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神經幹細胞合併褐藻膠聚合物應用於大白鼠脊髓損傷神經修復之療效

研究成果報告(精簡版)

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Application of neural stem cells with alginate polymers to the treatment on spinal cord injury in rats

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Key words: Neural stem cells, Alginate polymers, G-CSF, Spinal cord injury

Abstract

Purpose: The purity and amount of neural cell transplantation was related to the regeneration in the spinal cord injury. Herein, we evaluate the utility of FGF 1 B to purify mouse neural stem cells as the basis in the transplantation of spinal cord injury . On the other hand, alginate is a good biocompatible material for cell delivery. Furthermore, G-CSF either alone or combined with neural stem cells on the spinal cord injury could increase the progenitor cells intrinsically or extrinsically. This study was to investigate the effect of FGF 1B mouse neural stem cells embedded in alginate concomitantly administrated with G-CSF in the spinal cord gap injury.

Material and Methods: Forty Sprague Dawley (n=10 in each group) rats weighing 250-300gm were used in this study. Transverse spinal cord resections over level T8-9 were carried out, leaving approximately a 2 mm gap between the distal and proximal end of cord. Neural stem cells embedded in the alginate either treated without or with G-CSF (50ug/Kg x5 days) (group III and IV) and bare alginate either without or with G-CSF (50ug/Kg x 5days) (group I and II) were delivered to gap of injured spinal cord and used for evaluation. Spinal cord regeneration was assessed by CatWalk, electrophysiology, histology and immunohistochemisty 3 months after injury.

Results: The transplantation of neural stem cells with alginate polymers revealed no augment the regeneration of spinal cord injury in this study. However, G-CSF

administration itself augmented the spinal cord injury either with or without co-administration of neural stem cells. There were significant expressions of Neu-N, MAP-2, and GFAP in those groups treated with G-CSF either with or without purified mouse neural stem cells. The increased G-CSF receptor expression was also observed in those groups treated with G-CSF. There was no significant expression of Neu-N, MAP-2, GFAP, and G-CSFR in those treated with purified mouse neural stem cells.

Conclusion: Neural stem cells with alginate polymers did not augment the regeneration of the spinal cord injury in this study. G-CSF administration alone enhanced the spinal cord regeneration. There was no combined effect when treated by neural stem cells with alginate polymers and G-CSF administration.

Introduction:

The exact numbers of spinal cord injury (SCI) patients are not well known in Taiwan, but the estimated annual incidence of SCI is around 8.8 per one million people (1). Quadriplegia or paraplegia resulting from SCI causes huge damage to a patient's life quality, his or her family, and affects society. The demand for long-term care services for SCI patients has thus become a major concern and emerged as a big social problem (2). Microscopically, SCI leads to axonal degeneration, demyelination, activation of microglia and astrocytes, and death of neurons and oligodendrocytes (3). To date, treatment strategies for SCI have included surgical, pharmacological and physical therapies (4). The surgical intervention was aimed at renormalizing cord anatomy and repairing the severed cord or nerve root by the procedure of decompression followed by instrumental fixation (5). Administration of methylprednisolone was recommended as a pharmacological therapy based on the National Acute Spinal Cord Injury Studies; indeed, it only had some benefits in neurological function recovery, arbitrarily, within narrow time window (6). In

addition, physical therapy contributes some positive effects to re-build neurological function, especially muscular status (7). Even though modern medicine has tried profusely, huge problems still exist in this field.

Current strategies employed by investigators to enhance regeneration in the injured spinal cord included neutralization of potential growth inhibitory molecules, transplantation of cells or tissue that support axonal elongation, and delivery of neurotrophic factors to promote axonal growth (8-10). Although transplantation of fetal tissue has resulted in functional recovery in Parkinson's disease and Huntington's disease, clinical use of fetal cells is limited by the availability of donor tissue as well as logistic, immunological and ethical consideration (11). Neural stem cells (NSCs) as a source of multipotent graft tissue may be considered to be an alternative to fetal derived cells. NSCs could be isolated from embryonic or adult brain tissue, as well as differentiated from embryonic stem (ES) cells. NSCs are able to proliferate in vitro through many passages without losing their multipotentiality and can be induced to differentiate into enriched populations of glial or neural progenitors (12). There have been enormous in vivo studies indicating that NSCs can differentiate into neurons and glial cells after being transplanted into impaired brains or spinal cords (13-16). The fates of engrafted cells were predominant differentiation into glial cells and failure to replenish lost neurons caused by ischemia brain or SCI (12).

