Comparison of the Effect of Granulocyte-Macrophage Colony Stimulating Factor by Intranasal and Subcutaneous Injection for Traumatic Brain Injury in Rats

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ABSTRACT

Background: Recent studies have shown that autologous adult stem cells may be a strategy for treatment of traumatic brain injury (TBI) in humans. The purpose of this study was to explore the effect of autologous bone marrow stem cell mobilization induced by intranasal administration of granulocyte-macrophage colony stimulating factor (GM-CSF) for TBI in rats.

Methods: Adult Sprague-Dawley (SD) rats were assigned to three groups: control group (TBI only), TBI+GM-CSF subcutaneous injection and TBI+GM-CSF intranasally. Neurological scores and testing were done in all rats before TBI, and at 1, 7, 14, 28, 42 days post-TBI. Simultaneously, immunohistochemical labelling and Tunel terminal deoxynucleotidyl transferase dUTP Nick-End Labelling (TUNEL) assay were performed at the given time.

Results: From two-weeks post-TBI on ward, animals treated with GM-CSF subcutaneous injection or GM-CSF intranasally had lower neurological soft signs (NSS) scores than the control group. Spatial memory (Y-maze) of the treated group were significantly better than those in the control group at two weeks. After sacrifice, compared with the control, rats treated with GM-CSF subcutaneous injection and GM-CSF intranasally, significantly increased expressions of Brdu/GFAP, BDNF, Nestin, Factor VIII, and reduced expression of apoptotis cells around the lesion site. These findings indicate that GM-CSF subcutaneous injection and GM-CSF intranasally in a TBI model achieved similarly positive effect on functional and histologic recovery.

Conclusions: Intranasal delivery of granulocyte-macrophage colony-stimulating factor provides a potential protective effect in traumatic brain injury.

Keywords: BMMCs, functional recovery, GM-CSF, transplantation, traumatic brain injury

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INTRODUCTION

Traumatic brain injury (TBI) is one of the leading causes of death and chronic neurological dysfunction worldwide (1). The primary injury can be focal or diffuse and initially produces haemorrhage and axonal injury followed by secondary effects such as oedema and ischaemia arising from the activation of biochemical pathways including inflammation, cell apoptosis and gliosis (2). These secondary-phase effects begin to widen the area of injury and can persist for weeks to months, leading to profound behavioural deficits. Aggravated secondary brain damage is considered to impair the ability of resident neuronal stem cells to regenerate the lost neurons (3). Although the development of neurochemical, histopathological and molecular techniques to study human TBI has enabled researchers to begin to elucidate the pathological sequelae following TBI, there are no FDA-approved drugs specific for TBI treatment and medical intervention is limited to supportive care. Thus, the ideal therapy for TBI would have the combined effects of reducing the initial injury as well as limiting the secondary inflammatory responses while simultaneously promoting regeneration and replacement of lost neural tissue (4). Meanwhile, stem cell therapy has presented a potentially powerful solution for TBI.

Bone marrow cells possess ethical and legislative priorities over other types of stem cells that make them ideal candidates for treating TBI (5–7). Accumulating evidence has suggested that bone marrow cells contain haematopoietic stem cells, mesenchymal stem cells (MSCs), and other cells that supply multiple growth factors and stimulate neurogenesis and angiogenesis, which accelerate regeneration of the injured central nervous system [CNS] (5–7). Furthermore, autotransplantation avoids immunological rejection. A number of clinical

trials are going to access its therapeutic potential in TBI (5–8). Granulocyte-macrophage colony-stimulating factor (GM-CSF) is primarily defined as a haematopoietic growth factor, which has been used extensively in the treatment for chemotherapy-induced neutropenia, bone marrow reconstitution, and stem cell mobilization in humans (9, 10). It is known that GM-CSF can mobilize progenitor cells from the bone marrow into the peripheral blood, and thus has the capacity to migrate into and stimulate neurogenesis and angiogenesis for the neuronal lesion site (11). Granulocyte-macrophage colony-stimulating factor, has been demonstrated to also have a function in the central nervous system, because GM-CSF can cross the blood-brain barrier and both GM-CSF and its receptor are expressed by various brain cells (12, 13). A previous study has shown that the administration of GM-CSF improves functional recovery in rats with TBI (5–8). Moreover, GM-CSF itself may have anti-apoptotic and anti-inflammatory effects on neuronal cells as well as having a positive effect on neovascularization (8, 14, 15).

