

# Comparative effect of immature neuronal or glial cell transplantation on motor functional recovery following experimental traumatic brain injury in rats

FU-SHI QUAN, JIAN CHEN, YUAN ZHONG and WEN-ZHI REN

Laboratory Animal Center, College of Animal Sciences, Jilin University, Changchun, Jilin 130062, P.R. China

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**Abstract.** The present study evaluated the comparative effect of stereotactically transplanted immature neuronal or glial cells in brain on motor functional recovery and cytokine expression after cold-induced traumatic brain injury (TBI) in adult rats. A total of 60 rats were divided into four groups (n=15/group): Sham group; TBI only group; TBI plus neuronal cells-transplanted group (NC-G); and TBI plus glial cells-transplanted group (GC-G). Cortical lesions were induced by a touching metal stamp, frozen with liquid nitrogen, to the dura mater over the motor cortex of adult rats. Neuronal and glial cells were isolated from rat embryonic and newborn cortices, respectively, and cultured in culture flasks. Rats received neurons or glia grafts ( $\sim 1 \times 10^6$  cells) 5 days after TBI was induced. Motor functional evaluation was performed with the rotarod test prior to and following glial and neural cell grafts. Five rats from each group were sacrificed at 2, 4 and 6 weeks post-cell transplantation. Immunofluorescence staining was performed on brain section to identify the transplanted neuronal or glial cells using neural and astrocytic markers. The expression levels of cytokines, including transforming growth factor- $\beta$ , glial cell-derived neurotrophic factor and vascular endothelial growth factor, which have key roles in the proliferation, differentiation and survival of neural cells, were analyzed by immunohistochemistry and western blotting. A localized cortical lesion was evoked in all injured rats, resulting in significant motor deficits. Transplanted cells successfully migrated and survived in the injured brain lesion, and the expression of neuronal and astrocyte markers were detected in the NC-G and GC-G groups, respectively. Rats in the NC-G and GC-G cell-transplanted groups exhibited significant motor functional recovery and reduced histopathologic lesions, as compared with the TBI-G

rats that did not receive neural cells ( $P < 0.05$ , respectively). Furthermore, GC-G treatment induced significantly improved motor functional recovery, as compared with the NC-G group ( $P < 0.05$ ). Increased cytokine expression levels were detected in the NC-G and GC-G groups, as compared with the TBI-G; however, no differences were found between the two groups. These data suggested that transplanted immature neural cells may promote the survival of neural cells in cortical lesion and motor functional recovery. Furthermore, transplanted glial cells may be used as an effective therapeutic tool for TBI patients with abnormalities in motor functional recovery and cytokine expression.

## Introduction

Traumatic brain injury (TBI) remains one of the most serious types of neurologic degeneration in humans and the deleterious effects occur during distinct primary and secondary periods (1). Primary injury, which is induced by initial mechanical force trauma, results in the tearing and shrinkage of neural and vascular tissue. Following primary injury, the secondary periods are induced by ischemia, edema, ionic imbalances and energy metabolic changes (1,2) TBI results in motor and cognitive deficits that may persist for a prolonged period of time after the traumatic event (2).

Potential therapeutic strategies have been investigated for the treatment of patients following TBI (3). It has previously been established that the multifaceted derangements of cell function and corresponding cell death after TBI were the most important events that resulted in cognitive and emotional disabilities (3). Previous research has focused on the clinical treatment to restrain these derangements and improve cognitive and motor recovery (4). Additionally, an increasing number of studies have investigated the potential therapeutic applications of lactate (4), progesterone (5,6), ginseng extract (7) and resveratrol (8). Although various approaches have been established to protect neurons and improve the cognitive and motor recovery (7), the adverse effects and complicated purification techniques have restricted the clinic application of these techniques to date (8).

These approaches have only been demonstrated to improve the neurons that remain following TBI. Therefore, successful restoration of the injured brain must be accompanied by the regeneration or transplantation of neural cells. Previous studies

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*Correspondence to:* Dr Wen-Zhi Ren, Laboratory Animal Center, College of Animal Sciences, Jilin University, 5333 Xi'an Road, Changchun, Jilin 130062, P.R. China  
E-mail: renwenzhi\_l@163.com

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have suggested that genetically engineered cells, including brain tissue, embryonic stem cells, adult stem cells and neural stem cells, are capable of integrating and differentiating to restore brain function (9-12). More recently, neural stem cells have been investigated and the results demonstrated that these cells survived and grafted into the host architecture following transplantation into newborn or adult mice or rat brains, and were capable of differentiating into neurons and glia lineages, replacing lost neural cells, improving clinical outcomes after experimental TBI and providing a platform for gene-based therapies (13-19).

Conversely, another previous study found that stem cells differentiated into neural cells and increased cytokine levels in host brains (20). These contrasting findings question the ability of stem cells to replace the damaged brain architecture and suggest that the recovery of function following stem cell transplantation in TBI may be associated with the cytokine production abilities of neural cells, which differentiate from transplanted stem cells.

Although various cells, including embryonic stem cells, adult stem cells and neural stem cells, have been demonstrated to improve functional recovery and protect remnant neurons in various animal models (20-24), the precise mechanism remains unknown. Notably, when injected with embryonic stem cells during a previous study, some rats developed teratoma at the implantation site (21), which fuelled the heated ethical debates over the use of embryonic stem cells. Adult stem cells can effectively avoid these problems, however the multiplication and conservation of stem cells remains very difficult (25). Furthermore, the source of neural stem cells limits its use as only the brains of fetuses aged >12-14 weeks old contain neural stem cells (26). Alternatively, cultured immature neural cells have higher security, wide source and easy propagation *in vitro* and have no problem of immunity and ethic (23). Nevertheless, the comparative effects of immature neurons and glia on motor functional recovery after TBI following direct administration into the brain have rarely been reported.