Granulocyte Colony-Stimulating Factor (G-CSF), a key hematopoietic factor of myeloid lineage, has been extensively used for more than 10 years in the treatment of neutropenia as well as for bone marrow reconstitution and stem cell mobilization (17). Apart from these, a series of reports have demonstrated that G-CSF displays immunoregulatory properties: G-CSF was able to expand the monocyte/macrophage subset and promote an anti-inflammatory pattern conferring protection in murine

endotoxemia (18). A similar shift in cytokine production pattern has been recently confirmed in human peripheral blood T cells (19). Ha and associates reported that G-CSF gave neural protection against apoptosis in spinal cord injury and exerted its effects through G-CSF receptor on the neural cell (20). The reduced apoptosis of transplanted stem cell by G-CSF administration in peripheral nerve injury was also presented in our previous study (27)

Appropriate intervention in spinal cord injury should depend on the nature, extent, and duration of a particular disease state, as pathophysiology can dramatically evolve over time. Immediately after spinal cord injury, the initial mechanic damage occurred followed by a cascade of potentially harmful secondary events that include the formation of free radicals, detrimental inflammatory response and death of neurons and glia. Attenuation of proimflammatory cystokines such as TNF-α, interleukin 1 β after could promote neurogenesis and lessen gliosis (21). At later time point, interventions must address those processes to target functional regeneration of severed axons and restoration of interrupted axons. Iwanami and associates reported that grafted human neural stem cells survived and differentiated into neurons, astrocytes, and oligodendrocytes to restore connection of spinal cord (22). Thus, concomitant treatment with anti-apoptotic and anti-inflammatory agent such as G-CSF supplemented with neural stem cells not only has effects on suppression of detrimental effects due to injury but also supplies progenitor cells to restore cell loss. This combined effect on the repair of cord injury was shown in our previous study (28).

For neural repair, alginate could contribute to reducing the barrier composed of connective tissues and reactive astrocytic processes, and serve as a scaffold for the outgrowth of regenerating axons and elongation of astrocytic processes (29). Furthermore, alginate encapsulated BDNF-producing fibroblasts (Fb/BDNF) had

survived in culture successfully, made bioactive neurotrophins, survived transplantation into the injured spinal cord in the absence of immune suppression, and provided a permissive environment for host axonal growth (29). Taken together, alginate is a good biocompatible material to encapsulate cells for transplantation.

In this study, we will investigate the application of alginate encapsulation in stem cell therapy for spinal cord injury. Alginate will be fabricated into microspheres to encapsulate novel neural stem cells. Survival, migration and differentiation of the encapsulated stem cells will be evaluated by immunohistochemistry. The novel F1B-GFP neural stem cells will be provided by Dr. Ing-Ming Chiu, the Director of Stem Cell Research, National Health Research Institutes(30). The encapsulation technique will be provided by Professor Shan-hui Hsu at National Chung Hsing University(31). Additionally, the observation period will be extended to 90 days to examine the long-term effect of the treatment. We hope data collected from this study will fast the clinical application of NSCs with alginate polymers transplantation with G-CSF and help the SCI patients with paraplegia to stand up again.

Material and methods:

Preparation of Neural Stem Cells (NSCs)

Mouse brains from two-month old are minced and gently triturated through a series of descending-diameter Pasteur pipets to make a single-cell suspension. Minced cells are seeded in culture dishes without supplementary substrate or adhesion factors in DMEM/F-12 (1:1) medium containing 10% FBS. After two days, most cells died. Cells are pooled together and continue dividing another 2-3 days. At 6 to 8 days, sphere of proliferating cells are formed and then transferred to poly-L-ornithine-coated multi-chamber slides. Cells are transfected with F1B-GFP on

a two-day interval to determine a time point that could achieve the highest percentage of GFP-positive cells. The stable cell lines are obtained by selection with 400 g/ml G418. The G418-resistant mouse neural stem cells are pooled (approximately 30 colonies per plate), and expanded. GFP-positive mouse brain cells are enriched using fluorescence activated cell sorter repeatedly until greater than 95% purity is reached. These GFP-positive cells are also shown to have the self-renewal and pluripotent properties of neural stem cells.

Preparation of alginate-based microspheres

Isolated neural stem cells are centrifuged and resuspended in low viscosity alginate (or a mixture with other type of polymers) at a concentration of a few million cells per ml. Alginate-based microspheres are formed by passing the cell suspension through a small-gauge needle into a bath of $CaCl_2$ with gentle agitation. The microspheres are allowed to cure at room temperature, washed in saline, and then washed in DMEM-HG. The cells are grown in neural stem cell culture media in a humidified environment at 5% CO2 and 37°C for a period of time. The culture medium is replaced every three days.

Spinal Cord Injury and Transplantation of NSCs or G-CSF Treatment

Adult Sprague-Dawley rats (250-300 g) were anesthetized with 4% isoforane in induction and 2% for maintenance. Body temperature was monitored and maintained by thermal blanket. Using aseptic surgical techniques, a dorsal incision was made from the mid to low thoracic regions. Laminectomy over T8 and T9 of the spine was performed followed by exposing the spinal cord assisted with the microscope. Spinal cord injury was performed by transection of the cord at the level T8-T9 leaving 2 mm gap between the proximal and distal end of resected cord. The method of delivery of

stem cells into the injured spinal cord was by using stem cells embedded in fibrin glue (24). Ten μl of NSCs (500,000 cells/μl) or normal saline were injected into the spinal cord gap. The gap was subsequently filled with fibrin glue and covered by a piece of gelfoam. The surgical wound was closed layer by layer and the animals were allowed to recover. The animals were subcategorized into four groups. In group1 (n=10**)** as the control, normal saline (10 μl) was injected to the spinal gap sealed with alginate. In group II (n=10), normal saline (10 μl) was injected to the spinal gap sealed with alginate and the animal received subcutaneous G-CSF injection for five consecutive days (50μg/kg per day; Amgen Biologicals). In group III (n=10), 10 μl of NSCs (500,000 cells/μl) were slowly injected to spinal cord gap and sealed with alginate. In group IV, 10 μl of NSCs (500,000 cells/μl) were slowly injected to the spinal cord gap and sealed with alginate and the animal received subcutaneous G-CSF injection for five consecutive days (50μg/kg per day; Amgen Biologicals). Postoperative care included manual bladder compression 2-3 times per day, injection of lactated Ringer's saline for dehydration, food supplement for weight loss, administration of antibiotics for bacterial infection, and visual inspection for skin irritation or decubitus ulcers.

