing from 200 to 250 g were provided by the Laboratory Animals Department of Tongji Medical College, Huazhong University of Science and Technology (China) and maintained in temperature-and-humidity-controlled rooms (22–25°C, 60%–70%) with 12 h light-dark cycles. All rats had free access to laboratory chow and tap water but were deprived of solid food for 12 h before surgery. All rats were divided randomly into 6 groups: 5 I/R groups and one control group (sham group). According to the execution time after exposure to cerebral ischemia (1 h, 6 h, 12 h, 24 h, 3 days), I/R groups were subdivided into IR-1 h, IR-6 h, IR-12 h, IR-24 h, IR-3 day. Every single group includes 6 rats except that IR-3 day and sham groups consisted of 21, 12 ones respectively.

1.3 Establishment of Animal Models

Referring to the thread occlusion model of MCAO (introduced by Longa *et al*[11]), a 3-0 monofilament nylon suture was introduced into the right middle cerebral artery (MCA) lumen with a depth of (20±3) mm through a puncture at the internal carotid artery (ICA) after the rats were anesthetized with 10% chloral hydrate (350 mg/kg). The suture was withdrawn from the ICA at the 1st h after surgery. All rats were scored for the extent of neurological dysfunction after recovery from anesthesia: 0 score: no neurological function deficiency; 1 score: failing to extend contralateral forelimbs fully; 2 scores: contralateral circling while moving about; 3 scores: falling down to the contralateral side while moving about; 4 scores: inactive or unconscious. Those rats with 1–3 scores were effective models, and those died and eliminated rats were supplied randomly during observation period. Carotid artery was not inserted with fishing line in sham group, and the rest procedures ware the same as the surgical group.

1.4 Immunoblotting

Rats were sacrificed on ice after anesthetization with an intraperitoneal injection. Brains were removed and right parietal cortex was preserved in refrigerator (−70°C). Samples were homogenized and lysed on ice and then centrifuged at 15 000×*g* for 15 min. Supernatants were collected for quantification (BCA method). Samples were separated on 10% gradient SDS-polyacrylamide gels and electrophoretically transferred to a nitrocellulose membrane. Membrane was blocked with 10% bovine serum albumin (BSA) and subsequently incubated with primary antibodies overnight (4°C) followed by incubation with the corresponding peroxidase-conjugated secondary antibody for 2 h. Immunoreactivity was visualized by enhanced chemiluminescence detection. Densitometric analysis was performed using Image J software. The experiment was repeated three times.

1.5 Detection of Undissolved tau

A total of 20 mg right frontoparietal cortex was homogenized on ice in 100 μL of RIPA buffer (50 mmol/L Tris-HCl, pH 8.0, 150 mmol/L NaCl, 20 mmol/L EDTA, 1% Nonidet-P40 [v/v], 50 mmol/L NaF, 0.25% Na-Deoxycholate, and 1 mmol/L PMSF) and shaken at 4°C for 1 h before centrifugation at 11 300×*g* for 20 min. Precipitates were washed with RIPA buffer and re-suspended in 50 μL (or half volume of RIPA buffer) of 70% formic acid (FA). The re-suspended samples were centrifuged at 11 300×*g* for 20 min at 4°C, and then supernatants were collected. After dried by a vacuum pump, those sediments were re-suspended and dissolved with 50 μL of SDS-PAGE buffer (240 mmo/L Tris-HCl, pH 6.8, 6% SDS, 30% glycerol, 0.06% bromophenol blue), which were marked as "FA parts". The "FA parts" represented the relatively undissolved tau. Immunoblotting analysis was carried out after the "FA parts" were separated on 10% gradient SDS-polyacrylamide gels. The experiment was repeated three times.

