Nrf2 knockout: The effect on neurological dysfunction and the activation of glial cells of mice after brain injury

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Abstract: To investigate the protective role and possible mechanisms of Nrf2 gene in cerebral trauma in mice. The types Nrf2(-/-) and Nrf2(+/+) mice were confirmed by PCR, and the model of closed head injury was established. The severity of injury and the effect of the injury on neurological status were assessed by Neurological Severity Score (NSS) and fatality rate, and the activated conditions of microglia and astrocyte around the injured area were observed by immunohistochemical method. Compared with Nrf2(+/+) mice, the nerve dysfunction of the Nrf2(-/-) mice was obviously more severe ($P<0.01$). On the first day after injury, the activation of microglia around the injured area increased significantly in Nrf2 (-/-) mice, the difference was more significant on the third day, and there was still statistical difference until the 7th day ($P<0.05$). Moreover, On the days 1, 3, 7 after injury, the activation of astrocyte around the injured area also increased in Nrf2(-/-) mice, however, there was statistical difference only on the 3rd day ($P<0.05$). Nrf2 gene knockout can aggravate the nerve dysfunction after cerebral trauma, and this effect is achieved, at least partly, possibly via the effect of Nrf2 on glial activation.

Keywords: Craniocerebral trauma; nerve dysfunction; glia activation; Nrf2 gene.

INTRODUCTION

Transcription factor NF-E2-related factor 2 (Nuclear factor E2-related 2, Nrf2) is a transcription factor. Studies have shown that brain injury can activate Nrf2-ARE pathway (Yan *et al*., 2008). Yan *et al* (2008) adopted Feeney free fall method to made with traumatic brain injury model of rats, found a significant increase in Nrf2 protein after injury for 24 hours, and mRNA of the two downstream protein H0-1 and NQ01 regulated by Nrf2 increased significantly. At the same time, they found that Nrf2 and H0-1 located in the same cell type through immunohistochemistry method, and the researchers believe that there is activate Nrf2-ARE system after brain injury. When study on traumatic brain injury in knock on the Nrf2 gene mice, compared to wild type mice, Jin and Yan (Tsenter *et al*., 2008, Calkins *et al*., 2010, Innamorato *et al*., 2008 found that neurological deficit symptoms, neuronal apoptosis and brain edema of the knock gene mice after injury were clever increased than the wild-type significantly, showing that Nrf2 had effect on the neuroprotective of body. Nrf2 plays an important role on reducing nerve damage and promoting recovery of neurological function. Now we use closed head injury model of mice to assess the extent of injury and neurological status at each time point after injury with neurological deficits (neurological severity scores, NSS), and immunohistochemical method is used to detect trauma peri microglia cells and activation of astrocytes.

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MATERIALS AND METHODS

Animals experiment

In this study, Nrf2 of male in ICR knockout germline homozygous Nrf2 (-/-) and wild-type homozygotes Nrf2 (+/+) mice (they were provided by the United States Kensler kindly Laboratory at Johns Hopkins University). The weight is 28~32g. Using inbred and backcross breeding methods to sire, clip the tip of the tail to extract DNA before the experiment, and use PCR methods for gene identification.

Primary reagents

Rat anti-mouse CD11b antibody (MCA711, Serotec), rabbit anti-mouse antibody Iba1 (Cat. # 019 -1974, Wako), rabbit anti-mouse GFAP antibody (ab16997, Abcam), immunohistochemistry SP reagent boxes and DAB color kit (Beijing Biosynthesis Biotechnology Co., Ltd.).

Establishment and grouping of animal model

The study used Nrf2 (+/+) mice in the number of 70 and Nrf2 (-/-) mice in the number of 70. Randomly selected 6 mice as the control group from the two genotypes mice, respectively. Take the others into the experimental group. The experimental group establish modified closed head injury model of the mice (Flierl *et al*., 2009), and the control group only cut scalp sutured. Adopt NSS scores (table 1) and score is related to the degree of injury. The higher of the score, and the more severe neurological dysfunction (Tsenter *et al*., 2008).