In the present study, we compared GM-CSF subcutaneous injection and GM-CSF intranasal delivery, aimed to evaluate the effects of morphological and functional recovery in rats subjected to acute TBI. Furthermore, we hypothesized that intranasal GM-CSF would play a more useful neuroprotective role in TBI.

MATERIALS AND METHODS

Animals

All experimental procedures were performed in agreement with the Guidance Suggestions for the Care and Use of Laboratory Animals, issued by the Ministry of Science and Technology of China. Animals were housed under standard conditions with the light period of the light/dark cycle and allowed to eat and drink ad libitum. A total of 75 adult male Sprague-Dawley (SD) rats, weighing approximately 250 g–300 g, were the subjects of this study. The rats were anaesthetized *via* intraperitoneal injection of 3.6% chloral hydrate for all surgical procedures. Quantitative analyses and behavioural tests were conducted by investigators who were blinded to the experimental conditions.

Establishment of the traumatic brain injury model

A controlled cortical impact model of TBI in the rat was established according to Feeney's method (18). Male SD rats were fasted for 16 hours before operation. During the experiment, the rats were anaesthetized by intraperitoneal injection of 100 g/L chloral hydrate (0.35 g/kg). Then, they were mounted in the prone position. After the skin of rats were sterilized, a median incision was made on the scalp and periosteum was striped and the right parietal bone was exposed. A 5 mm diameter hole was drilled at 1.5 mm posterior to the frontoparietal suture and 2.5 mm lateral to the median line with integrity of the Dura mater. A 20 g counterweight was dropped vertically from a 30 cm long copper tube to strike the Dura mater and cause right parietal lobe TBI. Five drops of gentamicin (4×10^7 IU/L) were dropped on the cut, bone wax was used to seal the hole and the scalp was sutured. Animals were randomly assigned into three groups one day after TBI (25 in each group): group 1 (control group)

received phosphate buffered saline (PBS) by subcutaneous injection and intranasally daily for seven days; group 2 received recombinant human GM-CSF (20 μg/kg/ day, Amgen) by subcutaneous injection for seven subsequent days; group 3 received GM-CSF (20 μg/kg/day, Amgen) by intranasal administration daily for seven days. Experimental rats treated with either GM-CSF or PBS were injected intraperitoneally with Brdu (50 mg/kg, Sigma) daily for seven consecutive days after induction of TBI.

Neurological functional evaluation

Neurological function in the rats was assessed using the neurological severity scores (NSS) (19). The NSS is composed of motor (muscle status, abnormal movement), sensory (visual, tactile and proprioceptive), reflex and beam walking tests. The higher the NSS score is, the more severe the injury. The evaluation of all rats was started before TBI and performed after TBI once a day.

The cognitive capacity of rats was evaluated with a Y-electric maze (20). Briefly, rats were placed in the maze, and tasks were recorded in the following manner: after electric shock, the rat escaped from the starting zone to the safety zone within 10 seconds, *ie,* correct reaction. If the rat did not reach the safety zone within 10 seconds, an error reaction was recorded. Error reaction times were recorded once daily and all measurements were performed by observers blinded to individual treatment.

Histology and immunohistochemistry

The rats were sacrificed for histologic and morphologic analysis after TBI. At the end of the given time, the rats were deeply anaesthetized after assessment of neurological outcome, then transcardially perfused with 4% paraformaldehyde in phosphate buffered saline (pH7.4). The

brains were removed carefully and post-fixed in the same fixative. The brain tissues from each group were processed for preparation of paraffin sections, which were used for histological analysis and immunohistochemical staining.