1.6 TUNEL Staining and Immunofluorescence

Fifteen rats in IR-3 day group were anesthetized with an intraperitoneal injection of chloral hydrate and perfused transcardially with 4% paraformaldehyde. Brains were harvested by cutting heads and post-fixed for 6 h. After cryoprotected in 30% sucrose solution, fixed brains were dissected coronally into 10-μm thick slices on a freezing microtome. Sections with a maximal transverse diameter were selected and incubated with 0.1% Triton X-100 for 15 min. After washing twice in PBS, the samples were mixed respectively with tau (pTyr231), tau (pSer396) or bax antibody (at a dilution of 1:100) for an overnight incubation at 4°C. Then fluorescence secondary antibody was incubated with samples for 60 min in a dark chamber at 37°C, and these sections were sealed with cover slips after rinsed with PBS. As for apoptosis detection, an additional step was made that samples were incubated with 50 μL of TUNEL inspection fluid for 60 min before rinsed three times with PBS. Photographs were taken with a laser scanning confocal microscope. Five high power fields were randomly sampled and positive cells were calculated for every slice and three slices from each brain.

1.7 Statistical Analysis

All values represented $\bar{x}\pm s$ of the number of separate experiments performed in duplicate, as indicated in the corresponding figures. Comparisons between groups were made using one-way analysis of variance (ANOVA). The

correlation between two groups was tested using linear correlation and regression analysis. All data were analyzed by SPSS12.0 software package with $P<0.05$ as the minimum significant level.

2 RESULTS

2.1 Immunoblotting

2.1.1 Detection of tau Phosphorylation The immunoblotting results of tau (pTyr231), tau (pSer396), tau (pSer404) and tau (pSer199/202) at different time points after I/R showed that there was an enhancement of tau phosphorylation at 6 h after reperfusion, which were significantly different from controls. The phosphorylation reached the peak at I/R 24 h and showed no descent at IR 3 day. The total amount of tau detected with tau 5 at different time points exhibited no significant difference (fig. 1).

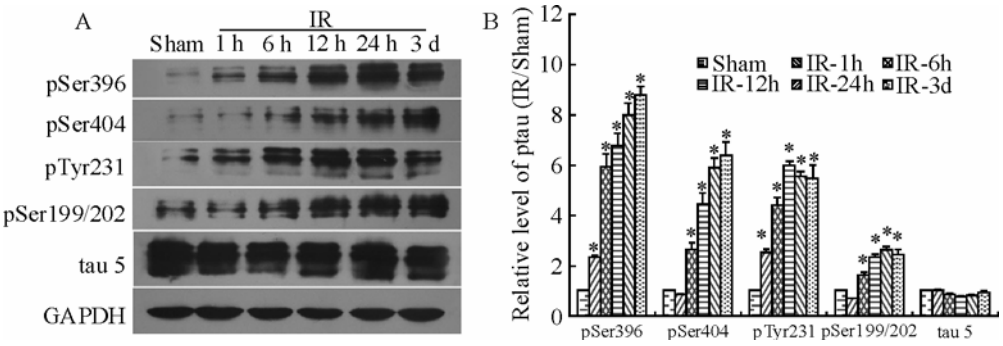

Fig. 1 The phosphorylation of tau at different time points after I/R and the expression change of tau 5
A: Electrophoregram; B: Consequence analysis chart. $^{*}P<0.05$ *vs* sham group

2.1.2 Detection of Undissolved Phosphorylated tau The "FA parts" (undissolved tau extracted by formic acid) in IR-3 day group and control group were detected with tau (pTyr231) and tau (pSer404) antibodies. Immunoblotting results revealed that the gray intensity representing the amount of undissolved tau was significantly higher in IR-3 day group than in controls (fig. 2).