All animals were anesthetized with diethyl ether, and the experimental group cut off the head of hair in mice after anesthesia effect. Cut in the scalp at median sagittal, exposing the top, suture needle with a syringe cross mark against the skull surface point to the left of midline with 2 mm, in front of the coronal with 1mm. 100g cylindrical striking hammer freefall from 6~10cm height, along the vertical metal bars, to impact plastic gasket against the left point on the surface of the skull, causing depressed skull fracture, suture the scalp and put it back into the cage after sterilization. Use NSS scores to assess the extent of injury after 1h, excluding death, NSS≤4 points and NSS≥9 points and reserved 1h NSS with score 5-8 points (moderate traumatic brain injury). Finally, 43 Nrf2 (+/+) mice and 42 Nrf2 (-/-) mice met the inclusion criteria. Only make death mortality statistics after the mice were selected, and reject its NSS score, remaining mice statistical its NSS score (1h and 1, 2, 3, 7, 14, 21, 28 d).

Specimen preparation

In order to do immunohistochemistry check, the experimental group randomly selected 18 mice from injury Nrf2 (+/+) type and Nrf2 (-/-) mice, divided into Nrf2 (-/-) group type injury after 1, 3, 7d and Nrf2 (+/+) group type injury after 1, 3, 7d. There were 6 mice in each group. Use sodium pentobarbital (80mg/kg) by intraperitoneal injection of anesthesia, rapid thoracotomy through the left ventricle into the ascending aorta cannulation, saline with 150ml of normal, followed by paraformaldehyde solution in 4□ to slow down perfusion after 2h fixation. Take the brain in a 20% sucrose solution overnight until brain tissue sink to the bottom. Wash it with 0.01mol/L PBS next day. Coronal cut brain tissue in containing contusion, and embedded in paraffin spare with conventional 4μm thick slices.

Immunohistochemical detection

Slice conventional dew axing to water, 0.01mol/L (pH 6.0) citrate buffer antigen retrieval, 3% H_2O_2 at room temperature with 20min, 10% normal goat serum closed at room temperature in 20min, added the murine anti-CD11b (1:100), rabbit anti Iba1 (1:200) and rabbit anti-GFAP (1:200), overnight at 4□, PBS rinse, do it according to immunohistochemistry kit and DAB color kit manual steps. Finally graded dehydrate with ethanol, xylene and neutral resin mounted. During incubation use 0.01mol/L PBS instead of primary antibody as a negative control.

Immunohistochemical image analysis

Use an image acquisition system to take six to nine vision in bruising around the stove at 400times magnification field of vision. Deal with each group average optical density of positive cells through image analysis software named Image pro-plus (US Media Cybermetics Inc.).

STATISTICAL ANALYSIS

Analyze date through SPSS13.0 statistical software, each set of data represent in x ± s. NSS score were analyzed by the first two-factor repeated measures of variance; If P< 0.05, do nonparametric Mann-Whitney U test for each time point between the two groups. Mortality used Fisher's exact test to check, results of immunohistochemistry were analyzed by independent sample t-test. Made α=0.05 as the level of the test and P <0.05 was considered statistically significant.

RESULTS

The effects ofNrf2 knockout mice after brain injury on neurological status and mortality (Fig. 1)

NSS score of Nrf2 (+/+) mice was (6.58±1.00) points, and that of Nrf2 (-/-) mice was (6.67±1.07) points in the experimental group mice after injury 1 h; The difference was not statistically significant (P>0.05), so two genotypes showed similar degree of injury in mice. Over time, neurological disorders in both genotypes of mice were improved (NSS score decreased). It reached a plateau at 7~14 d, but compared with Nrf2 (+/+) mice, NSS score of Nrf2 (-/-) type mice decreased to a lesser extent with 44.2%, while Nrf2 (- / -) mice only fell with 27.6 percent, two-factor repeated measures analysis of variance showed: The difference in NSS score of two genotypes Mice was statistically significant (P<0.01). It was also statistically significant (P<0.01) in the difference of NSS score between two genotypes mice at 7, 14, 21, 28 d through the non-parametric Mann-Whitney U test. After the experimental group was injury, Nrf2 (-/-) mice died seven (16.7%), when Nrf2 (+/+) mice died four (9.3%). After the Fisher exact test, the difference was not statistically significant (P>0.05). Two genotypes of mice in the control group had no deaths, and there was no obvious neurological dysfunction (NSS = 0 points).