The slices were stained with haematoxolin and eosin (H & E) to identify the morphological changes of the brain lesions. For immunohistochemical analysis, the slides were treated with 3% hydrogen peroxide in methanol to block endogenous peroxidase activity. After PBS washing, sections were then incubated with antibodies directed against Nestin (1:50, Santa Cruz, CA), Factor VIII (1:300, lab vision, Fremont, CA) or BDNF (1:250, Santa Cruz, Santa Cruz, CA) at 4°C overnight. The sections were visualized by the avidin-biotin-peroxidase complex method and developed in diaminobenzidine (DAB). The percentage of Nestin and BDNF positive cells of each group were counted in five random microscopic fields (×200 magnification). The numbers of Factor VIII-positive blood vessels were calculated throughout each field of view.

For BrdU/GFAP immunofluorescence analysis, the paraffin-embedded sections were de-paraffinized in Histoclear and rehydrated in a 100–70% ethanol gradient, followed by distilled water. Endogenous peroxidase was inhibited by incubating the sections in 0.3% hydrogen peroxide at room temperature for 20 minutes. The sections were then incubated, the slides were treated with 0.3% hydrogen peroxide at room temperature for 20 minutes. After PBS washing, sections were then incubated with primary rabbit BrdU polyclonal antibody BrdU (1:80, Sigma) at 4°C overnight. After three PBS washes, the sections were incubated in fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G [IgG] (1:30, Roche, Indianapolis, IN, USA) in PBS at 37°C for two hours. After three PBS wash steps, the

sections were incubated in goat anti-mouse GFAP (1:100, Roche) at 4°C overnight. Finally, sections were washed three times with PBS and incubated in Cy3-conjugated mouse anti-goat IgG (1:30, Roche) in PBS at 37°C for two hours. Cover slips were washed and mounted with 50% glycerin in PBS before imaging (fluorescein microscope, Leica, Solms, Germany). BrdU-GFAP positive cells were quantified in five random areas in the lesion boundary zone under fluorescence microscope.

Apoptosis assay

The apoptotic cells were analysed by an *in situ* Cell Death Detection Kit, AP (Roche), following the procedure specified by the manufacturer. DNA fragmentation of *nuclei* in the injured areas was stained by *in situ* TdT mediated dUTP nick end labelling (TUNEL) method. Means of the percentage of TUNEL-positive cells were counted under a light microscope from three randomly chosen fields (×200 magnification).

Statistical Analysis

Statistical calculations were carried out using the SPSS software (version 13.0, SPSS Inc, Chicago, IL, USA). Inter-group differences were determined using one-way analysis of variance (ANOVA) and Student-Newman-Keuls (SNK) test. All values are reported as the mean ± standard error of the mean (SEM). The value of $p < 0.05$ was considered statistically significantly different.

RESULTS

Neurological and motor functions in TBI rats

Neurological and motor function was measured using the NSS, which is sensitive for unilateral cortical injury, because it reflects multiple asymmetries, including postural, sensory, forelimb and hindlimb. All rats had a normal score, zero, before induction of TBI, and the score increased one day after the TBI. Neurological soft signs analyses demonstrated continuous improvement of motor function in the treated groups from one-week to six weeks (Fig 1). Both GM-CSF subcutaneous injection and TBI+GM-CSF intranasally treated rats showed significantly lower NSS scores, at days 28 and 42, than those in the control group ($p < 0.05$). It is interesting to note that there were no statistically significant differences between the GM-CSF subcutaneous injection and GM-CSF intranasally treated during the trial ($p > 0.05$; Table 1).

Cognitive function in TBI rats

The Y-maze was used to measure learning and memory in the rats. The Y-maze had been somewhat restored on the 7th day, and significantly restored with lower frequency of error in the treated group, compared to the control group by 14 days post-TBI, and the positive effect persisted for up to 42 days ($p < 0.05$). Statistical analysis showed no significant difference between the GM-CSF subcutaneous injection and the TBI+GM-CSF intranasal group ($p>0.05$; Table 2).