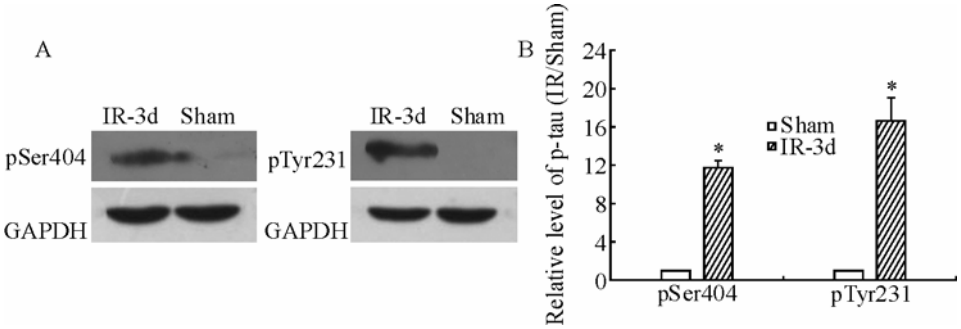

Fig. 2 Changes of undissolved tau at different time points after cerebral I/R
A: Electrophoregram; B: Consequence analysis chart. $^{*}P<0.05$ *vs* sham group

2.1.3 Expression of bcl-2 and bax There was significantly enhanced expression of bcl-2 at IR-24 h, and no descent at IR-3 day. The expression of bax was gradually enhanced after reperfusion and reached the peak at IR-24 h, which was not decreased at IR-3 day, whereas the ratio of bcl-2/bax began to reduce at IR-1 h, and a remarkable descent was shown from IR-24 h to IR-3 day (fig. 3).

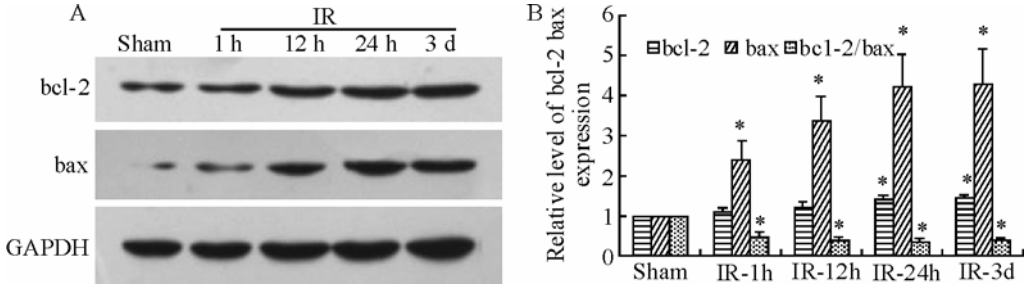

Fig. 3 The expression changes of bcl-2 and bax at different time points before and after reperfusion and change of bcl-2/bax ratio
A: Electrophoregram; B: Consequence analysis chart. $^{*}P<0.05$ *vs* sham group

2.2 Correlation between TUNEL Assay and tau Phosphorylation

Using tau (pTyr231), tau (pSer396) antibodies and TUNEL to research the conformational relationship between neurocyte apoptosis and phosphorylation of tau, it was found that there was an expressive co-localization

between phosphorylation of tau (pTyr231), tau (pSer396) and neurocyte apoptosis in IR-3 day group (fig. 4). The correlation analysis showed that there was a significant positive relationship between phosphorylation of tau (pTyr231), tau (pSer396) and neurocyte apoptosis. *R* value was 0.930 and 0.977 respectively (fig. 5).