The effects of Nrf2 knockout mice after brain injury on activation of microglial cells around contusion group (Figs. 2~4)

There is obvious aggregation and activation of microgliain cells around contusion after two genotypes mice injured 1d in the experimental group. After 3d, it was more obvious and immune markers slightly weakened after 7 d. Microglia activation and aggregation of Nrf2 (-/-) mice were higher than Nrf2 (+/+) mice, and quantitative analysis shows that the difference of the average optical density between the two genotypes mice were statistically significant in 1, 3, 7 d (P<0.05).

The effects of Nrf2 knockout mice after brain injury on activation of astrocytes cells around contusion group (Figs. 5-7)

The aggregation of astrocytes cells and GFAP coloring around contusion group in the experimental group was

Table 1: NSS score of mice

Task	Description
Out of Circle	After put the circle in the center, they can get out of the circle within the diameter of 30 cm in 2 min
Hemiplegia	Legs can buckle or against lateral pressure after lift tail
Walking a straight line	They can walk a straight line on the opening occasions
Startle reflex	Appear larger shock performance when noise appear suddenly such as clapping
Exploratory behavior	They have exploratory behaviors in a new situation
Side lever balance (5 mm)	Maintain balance 10 s in width of 5 mm square rod to
Balancing rod (5 mm)	Maintain a balance of 10 s in a round rod of diameter 5 mm to
Walking side bar (3 cm)	Walk 30 cm in width 3 cm square rod
Square bars Walking (2 cm)	Walk 30 cm in width 2 cm square rod
Walking side bar (1 cm)	Walk 30 cm in width 1 cm square rod

Note: get 0 points when each task is completed and get 1 point when it is in failure

more pronounced with time. Compared with Nrf2 (+/+) mice, the activation of astrocytes cells of Nrf2 (-/-) mice is more significantly. The difference of average optical density was statistically significant by independent sample t test after mice injured 3 d, ($P<0.05$).

DISCUSSION

The results showed that: Nrf2 (-/-) mice has a more significant neurological dysfunction after injured. Nrf2 gene has an important prognostic impact of traumatic brain injury, and a large number of mechanisms that could affect this process. This experiment studies the pathological process of glial cell activation. The group preliminary study showed that: Brain injury can activate Nrf2-ARE pathway and Nrf2 nuclear translocation is mainly in the glial cells (Yan *et al*, 2008). In addition, it has been reported in a variety of pathological model, the protective effect of Nrf2-mediated activation of glial cells is achieved mainly by regulating the implementation (Calkins *et al*., 2010; Innamorato *et al*., 2008). Therefore, the authors hypothesized: Nrf2 play a protective role in traumatic brain injury of mice, and this protective effect may achieve through regulate activation of glial cell.

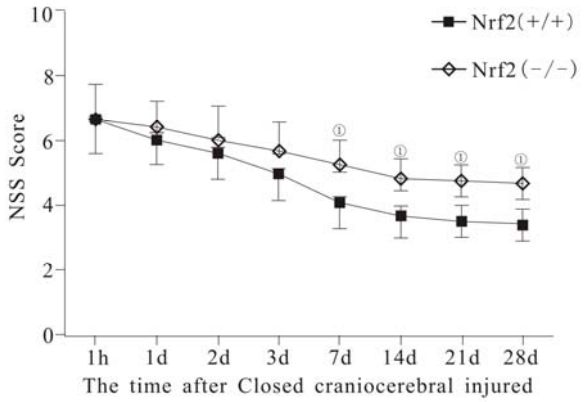

Fig. 1: NSS score of mice in the experimental group Nrf2 (+/+) type and Nrf2 (-/-) type after injured. Compared with Nrf2 (+/+) mice, $P<0.05$