Therefore, to explore the therapeutic potential of immature neural cell transplantation for brain repair, the present study was undertaken to examine the comparative effect of stereotactically transplanted neurons or glia on motor functional recovery in a rat model of TBI. Firstly, whether neurons or glia migrate into the focal injury area via brain tissue to protect the remnant neural cells and replace the lost cells was assessed. Secondly, cytokine levels were analyzed following cell transplantation to examine whether transplanted neural cells were capable of creating an environment that was conducive to functional recovery via cytokines production. Thirdly, the possible effective differences in motor functional recovery between neurons or glia transplantation were investigated.

## Materials and methods

**Animals and experimental groups.** A total of 60 male Sprague-Dawley rats, weighing ~220 g and aged 7 weeks $\pm$ 2 days, were purchased from the Experimental Animal Center of the College of Animal Sciences at Jilin University (Changchun, China) and were used in the present study. Rats were maintained at 22°C (humidity, 60%) with

a 12-h light/dark cycle and *ad libitum* access to food and tap water. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Jilin University.

Rats were divided into four groups (n=15/group): i) Sham (CON); ii) TBI plus neuronal cells-transplanted group (NC-G), rats were transplanted neuronal cells 5 days after TBI; iii) TBI plus glial cells-transplanted group (GC-G), rats were transplanted glial cells 5 days after TBI; iv) TBI only group (TBI-G), rats received TBI only. Five rats from each group were sacrificed at 2, 4 and 6 weeks after the graft via an overdose of sodium pentobarbital (30 mg/kg; Abbott Laboratories, Chicago, IL, USA).

**Isolation and neuronal and glial cell culture.** Cortical neuron cultures were harvested from the brains of 16-day-old rat embryos according to a modified procedure outlined by Freshney in 1987 (23). Briefly, cerebral hemispheres were isolated and placed into Ca<sup>2+</sup>/Mg<sup>2+</sup>-free Hank's balanced salt solution (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Brain tissue was dissociated in 0.025% trypsin for 10 min at 37°C and the proteolytic reaction was subsequently terminated by adding the same quantity of Dulbecco's modified Eagle medium (DMEM) and fetal bovine serum (FBS; both Gibco; Thermo Fisher Scientific, Inc.). Following centrifugation at 157 x g for 15 min at 4°C, the pellet containing the dissociated neuronal cells was resuspended in neurobasal media containing 400X L-glutamine (200 Mm), 50X B27, 100X penicillin and streptomycin antibiotics (all Gibco; Thermo Fisher Scientific, Inc.) and 200X glutamate (5 Mm; Sigma-Aldrich RBI, Natick, MA, USA). The concentration of the cells was adjusted to 2x10<sup>6</sup> cells/ml and the viability of the cells was 85%, as determined by the trypan blue dye exclusion method (24). Cells were seeded onto a poly-D-lysine (50 µg/ml; Sigma-Aldrich, St. Louis, MO USA) and laminine (1 µg/ml; Gibco; Thermo Fisher Scientific, Inc.)-coated 6-well plastic plate at a concentration of 1x10<sup>6</sup> cells/well and maintained at 37°C in an atmosphere containing 95% air and 5% CO<sub>2</sub>.

Mixed glial cell cultures were prepared from dissociated cerebral cortices of newborn rats. Dissociation of the cerebral hemispheres and cell culture were performed as described above for the cortical neuronal cells, although the components of the culture media were altered to DMEM supplemented with 10% FBS and 1% antibiotic solution (100X; Gibco; Thermo Fisher Scientific, Inc.).

**Preparation of cells for transplantation and microinjection.** Cultured neural cells were labeled with DiI fluorescent dye (1,1-dilinoyleyl-3,3,3',3'-tetramethylindocarbocyanine, perchlorate) prior to injection to ensure the cells appeared red. A total of 45 rats were randomly selected, anesthetized with 8% chloral hydrate (400 mg/kg) by intraperitoneal injection and placed in a stereotaxic frame. Microinjection coordinates (1 mm posterior, 1.5 mm right lateral, 2 mm ventral to the bregma) were selected according to a rat brain atlas and microinjection was performed using a 20 µl Hamilton syringe with a 22-gauge needle. Five days after TBI, rats received a microinjection of 10 µl cell suspension (~1x10<sup>6</sup> cells). The cell suspension was separated into 5 parts and injected slowly over 25 min with 5 min intervals for each 2 µl cell suspension.

**Surgical procedure for establishment of TBI models.** In the present study, a well-characterized traumatic injury model was used, as previously described (27). This injury model was selected as it is readily reproducible and allows for the comparison of affected (ipsi-) and non-affected (contralateral) hemispheres. Following intraperitoneal injection with 10% chloral hydrate (3.5 ml/kg) anesthesia, the head was fixed in a stereotaxic frame. Subsequently, the scalp was longitudinally incised and the incision was made on the right motor cortex (4 mm anterior from the lambdoid suture; 2 mm lateral from the midline). A probe (diameter, 4-mm) was pre-cooled with liquid nitrogen and used to touch the surface of the skull for 20 sec. Subsequently, the skin was sutured using 3.0 nylon in a running whipstitch and the rats were observed until they awoke.

**Assessment of motor function.** The modified rotarod test is widely used to evaluate the motor coordination of rodents, and is especially sensitive in detecting cerebellar dysfunction (26). All the rats underwent conditioning of the rotarod test for three trials a day for 5 days prior to TBI. Motor behavior was analyzed daily for three trials on days 1, 5, 7, 14, 28 and 42 following TBI by a blinded observer using rotarod apparatus (Med Associates, Inc, St. Albans, VT, USA). Rats were placed on a rotating rod which was set to slowly accelerate from 4-40 rpm over 5 min. The rotarod test requires the rat to walk and maintain balance as the revolving rod accelerates. The trial was terminated when the rat fell off the apparatus and tripped a plate which recorded the time.

**Tissue preparation.** At 2, 4 and 6 weeks following neural cell injection, rats were anesthetized with intraperitoneal injection of 10% chloral hydrate (3.5 ml/kg) and intracardially perfused with 150 ml normal saline and 300 ml ice-cold 4% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.4). The brains of the rats were removed and post-fixed in the same fixative overnight at 4°C. Brain images were captured using a digital camera. Necrotic areas were measured using morphometry software (Axiovision 3.0.6 SP4; Carl Zeiss AG, Oberkochen, Germany). For light microscopy studies, tissues were embedded in paraffin blocks.