Electrophysiological Study

The motor evoked potential (MEP) and conduction latency were investigated before the animals were sacrificed 3 months after injury. In brief, the rats underwent anesthetizing and a stimulating electrode was fixed on the dural surface of the motor area (3 mm lateral and 2 mm posterior to the bregma) 24 hours before sacrifice. Electrical stimulator was placed to touch the stimulating electrode and the reference electrode was placed on the opposite side skull; the conduction latency, and the compound muscle action potential were recorded with an active electrode needle 10mm below the tibia tubercle and a reference needle 20 mm from the active

electrode. The stimulation intensity and filtration range were 20mA and20-2000 Hz, respectively.

CatWalk-automated quantitative gait analysis (32)

Computerized gait analysis was performed as described by Hamers et al.. Briefly, animals had to cross a 100-cm-long glass floor plate, confined by Plexiglas walls that were spaced apart 8 cm located in a darkened room. Light from an otherwise completely encased white fluorescent tube enters one of the long edges of the glass floor. The light tube was placed 2 cm from the glass plate so that light entering the 5-mm-thick glass-plate is completely internally reflected. Only at those points where a paw touched the glass plate did light exit the glass-plate through scattering, thereby illuminating the points of contact of the paws. During crossings, the walkway was monitored from below by a Pulnix TM-62EX camera (Pulnix Inc. UK) equipped with a wide angle objective (Cosimar 8.5 mm). The camera registers the paw/floor contact in pixels of 1.23 mm2 whereby the intensity (gray value) of the signal depends on the applied pressure. Higher paw pressure results in a larger total area of skinfloor contact and a more intense scatter, that is, brighter pixels. The signal is digitized by a pcImage-SRGB frame grabber board (Matrix Vision GmbH, Oppenheimer, Germany) and subsequently acquired, compressed and stored by CatWalk software for further analysis. All areas containing pixels brighter than a preset analysis threshold are stored. Using the analysis component of the CatWalk program, these areas are assigned to one of the paws and data exported to a spreadsheet. Quantitative analysis of this data included the following parameters:

1. *Step sequence distributions*. Six different walking patterns or normal step sequence patterns can be discerned in rats, dependent on the sequential placement of the four paws that fall into three different categories (Table 1) (Cheng et al.,

1997)

2. *Regularity index (RI)*. This parameter is a measure of inter limb coordination. Inter limb coordination is considered normal when during uninterrupted locomotion only normal step sequences are used. The regularity index rates the degree of inter limb coordination as a percentage of complete coordination by the following equation: RI _ (NSSP _ 4/PP) _ 100%, wherein NSSP represents the number of normal step sequence patterns and PP the total number of paw placement. Consequently, extra paw placements and irregular walking on three paws will result in a decrease of RI.
3. *Print area*. This parameter was defined as the total floor area in pixels contacted by the paw during stance phase; this is the area that would be blackened if the animals' paw had been inked according to the walking test on paper of Kunkel-Bagden et al. Possible reasons for the hind limb print area to increase are paralysis of the lower limb leading to a deficiency in plantar stepping or paw/toe dragging during part of the step cycle. A decrease in this parameter can be indicative of mechanical allodynia .
4. *Base of support*. The distance in mm between the two hind paws was defined as the base of support. This distance is measured perpendicular to the direction of walking.
5. *Duration of swing and stance phase*. Since duration of stance or swing phase depends on the animal's walking speed and degree of dysfunction, these parameters are transformed to a fraction of total step duration according to the following formula: fraction stance or swing phase _ [time in stance or swing phase/(time single step)] _ 100%. Time of swing or stance phase and total step are expressed in seconds.
6. *Hind paw pressure*. This is the mean intensity of the contact area of the hind paw

at the moment of maximal paw-floor contact. This parameter is expressed in arbitrary units (a.u.).

Histology and Immunohistochemistry of the Spinal Cord

After the assessment of locomotor function and electrophysiologic tests, ten rats in each group received transcardial perfusion with 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4) after being reanesthetized. The spinal cords were removed and dissected by cutting into 1-cm segments rostral and caudal to the lesion center. These 1-cm tissue blocks were postfixed in 4% paraformaldehyde for 4 h and cryoprotected in 20% w/v sucrose in phosphate buffered saline (PBS) for 20 h. Six spinal cords in each group were embedded in Tissue-Tek OCT medium and cryosectioned at 10-μm thickness. After rinsing with PBS, the frozen sections were blocked with 5% skim milk or bovine serum (10%) in PBS for 30 min and then incubated overnight at 4°C with primary antibodies. The following antibodies were used in immunohistochemical studies: (1) microtubule-associated protein 2 (MAP2; 1:500; Neromarkers) for neurons; (2) glial fibrillary acidic protein (GFAP; 1:500; Becton, Dickinson and Company) for astrocytes; (3) Neu-N (1:1500; Chemicon) for neurons; (4) BrdU (5) G-CSF R (1:400; Boehringer Mannheim) for nuclear identification. Subsequently, the sections were rinsed with PBS and incubated with fluorescein isothiocyanate (FITC) - or rhodamine (TRICT) - conjugated secondary antibody for 1 h at room temperature. The immunohistochemical staining was examined under a fluorescence microscope. Among longitudinally consecutive spinal cord sections, five consecutive slices contiguous to those with the maximum diameter were selected for measurement. The targeted area chosen for calculating counts of Neu-N, GFAP, and MAP 2 were situated at that 5mm distal to injured area. Counts of BrdU were calculated directly at the injured site. The counts of immunohistochemical