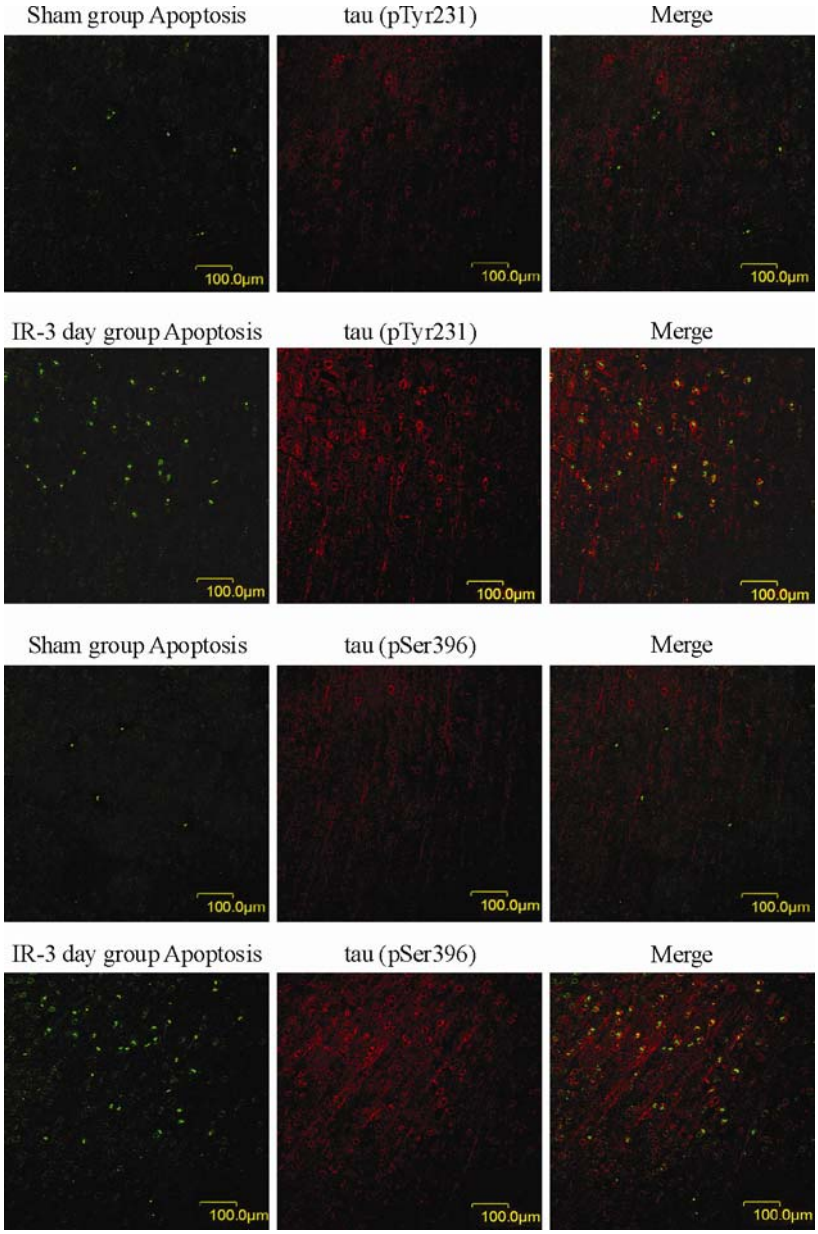

Fig. 4 Co-localization expression of tau (pTyr231), tau (pSer396) and apoptotic nerve cells in IR-3 day group

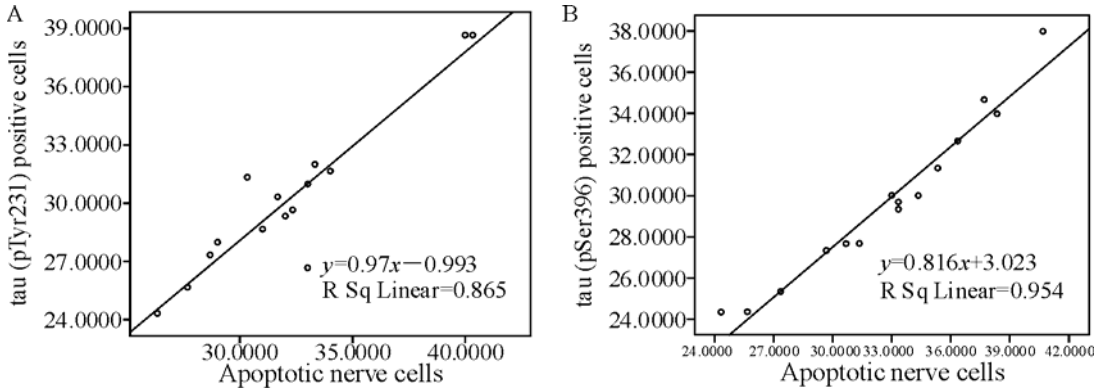

Fig. 5 Correlation analysis of tau (pTyr231)(A), tau (pSer396)(B) positive cells and apoptotic nerve cells in IR-3 day group

2.3 Immunofluorescence Double-labeling of bax Expression and tau Phosphorylation

Using tau (pTyr231), tau (pSer396) antibodies and bax antibody to research the conformational relationship between the expression of bax and phosphorylation of tau, it was found that there was an expressive co-localization between phosphroylation of tau (pTyr231), tau (pSer396) and bax expression in IR-3 day group (fig. 6). The correlation analysis showed that there was a significant positive relationship between phosphroylation of tau (pTyr231), tau (pSer396) and bax expression. *R* value was 0.914 and 0.903 respectively (fig. 7).