**Immunofluorescence.** For immunofluorescence imaging, 5  $\mu$ m-thick paraffin-embedded brain sections were cut, deparaffinized and rinsed in PBS. Sections were incubated in PBS supplemented with 3% bovine serum albumin (BSA), 10% normal calf serum and 1% Triton X-100 for 1 h at room temperature to block nonspecific binding. Subsequently, the sections were incubated overnight at 4°C with mouse anti-neuronal nuclei (NeuN; 1:500; ab18956; Abcam, Cambridge, UK) and rabbit anti-glial fibrillary acidic protein (GFAP; 1:1,000; MA191029; Chemicon International, Inc., Temecula, CA, USA) monoclonal antibodies in PBS containing 1% Triton X-100. Following washing in PBS for 30 min (6 times for 5 min), the sections were incubated with fluorescein isothiocyanate-conjugated, affinity-purified anti-mouse IgG and anti-rabbit IgG (both 1:200; both Jackson Immuno-Research Laboratories, West Grove, PA, USA) for 2 h at room temperature. Following washing in PBS for 30 min, the sections were mounted with mounting medium and examined under a fluorescence microscope (Olympus BX51; Olympus Corporation, Tokyo, Japan).

**Immunohistochemistry.** Sections (5  $\mu$ m) were cut, mounted on positively-charged slides, air-dried in an incubator at 40°C overnight and deparaffinized in xylene. Following rehydration with a graded alcohol series, the slides were incubated with 1% hydrogen peroxide diluted in methanol for 15 min to block endogenous peroxidase activity, and were subsequently rehydrated in distilled water and PBS. Following this, the slides were incubated with a blocking solution (PBS supplemented with 3% BSA and 10% normal calf serum) for 60 min at room temperature. This solution was removed from the slides using filter paper and the samples were incubated with the respective primary antibodies overnight at 4°C. The following primary antibodies were used: Mouse anti-glial cell-derived neurotrophic factor (GDNF; ab18956), rabbit anti-transforming growth factor (TGF)- $\beta$  (both 1:500; ab66043) and mouse anti-vascular endothelial growth factor (VEGF; 1:400; ab46154; all Abcam). Following removal of the unbound antibodies via several rinses with PBS followed by PBS containing 0.1% Triton X-100, the respective antibodies were detected using the avidin-biotin-peroxidase complex method (orb90540) and visualized using 3,3'-diaminobenzidine. Slides were lightly counterstained with hematoxylin.

Double-labeled sections were observed with an Olympus BH2 light microscope or an Olympus FV-1000 confocal laser-scanning microscope. The five darkest points of the images obtained by light microscopy were analyzed via densitometry using Multi Gauge 3.0 software (Fujifilm Life Science, Minato, Tokyo, Japan).

**Western blotting.** The cortex of each rat at the injury site was harvested on ice. Following washing with 0.9% sodium chloride, the clean tissue was preserved in liquid nitrogen until use. A protein preparation kit was used to extract the protein, and the concentration was measured by the Bradford method. Protein samples (20  $\mu$ g) from cortex of each rat at injury site were separated by 12% gel electrophoresis and transferred on to polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). Membranes were blocked in Tris-buffered saline with Tween 20 (TBS-T; pH 7.4) supplemented with 3% skimmed milk, and subsequently incubated overnight at 4°C with anti-TGF- $\beta$ , anti-GDNF and anti-VEGF primary antibodies (1:500). Following washing, the membranes were incubated with the following secondary antibodies: Anti-mouse IgG (CABT-ZC1010) for GDNF and VEGF and anti-rabbit IgG (CABT-ZC1022) for TGF- $\beta$  (1:2,000; Jackson Immuno-Research Laboratories) for 2 h at room temperature. All antibodies were diluted in TBS-T supplemented with 3% skimmed milk. The membranes were developed using enhanced chemiluminescent reagent and subjected to autoluminography for 2 min. Membranes were exposed on X-ray film (Eastman Kodak Company, Rochester, NY, USA). Blots were subsequently striped and re-blotted with mouse anti- $\beta$ -actin primary antibody (1:5,000; ab54724; Abcam), followed by incubation with anti-mouse IgG secondary antibody (1:2,000; PA128555; Thermo Fisher Scientific, Inc.).

**Statistical analysis.** Data were analyzed using SAS 8.1 (SAS Institute Inc., Cary, NC, USA) with analysis of variance.  $P < 0.05$  was considered to indicate a statistically significant



difference. Data are presented as the mean  $\pm$  standard deviation.

## Results

**Cell culture.** The isolated cells were successfully induced to differentiate into immature neurons and glia, respectively (Figs. 1A and B). Neurons and glia were stained with NeuN neuronal marker and GFAP astrocyte marker, respectively (Figs. 1C and D).

**Characterization of the brain cortex following TBI and post-transplantation.** Establishment of the rat model of TBI resulted in a wedge-like cold brain lesion which affected the motor cortex. The dorsal view of the brain 6 weeks post-TBI demonstrated that there was a marked difference in the injury area between the TBI-G and the NC-G and GC-G (Figs. 2A-C). The NC-G and GC-G exhibited decreased injury areas, as compared with the TBI only control group, TBI-G. Quantitative analysis of the lesion area 6 weeks post-TBI demonstrated that transplantation with neuronal and glial cells following TBI significantly reduced the injury area in the cerebral cortex ( $P < 0.05$ ; Fig. 1D).

**Body weight.** As compared with the rats in NC-G and GC-G, the mean body weight of the rats in the TBI-G was lowest from the day of TBI administration to 6 weeks post-TBI, and a notable decrease was observed at 2 weeks post-TBI (Fig. 3A). The body weight of rats in the CON-G remained significantly higher than other groups across all the time points ( $P < 0.05$ ). The NC-G and GC-G demonstrated significantly higher body weight increases, as compared with the TBI-G at 2 weeks post-TBI ( $P < 0.05$ ). Rats in the GC-G exhibited significantly increased body weight at 4 and 6 weeks post-TBI, as compared with the NC-G.