staining including Neu-N, MAP-2, GFAP, and G-CSFR were obtained at X 200 magnification by using the following sampling technique. Of 100 squares with a surface area of 0.01mm^2 each, 5 were randomly selected in an ocular grid and used to count immunohistochemical staining. The value was presented as mean± SE (standard error).

Part of resected tissues (n=4 in each group) were embedded in Epon by standard method and cut longitudinally with section of 6 um thick, stained with hematoxylin-eosin (H&E). All the histological examinations were performed by a pathologist who was blinded to the treatment method.

Statistical analysis

All values will be expressed as mean ± SEM (standard error). Differences among groups will be first compared by one-way or two- way analysis of variance (ANOVA) and then analyzed by Duncan's test. A $p<0.05$ is considered statistically significant.

Results:

CatWalk Analysis

The ultimate statistical vale of various Catwalks such as stance and swing duration relating to different time points is shown in Figure 1.There was no statistical improvement in group III as compared to that in group I. The statistical significance of improvement existed between group I and group II or IV. However there was no discernible improvement such as printed area and intensity.

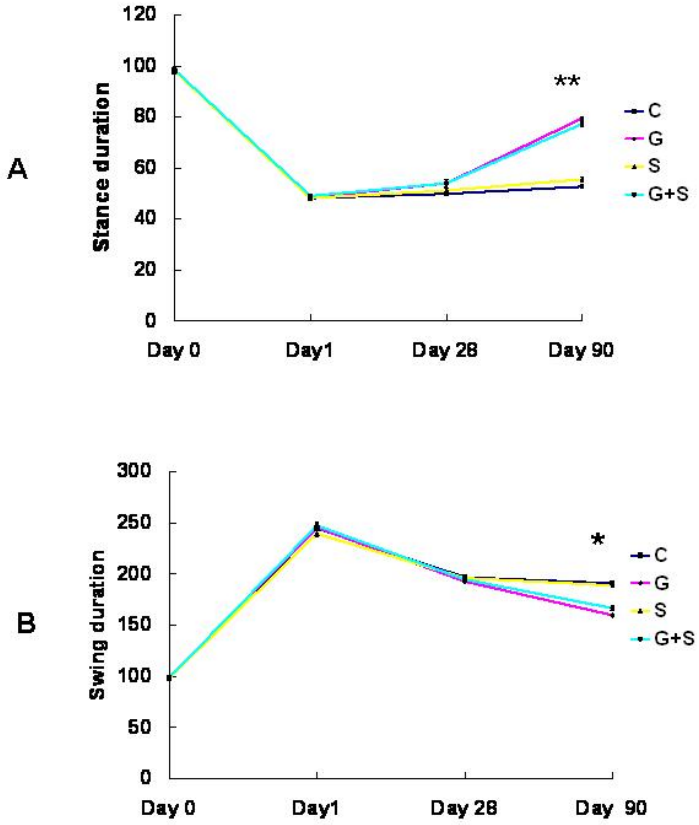

Figure 1: Illustration of stance and swing duration in four treatment groups. (A) Stance duration in four groups (B) Swing duration in four groups

C: control (I); G: G-CSF treated group (II); S: neural stem cells treated group (III); G+S: combination of G-CSF administration and neural stem cells (IV)

*: $p<0.05$; **: $p<0.01$

Electrophysiological examination

Previous studies have demonstrated that objective measurement of residual function in the injured rodent spinal cord could be obtained through behavior and MEP recordings (25, 26). Twelve weeks after injury, the motor evoked potential (MEP) in four respective groups were 32.7 (SE 2.4), 46.6 (SE 1.47), 37.75 (SE 2.11), and 48.7 (SE 1.54) mV illustrated in Figure 2 A. The discrepancy showed the statistical significance ($p<0.001$). The Post Hoc examination showed statistical significance between group I and II ($p<0.0001$), group I and IV ($p<0.00001$), group III and IV

(p=0.002). No significant difference existed between II and IV (p=0.34). The conduction latency in four respective groups were 1.9 (SE 0.11), 1.4 (SE 0.094), 1.76 (SE 0.042), and 1.33 (SE 0.042) msec shown in Figure 2B. The difference in conduction latency is statistically significant (p<0.001). The post Hoc test showed significant difference in group I and II (p=0.008), group I and IV (p<0.001) and group II and III (p=0.007), but there was no statistical significance between group II and IV (p=0.439).