Brain tissue pathology in TBI rats

A series of histological changes were apparently observed around the injured area, including haemorrhage, oedema, vasocongestion and inflammatory cell infiltration. At the second week after TBI, the extent of pathological lesions in rats treated with GM-CSF injection or

GM-CSF intranasally alone, were smaller, compared with control rats (Fig 1), suggesting a beneficial effect of both and GM-CSF on lesion repair.

Glial cell proliferation in brain tissues of TBI rats

The mature glial cell marker, glial fibrillary acidic protein (GFAP), was used in conjunction with 5-bromodeoxyuridine (BrdU) to identify newly generated glial cells by immunofluorescence double staining. At the second to the sixth week, the majority of BrdU/GFAP double-labelled positive cells could be observed in the lesion epicenter of the brain in all the rats. The number of cells positive for BrdU/GFAP was significantly less in the control group than that in the treated groups. However, there was little statistical difference between the two treatment groups ($p > 0.05$) [Table 3, Fig 2].

Brain-derived neurotropic factor expression in brain tissues of TBI rats

The expression of BDNF, a potent neuroprotective growth factor was observed in neurons and astrocytes at the fourth weeks. Figure 3 showed that both the treated groups exhibited significantly increased BDNF positive cells compared with the PBS treatment group ($p < 0.05$). There were no significant differences between the animals in the group that received GM-CSF by subcutaneous injection and the GM-CSF intranasal group ($p > 0.05$) [Table 4, Fig. 3].

Nestin expression in brain tissues of TBI rats

The presence of the immature cellular marker nestin was clearly detectable in the treated groups. Statistical analyses showed that at 7–42 days after TBI, the number of nestin-positive cells significantly increased in brain tissues of both treated groups compared with the control group ($p < 0.05$). There were no significant differences between the rats in the groups with

GM-CSF subcutaneous injection and GM-CSF intranasal group [$p>0.05$] (Table 5, Fig 4).

Factor VIII expression in brain tissues of TBI rats

To access the new capillary networks around the injury site of the brain, immunohistochemical staining for Factor VIII was performed. The results showed that the GM-CSF subcutaneous injection and GM-CSF intranasally treated group significantly enhanced the density of factor VIII—positive microvessels in set areas than that of the control group ($p < 0.05$). It is indicated that both GM-CSF subcutaneous injection and GM-CSF intranasally promoted significantly the proliferation of microvessels around the injured region of the brain (Table 6, Fig 5).

Apoptosis analysis

At the second week, TUNEL assay of GM-CSF subcutaneous injection and GM-CSF intranasally was investigated. Apoptosis positive cells of each group around the injury site of brain were counted and analysed. The percentage of apoptotic cells was significantly less in the GM-CSF (subcutaneous injection) and GM-CSF intranasal group than the control group.

No statistically significant differences were revealed between the GM-CSF subcutaneous injection and GM-CSF intranasal group (Table 7, Fig. 6). The data indicated that there was less apoptosis in rats post-TBI treated with GM-CSF (subcutaneous injection) or GM-CSF (intranasally).

DISCUSSION

In present studies, we compared GM-CSF subcutaneous injection and GM-CSF intranasal treatments in rats with TBI, and the results demonstrated that GM-CSF subcutaneous injection as well as GM-CSF intranasally, significantly accelerated the neurological functional improvement and regeneration following TBI, which was in parallel with histological recovery. There were significant increases in neurogenesis, angiogenesis, upregulation of brain-derived neurotropic factor and blocking of perihaematomal cell death within TBI. These findings suggest that administration of GM-CSF subcutaneous injection and GM-CSF intranasally have similar therapeutical effect for TBI, but the latter could be a more non-invasive feature in treating TBI.