**Assessment of motor function outcome.** No significant differences in mean rotarod scores were observed between the CON-G and the other groups prior to TBI (Fig. 3B). Following TBI, TBI-G, NC-G and GC-G demonstrated a significant decline in rotarod performance, as compared with their pre-TBI scores. Rotarod scores of rats in NC-G and GC-G were significantly higher than TBI-G at 2, 4, 6 weeks post-cell transplantation ( $P < 0.05$ ). Furthermore, the GC-G demonstrated a reduced decline in rotarod scores, as compared with NC-G and TBI-G, suggesting glial cell transplantation promoted the recovery of motor function. No significant changes in rotarod performance were detected for rats in the CON-G at any time.

**Migration of transplanted cells.** Migration ability of transplanted-neurons or transplanted-glia were observed via the longitudinal view of the brain at 2, 4 and 6 weeks post-cell transplantation. As shown in Fig. 4, transplanted cells migrated via the corpus callosum from the injection site to the TBI lesion at 2 weeks. The number of transplanted-neurons and transplanted-glia at injection site decreased, whereas the number of these cells increased with time at the TBI site. The number changes of neurons and glia with labeling DiI in cortex and corpus callosum were similar.

**Histological characterization of the TBI lesion.** DiI pre-labeled neuronal or glia were observed at the injection site and surrounding areas of TBI injury 2, 4 and 6 weeks post-transplantation. In NC-G, DiI-positive cells exhibited immunoreactivity for the NeuN neuronal marker and substantial co-expression, as shown in Fig. 5; however these cells did not exhibit immunoreactivity for GFAP. In GC-G, the pre-labeled glia were immunoreactive for the GFAP astrocytic marker but did not exhibit immunoreactivity for NeuN. These results demonstrated that the transplanted neurons and glia, although small in population, successfully migrated from the transplant site to the TBI lesion and differentiated into neural cells *in vivo* following TBI.

**Immunohistochemistry.** GDNF expression levels decreased in a time-dependent manner; whereas TGF- $\beta$  and VEGF expression levels increased with time (Fig. 6). Quantitative analysis demonstrated that TBI-G, NC-G and GC-G exhibited variable TGF- $\beta$  ( $P < 0.05$ ), GDNF ( $P > 0.05$ ) and VEGF ( $P > 0.05$ ) expression levels at 2, 4 and 6 weeks post-transplantation. NC-G and GC-G exhibited significantly increased secretion of the three cytokines, as compared with TBI-G at the same time points. Moreover, GC-G treatment significantly increased the expression of TGF- $\beta$ , GDNF and VEGF, as compared with NC-G.

**Western blot analysis.** The expression levels of TGF- $\beta$ , VEGF and GDNF in each group, as detected by western blotting, are shown in Fig. 7. The expression levels of CON-G, TGF- $\beta$ , VEGF and GDNF were increased in NC-G and GC-G, as compared with the CON-G. TGF- $\beta$ , VEGF and GDNF expression levels were increased in GC-G, as compared with NC-G.

**Confocal microscopy.** Confocal microscopy demonstrated the expression of TGF- $\beta$ , VEGF and GDNF in neural cells at the injury site (data not shown). TGF- $\beta$ , VEGF and GDNF expression was observed in transplanted-neurons and transplanted-glia. These results indicated that transplanted-neurons and glia may secrete TGF- $\beta$ , VEGF and GDNF.

## Discussion

The results of the present study demonstrated, when transplanted after the induction of TBI in rats, immature neurons or glia migrated from the injection site to the TBI lesion and significantly improved motor functional recovery, as assessed by rotarod test at 2, 4 and 6 weeks. In NC-G and GC-G, respective neuronal and glial cell transplantation significantly reduced the injury area in the ipsilateral cerebral cortex and significantly increased the expression levels of TGF- $\beta$ , GDNF, and VEGF in the surrounding TBI lesion, as compared with the TBI-G. Moreover, glial cell transplantation markedly reduced the initial motor impairments induced by TBI, as compared with neuronal cell transplantation. Following cell transplantation, the rotarod scores of NC-G and GC-G increased, as compared with TBI-G. Therefore, the GC-G group resisted the decline of rotarod scores and promoted the recovery of motor function, compared with NC-G and TBI-G.

Previous studies have investigated functional recovery following stem cell transplantation in models of TBI. Undifferentiated stem cells, including bone marrow (28),

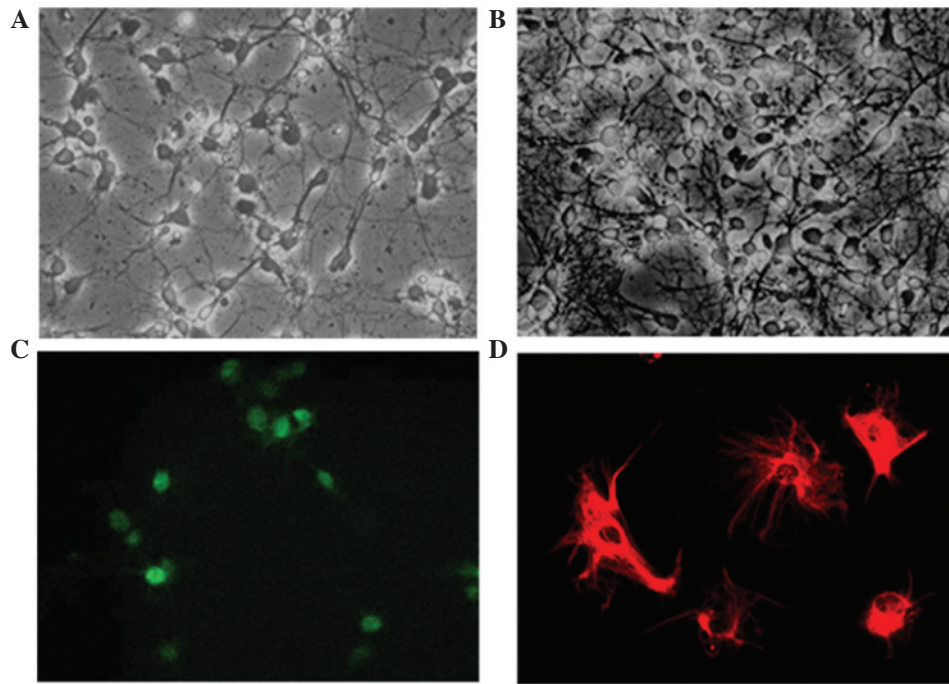


Figure 1. Newly isolated cells in the culture dish differentiate into both (A) immature neurons and (B) astrocytes. Confocal microscopic images demonstrated immature cells stained with the (C) NeuN neuronal marker (green) and the (D) glial fibrillary acidic protein astrocytic marker (red) at 5 days following culture. Magnification, x400.