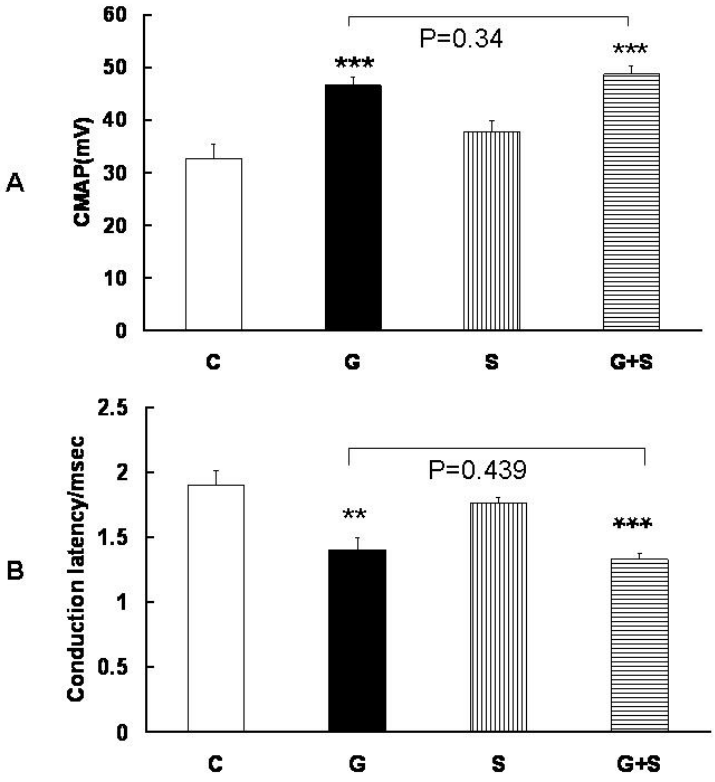

Figure 2: Illustration of electrophysiological study 12 weeks after treatment (A) compound muscle action potential in four treated groups (B) Conduction latency in four treated groups.

C: control (I); G: G-CSF treated group (II); S: neural stem cells treated group (III); G+S: combination of G-CSF administration and neural stem cells (IV)

*: p<0.05; **: p<0.01; ***: P<0.0001

Immunohistochemistry in distal part of injured cord

The immunohistochemical studies over the distal end of injured spinal cord including Neu-N, MAP-2, GFAP, and G-CSFR are illustrated in Figure 3, 4, 5, and 6. Higher expression of Neu-N (Figure 3) and MAP-2(Figure 4) was observed in group I and IV as compared to group I and III. Increased GFAP expression was also shown in group II and IV (Figure 5). G-CSF expression was escalated in group II and IV (Figure 6). There was no statistical increment such as Neun-N, MAP-2, GFAP, and G-CSFR observed in group III as compared to group I.

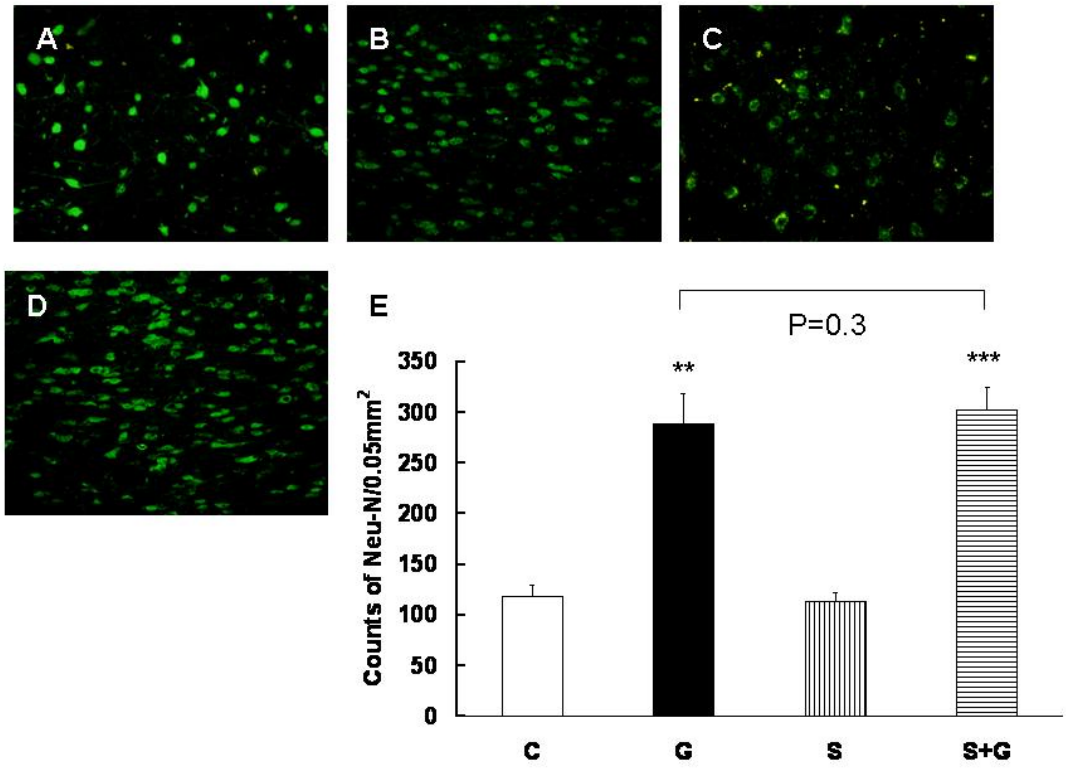

Figure 3: Expression of Neu-N in the distal end of the spinal cord (A) expression of Neu-N in group I (B) expression of Neu-N in group II (C) expression of Neu-N in group III (D) expression of Neu-N in group IV (E) Quantitative analysis of Neu-N