In these studies, we used the brain contusion model to study the efficacy of TBI therapeutic interventions evaluated by both NSS and the Y-electric maze test outcomes. Previous preclinical studies (21, 22) analysed the therapeutic efficacy of xenogeneic bone marrow-derived mesenchymal stem cells (BMSCs) transplantation, but these methods require immunosuppressive treatment. The present study demonstrated that intranasal administration of GM-CSF improved sensorimotor function after TBI. To the best of our knowledge, this is the first study to demonstrate that intranasal administration of GM-CSF affects deficits in spatial learning and sensorimotor functions after TBI. Neurological soft signs results and the times of error reactions in the Y-electric maze test demonstrated that intranasal administration of GM-CSF promoted recovery of sensorimotor functional deficits. Granulocyte-macrophage colony stimulating factor subcutaneous injection also resulted in significant treatment effects on NSS at later time points compared with the control group. The data showed that GM-CSF subcutaneous injection in the acute stage after TBI significantly enhanced neuroplasticity and

thereby improved neurological outcome in rats; similar results were seen in TBI rats with GM-CSF intranasal treatments, which were consistent with those of previous studies (8, 23). Morphological analysis showed that the lesion sizes in the treated rats were smaller than those of the PBS-treated ones according to our assessment on brain slices with H&E staining. The two treatment groups markedly improved histological recovery, which may contribute to functional outcome.

The clear molecular and cellular mechanisms underlying the effect of GM-CSF on TBI were not fully elucidated. In this study, Brdu immunoreactive cells were observed at the lesion site in the chronic stage of TBI after GM-CSF treatment. Our experimental results demonstrated higher expression of the neuronal markers, Nestin and Brdu/GFAP, in GM-CSF treated group than in the control group, which might have partly contributed to functional recovery through neurogenesis. Angiogenesis has been reported to be important in reducing neurological deficits after TBI (6). Other authors have noted that neurotropic growth factors were vital for facilitating angiogenesis and reducing parenchymal cell apoptosis (24–26).

Previous studies reported that GM-CSF, the prototypical mobilizing agents, have the capability of mobilizing bone marrow-derived stem cells (BMSCs) composed of haematopoietic progenitor cells and bone marrow stromal cells. The beneficial effect of GM-CSF on stroke has been well understood (27). Granulocyte-macrophage colony stimulating factor facilitates endogenous neuronal and vascular endothelial trans-differentiation of BMSC, thus it plays a key role in potentiating intrinsic neural self-repair processes at the injured site (23), which has been supported by our previous

findings. In our study, it was important to note that GM-CSF increased levels of BDNF, which plays a key role in the neuroprotective effect of TBI. It was likely that expression of BDNF in the lesions zone was another beneficial factor for neuro-restoration in TBI. Furthermore, GM-CSF has been known to have the anti-apoptotic effect (8), which was consistent with our observation in the TUNEL assay. Granulocyte-macrophage colony stimulating factor was found to exert direct effect on attenuating cells' death through the GM-CSF receptors expressed on neurons and glial cells by triggering downstream signalling pathway. It is suggested that GM-CSF induced activation of the JAK2/STAT3 signalling pathways and the apoptosis regulation factors (28).

In summary, the current study found that GM-CSF intranasal administration was similar to GM-CSF subcutaneous injection on functional benefits in a TBI model. The use of GM-CSF may greatly simplify the clinical application of mobilizing stem cells, and then delivering them to the injured site, whereas GM-CSF is noninvasive, convenient, and time saving for patients. Thus, GM-CSF is viable alternative for use in future studies of TBI patients.

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Table 1: Comparison of neurological severity scores in TBI rats

Group	1d	7d	14d	28d	42d
Control	13.80 ± 0.84	8.93 ± 1.31^{b}	6.78 ± 1.64^{b}	4.82 ± 0.84^{b}	2.31 ± 0.65^{b}
GM-CSF injection	13.83 ± 1.12	8.27 ± 1.25^{b}	5.32 ± 1.45^{b}	$2.88 \pm 0.53^{a,b}$	$1.10 \pm 0.82^{a,b}$
GM-CSF intranasal	13.81 ± 0.93	8.20 ± 1.32^{b}	5.11 ± 1.37^{b}	$2.63 \pm 0.56^{a,b}$	$0.98 \pm 0.86^{a,b}$

Data are expressed as mean ± SD from 25-5 rats in each group at each time point. High scores represent severe injury. $^{a}p < 0.05$, *vs*. the control group; $^{b}p < 0.05$, *vs*. the 1std after TBI using two-tailed independent sample *t*-test.