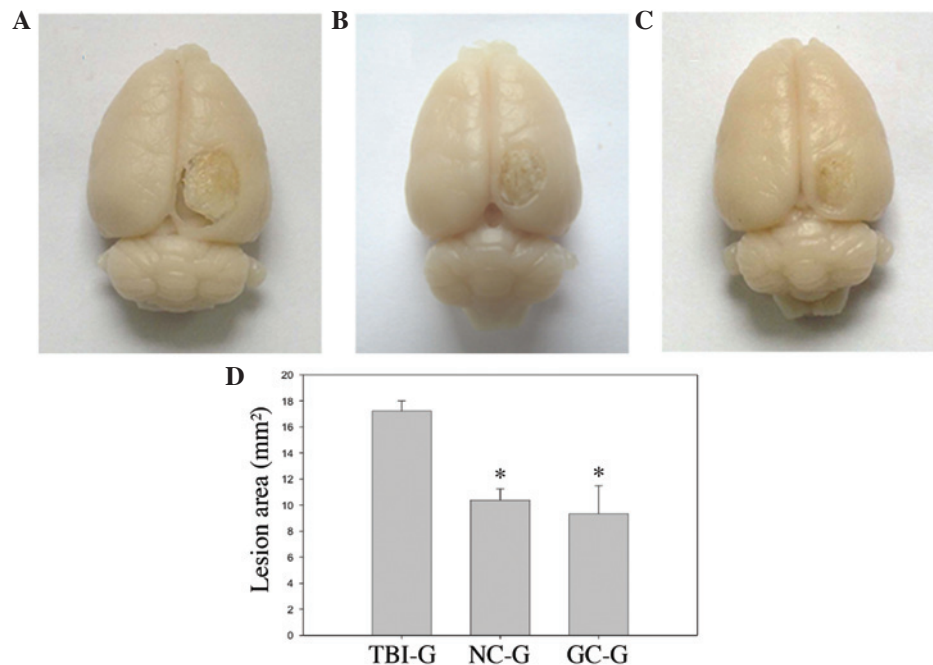


Figure 2. Effects of neural cell transplantation on a cold-induced brain lesion at 6 weeks post-cell injection in the (A) TBI, (B) NC-G and (C) GC-G groups. (D) Quantitative analysis of the areas of the respective brain lesions demonstrated that the rats injected with (B) neuron or (C) glia cell transplants exhibited decreased lesion areas, as compared with (A) the sham TBI control. Data are expressed as the mean  $\pm$  standard error of the mean. \* $P < 0.05$ , as compared with the TBI-G control ( $n = 5/\text{group}$ ). TBI, traumatic cold brain injury; NC-G, TBI plus neuronal cells-transplanted group; GC-G, TBI plus glial cells-transplanted group; TBI-G, TBI only group.

embryonic (29) and neural (30) stem cells, have the ability to differentiate into neurons and glial cells to replace lost neurons, improve outcomes after experimental TBI and provide a platform for gene-based therapies (14-17,31,32). In previous studies, a positive correlation was detected between the survival of transplanted cell and functional recovery (33,34). However, brain

trauma leads to the production of free radicals, excitotoxicity, calcium overloading, cytokine secretion, and growth-factor activation or withdrawal (35,36), which are not only harmful to brain cells but also to transplanted cells (37). Furthermore, teratoma development (21,34), ethical debates, multiplication and conservation difficulty (25) have limited its use.

In the present study, first, the survival and migration ability of transplanted immature neurons or glia were evaluated, which avoided the problem of undifferentiated stem cells, in a rat model of TBI. On day 5 after TBI was established, immature neuronal and glial cells were injected into the rats far from the injury site to enhance the survival of transplanted cells. DiI, which is a lipophilic membrane stain that diffuses laterally to stain the entire cells, was used as a marker for transplanted cells. It is possible that non-transplanted cells, which graft and migrate into the brain, may pick up DiI, and stain positively. Brain sections from rats in NC-G and GC-G were double-stained with NeuN or GFAP antibodies and DiI. Transplanted cells, which were positive for NeuN or GFAP and DiI, were observed in the cerebral cortex and corpus callosum of the injection site, which is the space from the transplanted site to the injured site. Previous studies have shown that ipsilateral and contralateral transplantation of neural stem cells or human mesenchymal stem cells resulted in migration from the injection site to the injury site and significantly improved motor functions (38). However, to the best of our knowledge, this is the first report of long distance migration of neural cells. The present results indicated that transplanted neural cells are also capable of migrating to an injured site via subcortical structures in response to brain injury. Furthermore, cell counting of brain sections from four different sites (injection site, 1/3 and 2/3 distance between injection and injury site, and injury site) demonstrated a decrease in the number of cells at the injection site and an increase at injury site at 2, 4 and 6 weeks post-transplantation. These results also demonstrated a temporal migration of transplanted cells from the injection site to the injury site as the assemble of transplanted cells were observed at a 1/3 distance from the injection site to the injury site at 4 weeks post-TBI and at a 2/3 distance at 6 weeks in the corpus callosum. The precise mechanism for the present finding that transplanted-neurons and glia are capable of migrating to injured lesions following TBI is yet to be fully elucidated.