**: $p<0.001$; *** $p<0.0001$

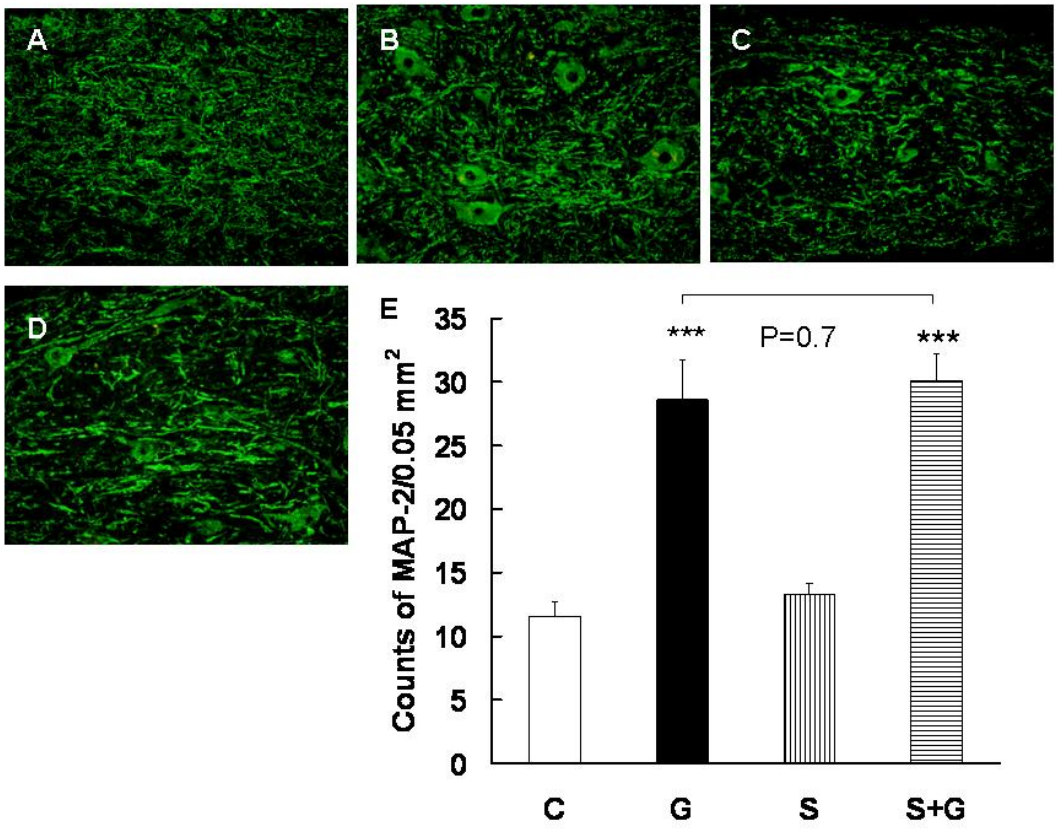

Figure 4: Expression of MAP-2 in the distal end of the spinal cord (A) expression of MAP-2 in group I (B) expression of MAP-2 in group II (C) expression of MAP-2 in group III (D) expression of MAP-2 in group IV (E) Quantitative analysis of MAP-2