To test this hypothesis, the present study further test the activation of microglia and astrocytes around contusion foci by immunohistochemical methods. Compared with Nrf2 (+/+) mice, the activation of microglia of Nrf2 (-/-) mice was significantly increased after mice were injured 1 d. This trend is more obvious after injured 3d and there is still Statistical significance after injured 7d. In addition, the activation of astrocyte in the type of Nrf2 (-/) mice also significantly enhanced after injured 3 d. These results indicate that: Nrf2 can adjuste the activation of glial cells around contusion foci after brain injured. Literature reporteded: Excessive and prolonged microglial activation plays toxicity role in the central nervous system diseases (Graeber, 2010; Floyd and Lyeth, 2007; Laird *et al*., 2008; Lettenmann *et al*., 2011). In the processes of the central nervous system in a variety of pathological, such as trauma and ischemia, activation and migrate to the damaged area of microglia and astrocytes is a common phenomenon. Activation of astrocytes and microglia bring to bear synergies and microglial cells can produce a variety of toxic substances, such as inflammatory cytokines, nitric oxide, reactive oxygen species (ROS), prostaglandins and chemokines (Kettenmann *et al*., 2011). Astrocytes may produce the above-described materials, but it may also play a protective role (Floyd and Lyeth, 2007). In addition, the microglia also secrete TNF-α and IL-1 promoting the activation of astrocyte, creating a vicious cycle continue to strengthen (Cederberg and Siesjo, 2010).

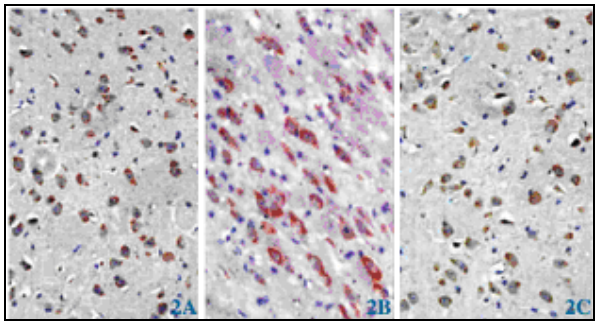

Fig. 2: The expression of positive cells CD11b of Nrf2 (+/+) mice around contusion peri after injured

(immunohistochemistry × 400) 2A injured with 1d; 2B injured with 3d; 2C injured with 7d.

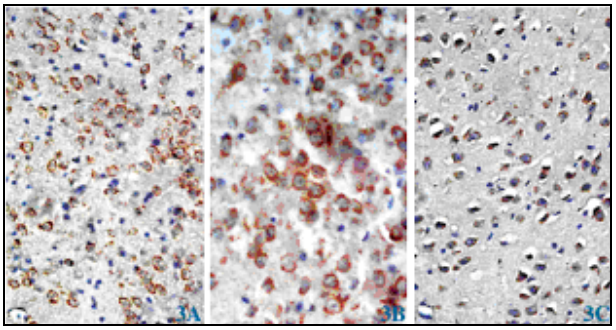

Fig. 3: The expression of positive cells CD11b of Nrf2 (-/-) mice around contusion peri after injured (immunohistochemistry × 400) 3A injured with 1d; 3B injured with 3d; 3C injured with 7d

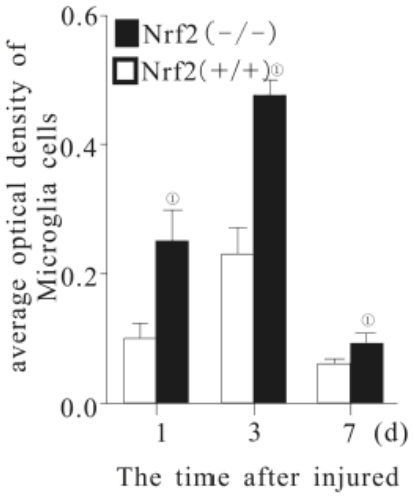

Fig. 4: The comparison of average optical density in experimental groups of mice around contusion peri 6A The average optical density of microglia cells