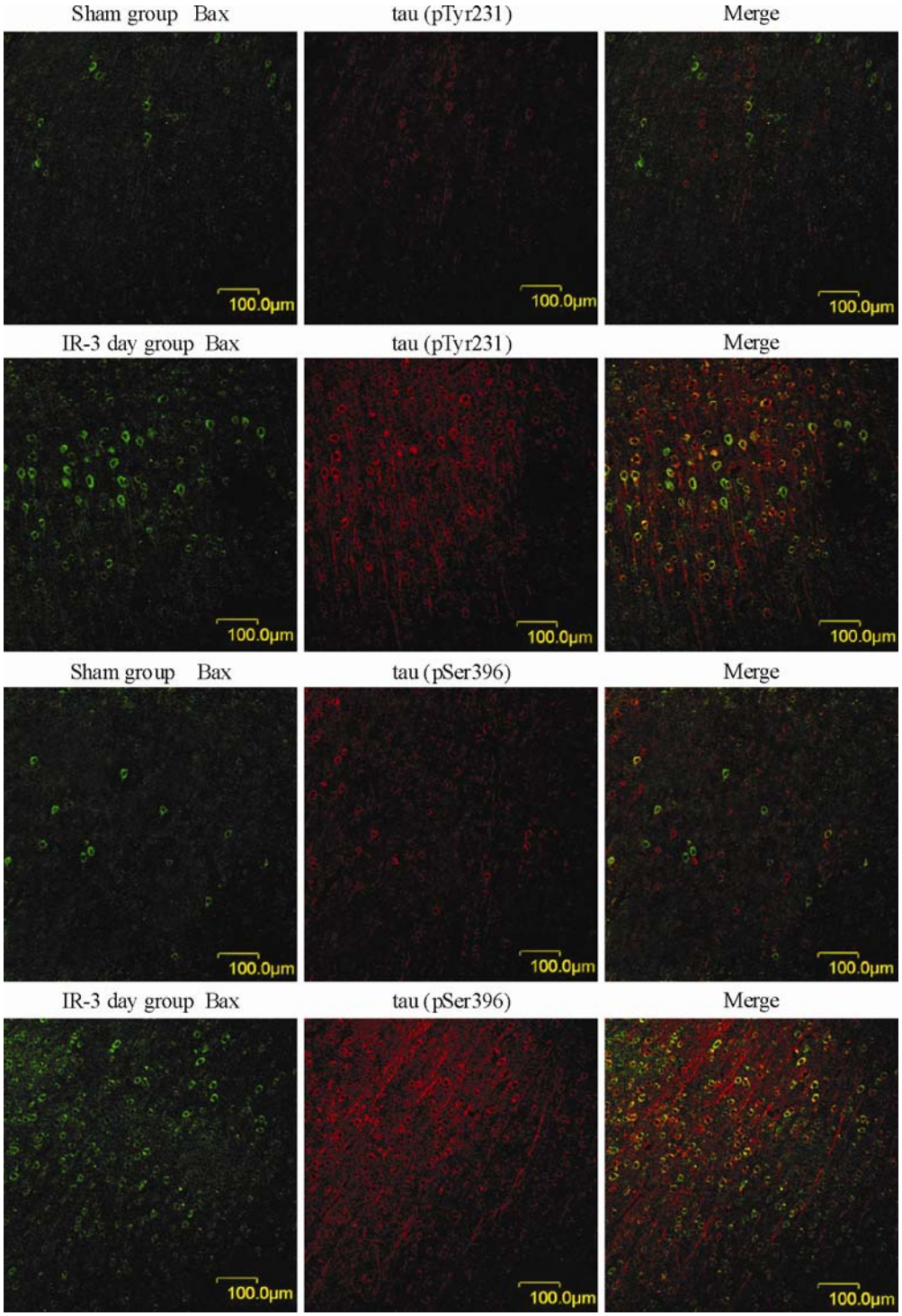

Fig. 6 Co-localization expression of tau (pTyr231), tau (pSer396) and bax expression