*** $p<0.0001$

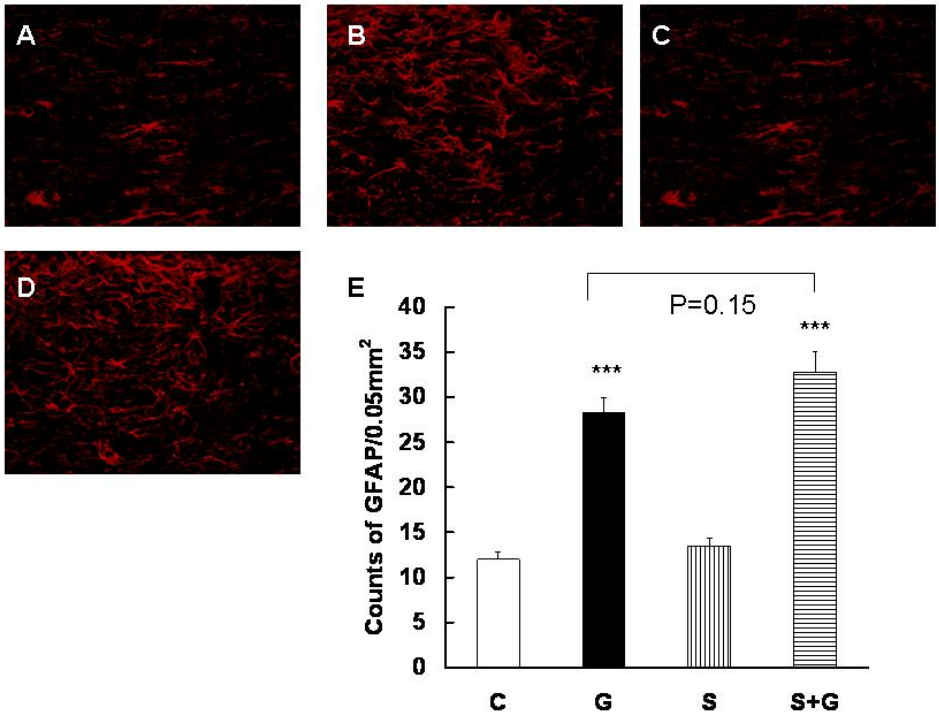

Figure 5: Expression of GFAP in the distal end of the spinal cord (A) expression of GFAP in group I (B) expression of GFAP in group II (C) expression of GFAP in group III (D) expression of GFAP in group IV (E) Quantitative analysis of MAP-2

*** $p<0.0001$

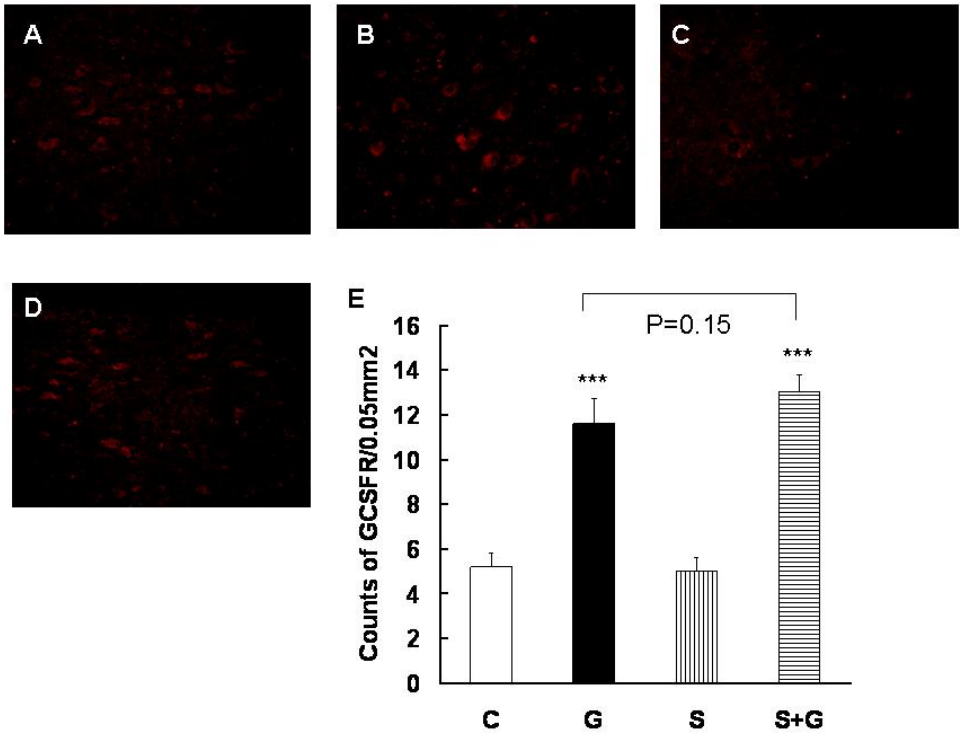

Figure 6: Expression of G-CSF receptor in the distal end of the spinal cord (A) expression of G-CSFR in group I (B) expression of G-CSFR in group II (C) expression of G-CSFR in group III (D) expression of G-CSFR in group IV (E) Quantitative analysis of G-CSFR

*** $p<0.0001$

Conclusion

Neural stem cells with alginate polymers did not augment the regeneration of the spinal cord injury in this rats study. On the contrast, G-CSF administration alone enhanced the spinal cord regeneration. There was no combined effect when treated with neural stem cells with alginate polymers and G-CSF administration.

Acknowledgments

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Reference

1. Chen H Y, Chen SS, Chiu WT, et al.. A nationwide epidemiological study of

spinal cord injury in geriatric patients in Taiwan. Neuroepidemiology 16(5): 241-7,1997

2. Legos JJ, Gopez JJ, Young WF. Non-surgical management of spinal cord injury. Expert Opin Investig Drugs 11(4): 469-82, 2002
3. Young W. Secondary injury mechanisms in acute spinal cord injury. J Emerg Med 11 Suppl 1: 13-22, 1993
4. Fehlings MG, Sekhon. Acute interventions in spinal cord injury: what do we know, what should we do? Clin Neurosurg 48: 226-242, 2001
5. Bailes JE Jr, Van der Veer CA. Surgical management of patients with sport-related spinal injuries. Clinical Neurosurgery 48: 243-259, 2001
6. Molloy S, Middleton F, Casey ATH. Failure to administer methylprednisolone for acute traumatic spinal cord injury-a prospective audit of 100 patients from a regional spinal injuries unit. Injury 33(7): 575-78, 2002
7. Jeffrey LJ, Jonas GJ, William YF. Non-surgical management of spinal cord injury. Expert Opin Investig Drugs 4: 469-482, 2004
8. Blesch A, Lu P, Tuszynski MH. Neurotrophic factors, gene therapy, and neural stem cells for spinal cord repair. Brain Res Bull 57: 833-838, 2002
9. Cheng H, Cao Y, Olson L. Spinal cord repair in adult paraplegic rats: partial restoration of hind limb function. Science 273:510-513, 1996.
10. Cheng H, Wu JP, Tzeng SF. Neuroprotection of glial cell line-derived neurotrophic factor in damaged spinal cords following contusive injury. J Neurosci Res 69:379-405, 2002
11. Björklund A, Lindvall O. Cell replacement therapies for central nervous system disorders. Nat Neurosci 3:537-544, 2000
12. Cao Q, Benton RL, Whittemore SR. Stem cell repair of central nervous system injury. J Neurosci Res 68:501-510, 2002
13. Studer L, Taber V, Mc Kay RD. Transplantation of expanded mesencephalic precursors leads to recovery in parkinsonian rats. Nat Neurosci 1:290-295,