Large number of documents showed that: in a variety of disease models, Nrf2 can play anti-inflammatory effects (Rangasamy *et al*., 2005; Thimmulappa *et al*., 2006; Osburn *et al*., 2007), but it remains unclear to Nrf2 for how to regulate the activation mechanism of microglia and astrocyte. Previous studies demonstrated: Nrf2 achieved anti-inflammatory effects by restoring redox dynamic equilibrium, but ROS also play an important role in brain injury. After brain injury, NADPH oxidase of glial cells were activated, and produce large amounts of endogenous and exogenous ROS, ROS as a second messenger strengthen the inflammatory response of microglia and astrocyte (Block *et al*., 2007). At the same time, ROS also caused Nrf2 transfer to the nucleus and then express a large number of antioxidant enzymes to maintain redox balance (Kraft *et al*., 2006).

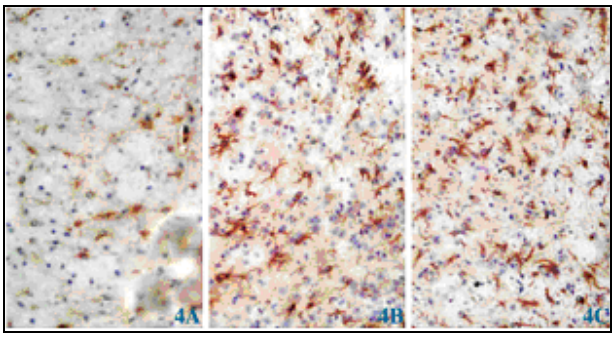

Fig. 5: The expression of positive cells GFAP of Nrf2 (+/+) mice around contusion peri after injured (immunohistochemistry × 400) 4A injured with 1d; 4B injured with 3d; 4C injured with 7d

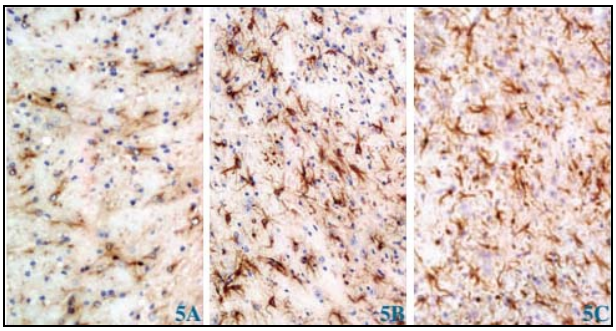

Fig. 6: The expression of positive cells GFAP of Nrf2 (-/-) mice around contusion peri after injured (immunohistochemistry × 400) 4A injured with 1d; 4B injured with 3 d; 4C injured with 7 d

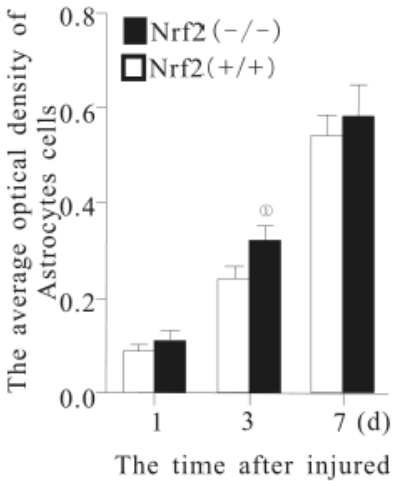

Fig. 7: The comparison of average optical density in experimental groups of mice around contusion peri Compated with Nrf2 (+/+) mice, P<0.05. 6B. The average optical density of astrocytes cells.

CONCLUSION

Therefore, the authors suggest: Nrf2 may cut the activation of glial cells through the maintenance of

dynamic redox equilibrium. Clinically can get a pathway by activating Nrf2-ARE, such as the use of drugs exert anti-inflammatory effects and thus treat a variety of neurological disorders.

This study provides genetic evidence: Nrf2 knockout mice after brain injury resulting in neurological dysfunction increased and the process is accompanied by the activation of glial cells around contusion foci strengthened. Future research will further clarify the Nrf2 anti-inflammatory mechanism and find the ideal Nrf2 activator, thereby providing a new treatment for brain injury.

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