Table 2: Comparison of the times of error reaction in Y-electric maze test in TBI rats

Group	7 d	14d	28d	42d
Control	20.60 ± 2.01	18.40 ± 2.60	14.80 ± 2.18^{b}	9.80 ± 1.20^{b}
GM-CSF injection	18.60 ± 3.72	$12.61 \pm 2.46^{a,b}$	$9.00 \pm 1.21^{a,b}$	$5.20 \pm 0.89^{a,b}$
GM-CSF intranasal	18.55 ± 3.32	$12.56 \pm 2.54^{a,b}$	$8.40 \pm 1.42^{a,b}$	$5.12 \pm 0.93^{a,b}$

Data are expressed as mean ± SD from 20-5 rats in each group at each time point. Frequency of error times represents severe injury. $^{a}p < 0.05$, *vs.* control group; $^{b}p < 0.05$, *vs.* seven days after TBI using two-tailed independent sample *t*-test.

Table 3: Number of BrdU/GFAP protein double-labeled cells in brain tissues of TBI rats

Group	7d	14d	28d	42d
Control	1.43 ± 0.76	0.62 ± 0.48^{b}	0.43 ± 0.78^{b}	0.57 ± 0.86^{b}
GM-CSF injection	6.96 ± 2.72^{a}	6.73 ± 2.86^{a}	$4.61 \pm 1.46^{a,b}$	$3.94 \pm 1.95^{a,b}$
GM-CSF intranasal	7.13 ± 2.28^{a}	7.20 ± 2.84^{a}	$4.80 \pm 1.56^{a,b}$	$4.32 \pm 2.17^{a,b}$

Data are expressed as mean ± SD from five rats in each group at each time point. $^{a}p < 0.05$, *vs.* control group; $^{b}p < 0.05$, *vs.* seven days after TBI using two-tailed independent sample *t*-test.

Table 4: BDNF expression in brain tissues of TBI rats

Group	7 d	14d	28d	42d
Control	14.40 ± 3.76	12.80 ± 2.98	13.40 ± 4.78	9.60 ± 5.86
GM-CSF injection	42.60 ± 6.28[a]	33.40 ± 5.84[a,b]	29.80 ± 2.86[a,b]	24.40 ± 3.17[a,b]
GM-CSF intranasal	43.20 ± 6.41[a]	35.60 ± 5.09[a,b]	30.53 ± 2.96[a,b]	26.21 ± 3.63[a,b]

Data are expressed as mean ± SD from 10 rats in each group at each time point. [a]$p < 0.05$, *vs* control group; [b]$p < 0.05$, *vs* seven days after traumatic brain injury using two-tailed independent sample *t*-test.

Table 5: Nestin expression in brain tissues of TBI rats

Group	7 d	14d	28d	42d
Control	4.40 ± 1.76	4.80 ± 0.98	3.40 ± 0.78	3.60 ± 1.86
GM-CSF injection	16.60 ± 2.28[a]	19.40 ± 2.84[a]	20.80 ± 2.56[a,b]	21.40 ±2.17[a,b]
GM-CSF intranasal	14.32 ± 3.79[a]	17.20 ± 2.41[a]	18.60 ± 2.09[a,b]	19.80 ±1.02[a,b]

Data are expressed as mean ± SD from five rats in each group at each time point. [a]$p < 0.05$, *vs.* control group; [b]$p < 0.05$, *vs.* seven days after TBI using two-tailed independent sample *t*-test.