To investigate whether changes at the cytokine level contribute to the recovery of motor function following TBI, the effects of transplantation with neurons or glia on the expression of cytokines were assessed in rat models of TBI. The results of the present study demonstrated that transplantation of neurons or glia induced increased cytokine expression at the injury site. It has previously been demonstrated that cytokines are associated with functional recovery following brain injury (33). GDNF has an important role in the differentiation of neurons during normal development and the survival and recovery of various populations of mature neurons (33). GDNF, which is a potent and relatively specific neurotrophic factor, has been demonstrated to have a protective effect on various injuries of central and peripheral nervous systems *in vitro* and *in vivo* (18). Wei *et al* demonstrated that the expression of GDNF mRNA and protein was first increased as early as 2 h after ischemia-reperfusion in peri-infarct cerebral cortex and striatum, followed by a decline and subsequent second increase at 72 h (39). Miyazaki *et al* (40) have previously shown that transient forebrain ischemia induced GDNF mRNA expression in the hippocampus from 3 h to 3 days after the ischemic episode, with peak expression detected at 6 h.

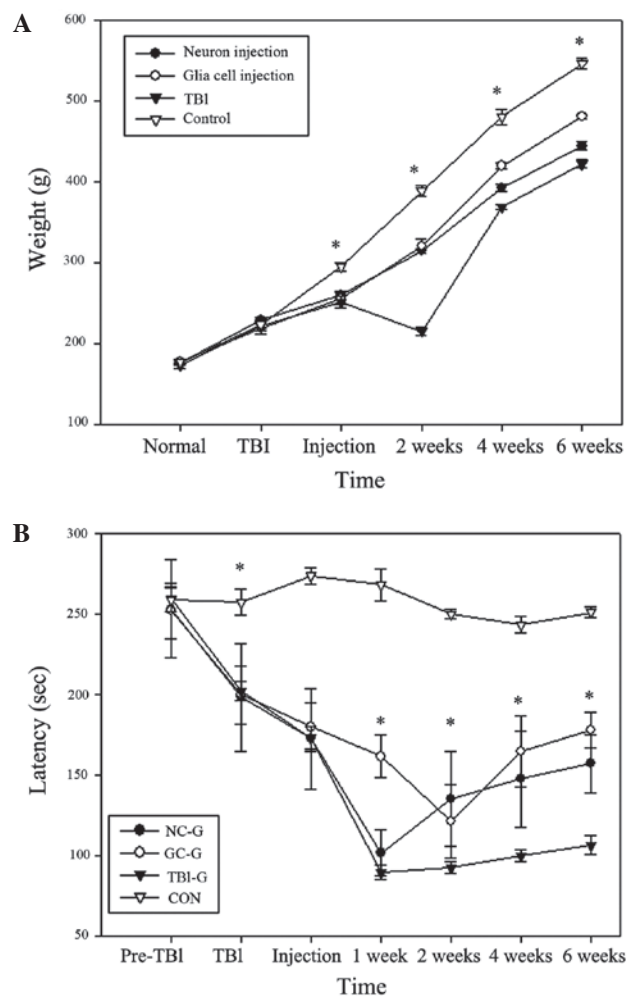


Figure 3. Effects of transplantation with neurons or glia cells on (A) body weight and (B) motor function recovery with rota rod test in a rat model of TBI rats. The NC-G and GC-G exhibited significant improved motor function recovery and body weight as compared with the TBI-G (\* $P < 0.05$ ;  $n = 5$ /group). Data are presented as the mean  $\pm$  standard deviation. TBI, traumatic cold brain injury; NC-G, TBI plus neuronal cells-transplanted group; GC-G, TBI plus glial cells-transplanted group; TBI-G, TBI only group; CON, control.

TGF- $\beta$  is a multifunctional peptide that controls proliferation, differentiation, and other functions in numerous cell types (41). TGF- $\beta$  has been demonstrated to be highly upregulated in the central nervous system following ischemia-induced brain damage (41). *In vitro*, TGF- $\beta$  protects neurons against excitotoxicity by inhibiting t-PA-potentiated NMDA-induced neuronal death through a mechanism involving the upregulation of plasminogen activator inhibitor type-1 in astrocytes (41,42). In addition, TGF- $\beta$  has been characterized as an antiapoptotic factor in a model of staurosporine-induced neuronal death through a mechanism involving the activation of extracellular signal-regulated kinase (Erk) 1/2 and a concomitant increase in the phosphorylation of the antiapoptotic protein B cell lymphoma-2-associated death promoter (42).

VEGF exerts neurotrophic effects which manifest as increased axonal outgrowth and improved cell survival in neuronal cultures (42,43). VEGF also protects neurons from hypoxia (44) and glutamate toxicity (45), which may explain its ability to reduce injury and improve clinical outcomes after experimental stroke (46-48). Conversely, defective VEGF