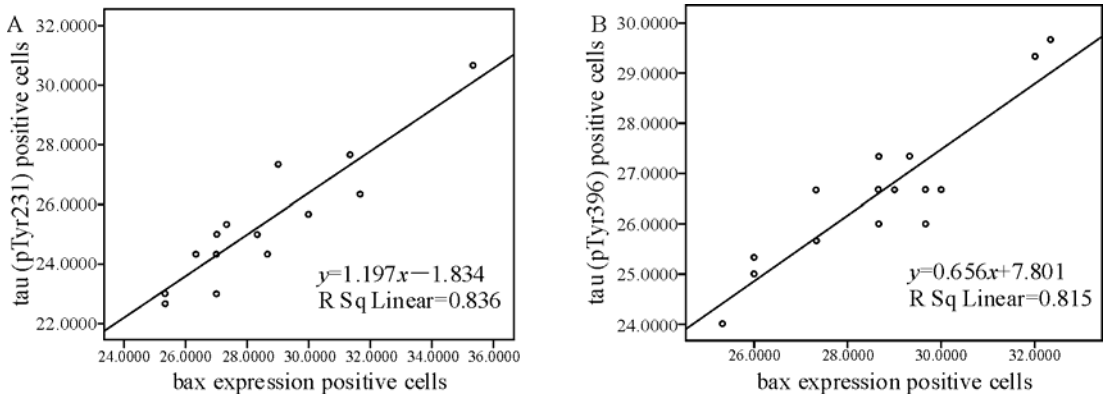

Fig. 7 Correlation analysis of tau (pTyr231)(A), tau (pSer396) (B) positive cells and bax expression in IR-3 day group

3 DISCUSSION

AD was regarded as a neurodegenerative disease which had no connection with cerebrovascular disorders formerly, whereas recent epidemiological studies and clinical researches supported that cerebral ischemia was correlated to AD onset. The role of ischemia played in this course is emphasized now. Recent clinical and fundamental researches found that the relationship between AD and cerebrovascular disorders was much closer than before. Cerebral ischemia can help AD progress by worsening the cognitive handicap of AD patients [12, 13]. Risk factors such as hypercholesteremia[14], artherosclerosis[15] and hypertension[16] etc, which aggravate vascular disorders, can also increase the morbidity of AD. NFTs formed by aberrant phosphorylated tau are the hallmark lesions of AD. In this study we found that tau proteins in rodents' cortex were aberrantly hyperphosphorylated and undissolved phosphorylated tau proteins were also detected after I/R injury, as indicated that cerebral I/R-induced AD-like pathological changes and cerebral ischemia might be a cause of AD.

There are three kinds of tau proteins in AD brains: (1) dissoluble, non-aberrant phosphorylated tau (C-tau); (2) dissoluble, aberrant phosphorylated tau (AD p-tau); (3) undissolvable PHF-form tau (PHF-tau). The biological activities of C-tau and normal tau are alike. AD p-tau is biologically inactive but doesn't form PHF. PHF-tau is aberrant phosphorylated tau extracted from NFTs[17]. So we further confirmed that tau proteins were not only hyperphosphorylated but also led to AD's NFTs-like changes after I/R. This suggested that stroke didn't merely promote the progression of AD, but could also be the initial factor of it, which provided basic evidence for studying the role of stroke played in AD's etiology. Former studies reported that the PHF-1 site of tau (Ser396/404) was found to be highly phosphorylated[18] after ischemic attack in rodent's hippocampus, and the discovery that AD's hallmark pathological changes–Alz50 existed in the adjacent area around cerebral infarction[19] supported our conclusion. We also confirmed that specific sites of tau would be aberrantly phosphorylated after I/R in rodents, which was reported by Wen *et al*.