Table 6: Comparison of the number of factor VIII-positive cells in brain tissues of TBI rats

Group	7 d	14d	28d	42d
Control	17.40 ± 5.73	21.80 ± 4.98	20.40 ± 7.78	28.60 ± 7.06
GM-CSF injection	29.60 ± 9.70[a]	36.00 ± 6.86[a,b]	40.60 ± 4.80[a,b]	43.80 ± 7.05[a,b]
GM-CSF intranasal	30.20 ± 8.28[a]	37.60 ± 4.40[a,b]	42.80 ± 6.39[a,b]	45.60 ± 7.49[a,b]

Data are expressed as mean ± SD from five rats in each group at each time point. [a]$p < 0.05$, *vs.* control group; [b]$p < 0.05$, *v*. seven days after TBI using two-tailed independent sample *t*-test.

Table 7: Quantification of TUNEL-positive cells in brain tissues of TBI rats

Group	**7 d**	**14d**	**28d**	**42d**
Control	53.63 ± 9.80	44.81 ± 7.30^{b}	38.43 ± 8.21^{b}	35.62 ± 8.30^{b}
GM-CSF injectino	19.66 ± 6.30^{a}	16.87 ± 5.80^{a}	17.14 ± 3.90^{a}	12.21 ± 3.50^{ab}
GM-CSF intranasal	18.20 ± 8.28^{a}	15.60 ± 4.40^{a}	16.80 ± 6.39^{a}	11.65 ± 5.49^{ab}

Data are expressed as mean ± SD from five rats in each group at each time point. $^{a}p < 0.05$, *vs*. control group; $^{b}p < 0.05$, *v*. seven days after TBI using two-tailed independent sample *t*-test.

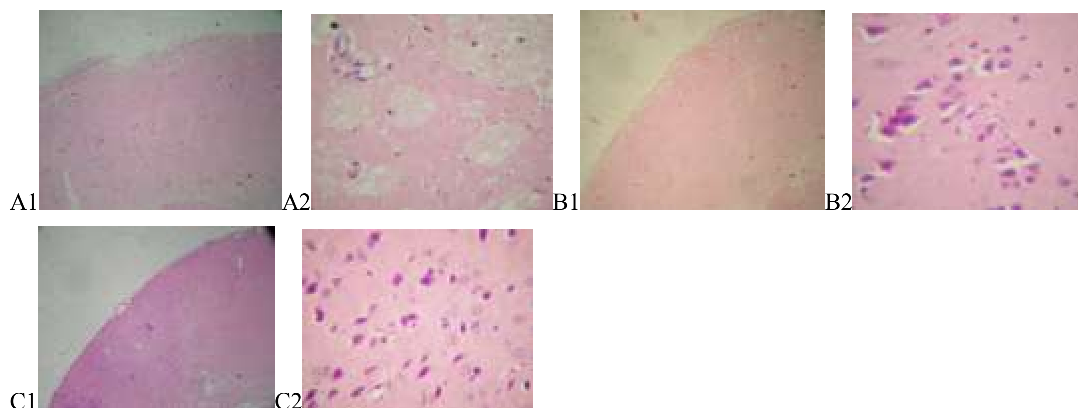

Fig. 1: Representative pathological morphology of TBI in each group at the fourth week after TBI. (A1, A2) control group; (B1, B2) GM-CSF subcutaneous injection; (C1, C2) GM-CSFintranasal. Compared with the control group, the other two groups showed much better reconstruction and less injured lesion in the TBI.

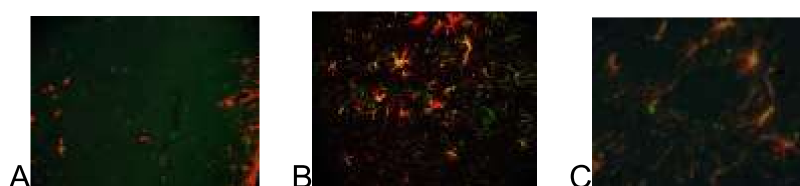

Fig. 2: Representative BrdU/GFAP expression in brain tissues at the fourth week after TBI (immunofluorescence double staining, yellow, fluorescent microscopy). (A1, A2, A3) Control group; (B1, B2, B3) GM-CSF injection; (C1, C2, C3) GM-CSF intranasal.