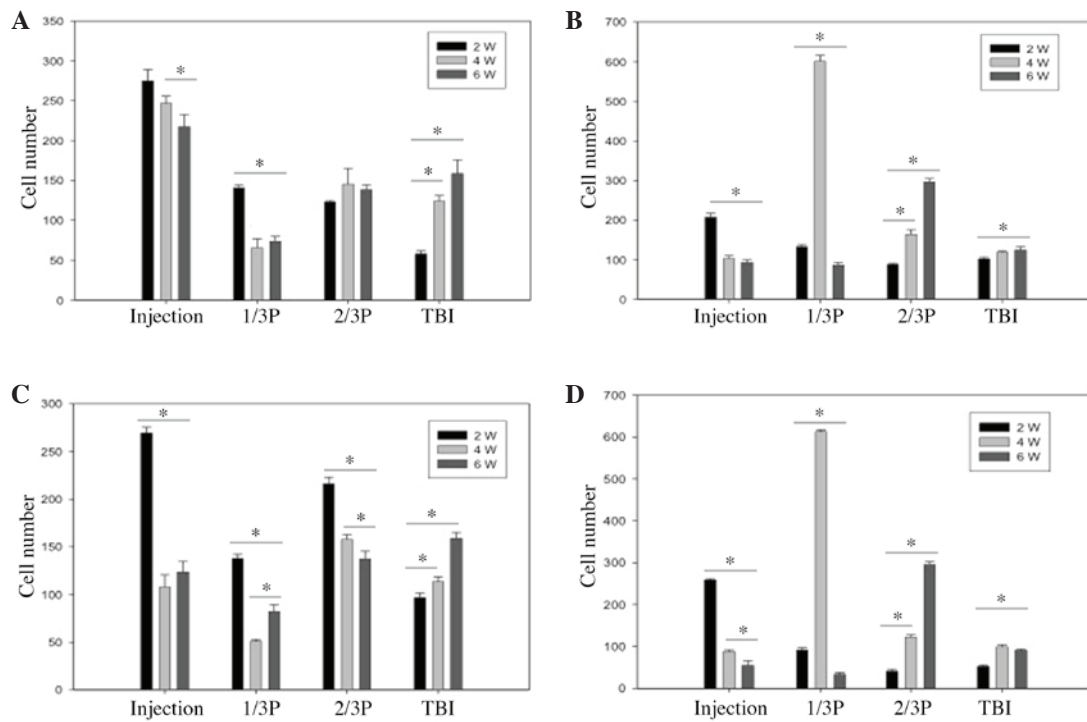


Figure 4. Number of transplanted neurons that survived up to 2, 4 and 6 weeks in different transverse planes of the (A) cerebral cortex and (B) corpus callosum and the number of transplanted glial cells that survived 2, 4 and 6 weeks in different transverse planes of the (C) cerebral cortex and (D) corpus callosum. Data are expressed as the mean  $\pm$  standard error of the mean. \*P<0.05 (n=5/group). W, weeks; 1/3P, the transverse plane at a 1/3 interval from the injection site to the TBI site; 2/3P, the transverse plane at a 2/3 interval from the injection site to the TBI site; TBI, traumatic cold brain injury.

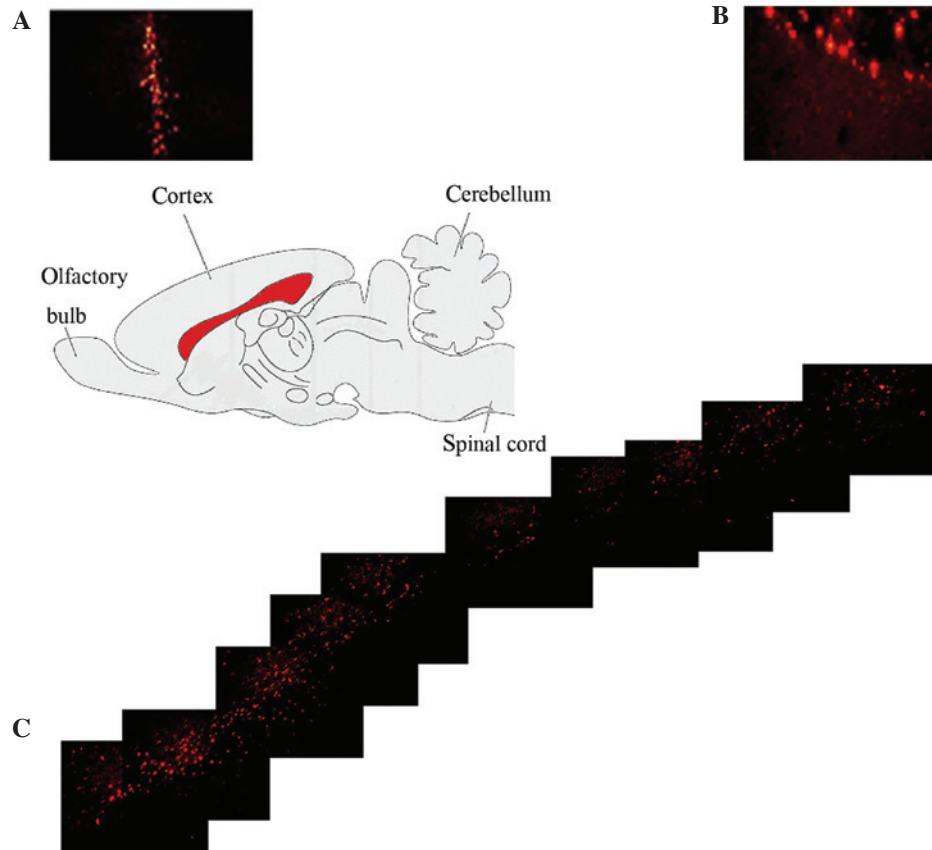


Figure 5. Brain median sagittal section showing the fluorescent dye DiI-labeling of transplanted neural cells via corpus callosum a successful migration from the (A) injection site to the (B) traumatic cold brain injury (TBI) site. (C) Confocal microscopic images of the TBI lesion at 2, 4, and 6 weeks following cell transplantation demonstrated double-labeling of injected cells with the neuronal nuclei (NeuN) neuronal marker and the glial fibrillary acidic protein (GFAP) astrocytic marker. DiI-positive cells (red) in the TBI lesion were co-stained with the NeuN neuronal or GFAP astrocytic (green) markers and merged as yellow.