Many researches proved that cerebral ischemic/hypoxic injury could not only induce neuronal necrosis, but also lead to neuronal apoptosis. The apoptotic cells contain not only neurons but also astrocytes. In the present study, hyperphosphorylated tau was found co-existing with apoptotic neurons at the 72nd h after reperfusion, and these apoptotic neurons were highly correlated to the tau hyperphosphorylation. Now we can not determine the causal relationship between neurocyte apoptosis and tau hyperphosphorylation, but hyperphosphorylated tau partly lose the ability to bind to microtubules and promote microtubule assembly, and aberrant tau proteins also prevent MAPs from binding to microtubules, which leads to microtubule depolymerization, axonal transport inhibition, neuronal cytoskeleton instability and acceleration of cell apoptosis[20]. Hyperphosphorylated tau only exists temporarily. It was dephosphorylated and degraded at the end of cell apoptosis. So, hyperphosphorylated tau and neurocyte apoptosis were correlated but not independent from each other. Tau hyperphosphorylation may probably be the initial factor of apoptosis.

Studies showed that cell apoptosis was involved in the course of AD onset, and the mechanism of neuronal loss was correlated to cell apoptosis[21], which co-existed with tau hyperphosphorylation[22]. The expression products of apoptosis inhibiting gene bcl-2 and apoptosis promoting gene bax were gathered in the senile plaques, and were highly expressed in the tau-labeled NFTs, suggesting that bax was involved in the NFTs formation[23, 24]. Guise *et al* found that hyperphosporylated tau inhibited the anti-apoptotic activity of bcl-2 and became an important factor promoting cell apoptosis during their studies in which they induced SK-N-SH neuroblastoma cells to express hyperphosphorylated tau[25]. Papers also reported that change of bcl-2/bax ratio was correlated to the Okadaic acid-induced tau hyperphosphorylation and neuronal apoptosis[26]. The apoptosis related genes bax[27], P53 and bcl-2 which took part in AD onset also regulated the post-ischemic cell apoptosis, and tau hyperphosphorylation as well as neurocyte apoptosis also happened after cerebral ischemia. The apoptosis-related genes bcl-2 and bax played important roles in the course of neuronal apoptosis in AD brains.

Bcl-2 and bax are apoptosis regulating genes

which are functionally contradictory. As a member of bcl-2 gene family, bax is regarded as the most important apoptosis promoting gene[28], which can inhibit the anti-apoptotic activity of bcl-2 and promote cell apoptosis by forming a dimer with bcl-2. Cell apoptosis is inhibited when bcl-2/bax ratio increases, and vice versa[29]. Both of their expression levels are enhanced after I/R but bax expression amplified larger than bcl-2, so the ratio of bcl-2/bax is declined and cell apoptosis is promoted. In this study, not only the AD-like pathological changes induced by hyperphosphorylated tau in rodents' cortex were observed, but also the expressive co-localization between hyperphosphorylated tau and neurocyte apoptosis (or bax expression) was confirmed. Cerebral ischemia takes part in the course of AD onset by inducing cell apoptosis through hyperphosphorylated tau. This, on the other hand, proved that the characteristic pathological changes of AD took place after cerebral I/R. The equilibrium system made up of bax and bcl-2 plays an important role in AD and cerebral ischemia treatment. However, bax and bcl-2 are not the only factors which promote or inhibit cell apoptosis. There are other apoptosis modulating genes participating in this course, and their relationships need to be further illustrated.

Thus, to sum up the points which we have just indicated, cerebral ischemia can induce and promote the AD-like pathological changes, which not only provides theoretical basis for the significantly increased morbidity of dementia after stroke, but also inaugurate a new way for the therapy of post-ischemic dementia. Active prevention from cerebral ischemia may slow down the progression of AD.

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