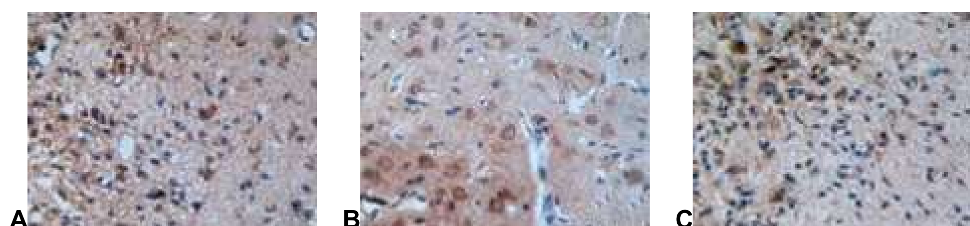

Fig. 3: Representative BDNF expression in brain tissues at 28 days after TBI (streptavidin-peroxidase; × 100). (A) Control group; (B) GM-CSF injection; (C) GM-CSF intranasal.

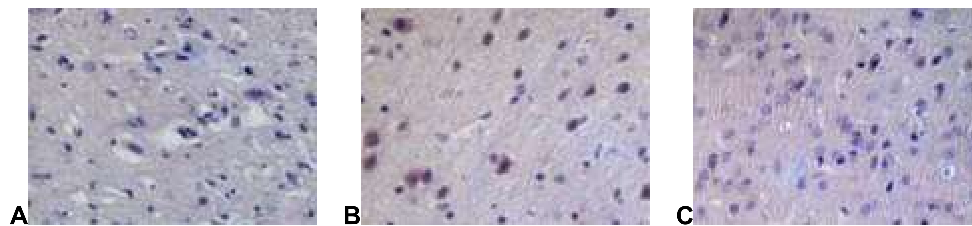

Fig. 4: Representative Nestin expression in brain tissues at 28 days after TBI (streptavidin-peroxidase; × 100). (A) Control group; (B) GM-CSF injection; (C) GM-CSF intranasal.

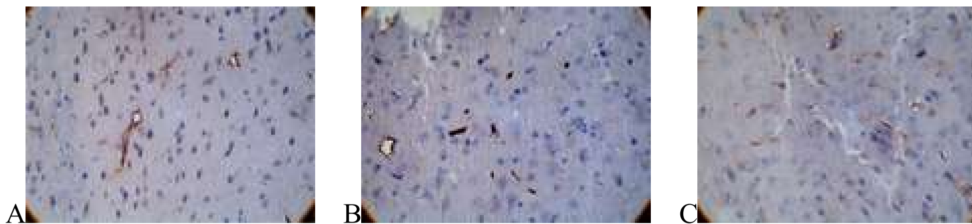

Fig 5: Representative Factor VIII antigen expression in brain tissues at 28 days after TBI (streptavidin-peroxidase; × 100). (A) Control group; (B) GM-CSF injection; (C) GM-CSF intranasal.

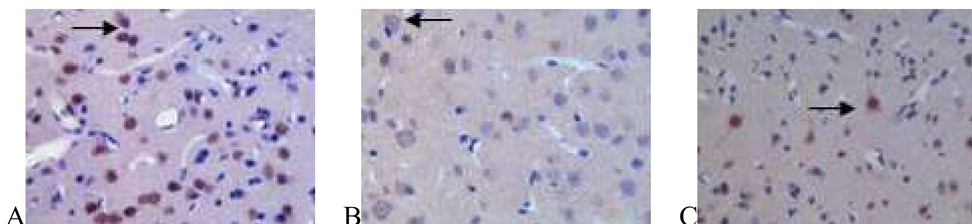

Fig. 6: Representative cellular apoptosis in brain tissues at 28 days after TBI (terminal deoxynucleotidyl transferase dUTP nick-end labeling, × 200). (A) Control group; (B) GM-CSF injection; (C) GM-CSF intranasal.