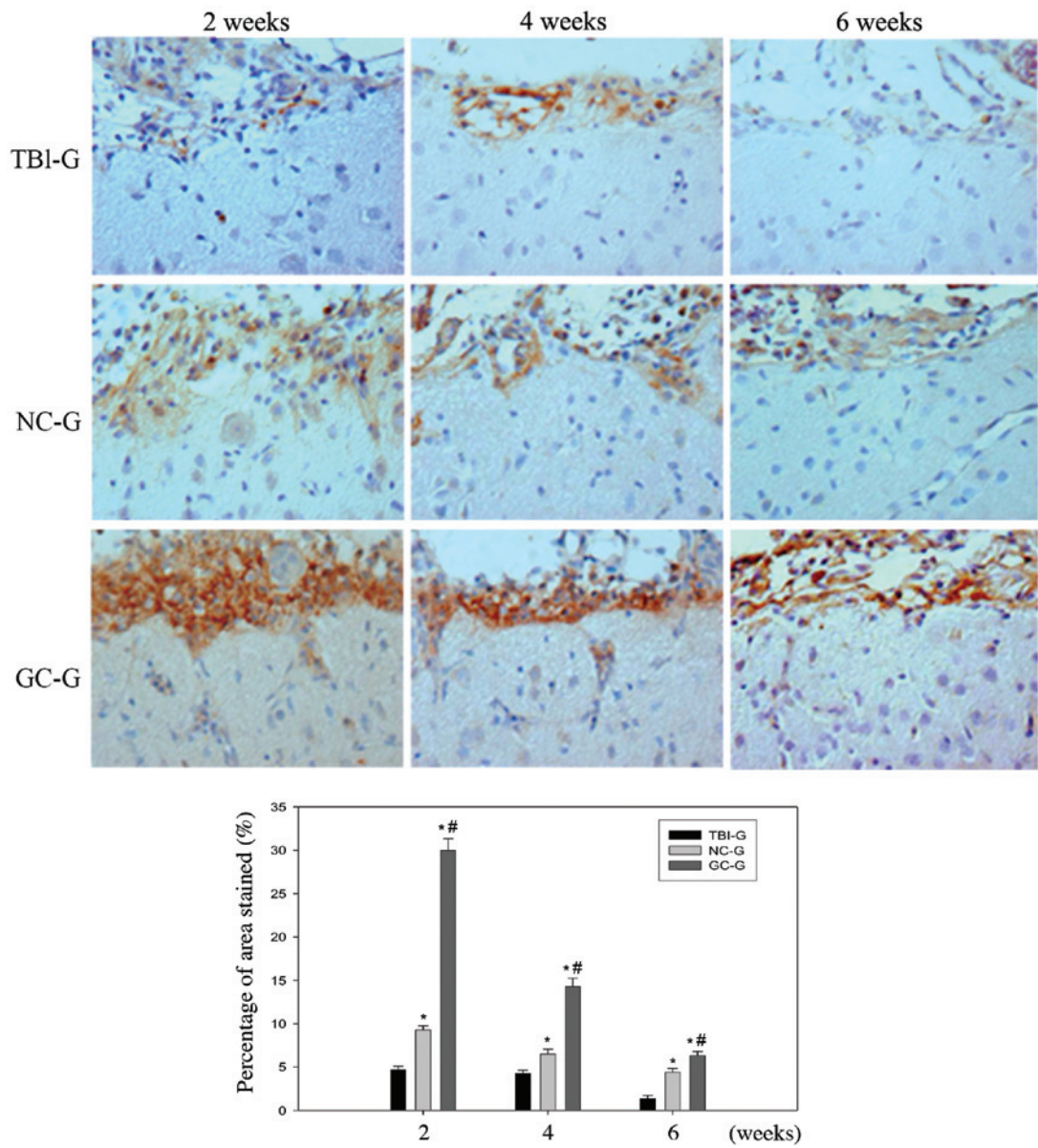


Figure 6. Effects of transplanted neurons or glia on the expression of TGF-β in brain cold injury rats. Injured rats transplanted with neurons or glia exhibited significantly increased TGF-β expression levels, as compared with the TBI-G rats. Compared to NC-G, the expression of TGF-β in the TBI lesion was significantly higher in the GC-G. No significance difference in the expression of GDNF and VEGF was detected between injured rats transplanted with neurons or glia. Notably, the confocal images showed the expression of TGF-β, GDNF and VEGF in neural cells at the injury lesion site. \*P<0.05 vs. the TBI-G; #P<0.05 vs. NC-G. TBI, traumatic brain injury; NC-G, TBI plus neuronal cells-transplanted group; GC-G, TBI plus glial cells-transplanted group; TBI-G, TBI only group..

signaling may contribute to motor neuronal disease (49-52). It has been demonstrated that VEGF stimulates neurogenesis, which is the production of novel neurons, in the adult song-bird (53) and rodent brains (54). Therefore, the functional benefits induced by cell transplantation may be due to the neurotrophic factors produced by the transplanted cells (55). In the present study, the expression levels of GDNF, TGF-β, and VEGF at the TBI site were observed in NC-G, GC-G and TBI-G via immunohistochemistry and western blotting. Confocal microscopy demonstrated that the transplanted neurons and glia contained GDNF, TGF-β and VEGF in their cytoplasm. Moreover the results of western blotting showed that GDNF, TGF-β and VEGF expression was induced at higher levels in NC-G and GC-G, as compared with TBI-G. These results suggested that the transplanted cells induced the secretion of these cytokines and may create a salutary environment for

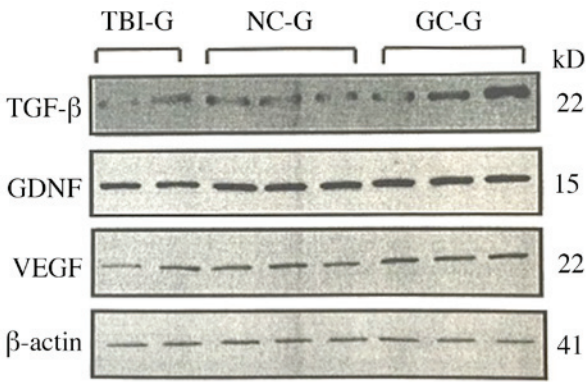


Figure 7. Western blot analysis was used to assess the expression levels of TGF-β, GDNF, VEGF and β-actin in the three groups. NC-G, TBI plus neuronal cells-transplanted group; GC-G, TBI plus glial cells-transplanted group; TBI-G, TBI only group; TGF, tumor growth factor.



the survival of neural cells and the proliferation of microvessels. In addition, the expression levels of the three cytokines induced by transplantation with neuronal cells were lower than the levels observed following transplantation with glial cells. These findings are consistent with the results of the rotarod test and the survival and migration of transplanted cells in the ipsilateral cortex. Taken together, these results provide strong evidence that the transplanted-neurons and transplanted-glia have an important role in the control of cytokine expression levels and motor functional recovery following TBI.

In conclusion, the results of present study suggested that: i) Transplantation of cultured immature neurons and glia may be a potential treatment for motor functional recovery following TBI; ii) transplanted cells may have an important role in the activation of the GDNF, TGF- $\beta$ , and VEGF expression; and iii) glial cell transplantation induces more beneficial effects on motor functional recovery following TBI, as compared with neuronal cell transplantation.

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