1998

14. Abe K. Therapeutic potential of neurotrophic factors and neural stem cells against ischemic brain injury. J Cereb Blood Flow Meta 20:1393-1408, 2000
15. McDonald JW, Liu XZ et al. Transplanted embryonic stem cells survive, differentiate and promote recovery in injuried rat spinal cords. Nat Med 5:1410-2, 1999
16. Tzeng SF. Neural progenitors isolated from newborn rat spinal cords differentiate into neurons and astroglia. J Biomed Sci 9:10-16, 2002
17. Gorgen I, Hartung T, Leist M et al.. Granulocyte colony-stimulating factor treatment protects rodents against lipopolysaccharide-induced toxicity via suppression of systemic tumor necrosis factor. J. Immunol. 149(3):918-924, 1992
18. Zavala F, Abad S, Ezine S, Taupin V, Masson A, Bach JF. G-CSF therapy of ongoing experimental allergic encephalomyelitis via chemokine- and cytokine-based immune deviation. J Immunol 168(4): 2011-2019, 2002
19. Sloand EM, Kim S, MacIejewski JP et al.. Pharmacologic doses of granulocyte colony-stimulating factor affect cytokine production by lymphocytes in vitro and in vivo. Blood 95(7):2269-2274, 2000
20. Ha Y, Kim YS, Yoon SH, Park SR, Kim EY, Park HC. Role of granulocyte-macrophage colony-stimulating factor in preventing apoptosis and improving functional outcome in experimental spinal cord contusion injury. J Neurosurg Spine 2:55-61, 2005
21. Pannu R, Barbosa E, Singh AK, Singh I. Attenuation of acute inflammatory response by atorvastatin after spinal cord injury in rats. 79:340-350, 2005
22. Iwanami A, Kaneko S, Nakamura M et al.. Transplantation of human neural stem cells for spinal cord injury in primates. J Neurosci Res 15:80(2): 182-190, 2005
23. Chiang YH, Silani V, Zhou FC. Morphological differentiation of atroglial progenitor cells from EGF-responsive neurosphere in response to fetal calf serum, basic fibroblast growth factor, and retinol. Cell Transplant 5:179-189,1996
24. Pan HC, Yang DY, Chiu YT, Lai SZ et al.. Enhanced regeneration in injured

sciatic nerve by human amniotic mesenchymal stem cell. Journal of Clinical Neuroscience 13:570-575, 2006

25. Basso DM, Beattie MS, Bresnahan JC. A sensitive and reliable locomotor rating scale for open filed testing in rats. J Neurotrauma 30:25-51,1995

26. Felings MG, Tator CH, Linden RD, Piper IR. Motor evoked potentials recorded from normal and spinal cord-injured rats. Neurosurgery 20:125-130, 1987

27. Hung-Chuan Pan, Chung-Jung Chen, Fu-Chou Cheng, Shu-Peng Ho, Mu-Jung Liu, Shiaw-Min Hwang, Ming-Hong Chang, Yeou-Chih Wang. Combination of G-CSF administration and human amniotic fluid mesenchymal stem cells transplantation promotes peripheral nerve regeneration. Neurochemical Research 2008.

28. Hung-Chuan Pan, Fu-Chou Cheng, Shu-Zhen Lai, Dar-Yu Yang, Yeou-Chih Wang. Enhanced regeneration in spinal cord injury by concomitant treatment of G-CSF and neural stem cells. J of Clinical Neuroscience 15:656-664, 2008

29. Kataoka K, Suzuki Y, Kitada M, Hashimoto T, Chou H, Bai H, Ohta M, Wu S, Suzuki K, Ide C. Alginate enhanced elongation of early regeneration axons in spinal cord of young rats. Tissue Eng. 10(3-4): 493-504, 2004

30 Chiu IM, Touhalisky K, Liu Y, Yates A, Frostholm A. Tumorigenesis in transgenic mice in which the SV40 T antigen is driven by the brain-specific FGF1 promoter. Oncogene. 14;19(54):6229-39., 2000

31. Hsu SH, Lin CH. The properties of gelatin-poly (gamma-glutamic acid) hydrogels as biological glues. Biorheology. 44(1):17-28, 2007.

32. Hendriks WTJ, Eggers R, Ruitrube William TJ, Ruitemberg J, Blitis B, Hamers FPT, Verhaagen J, Boer GJ. Profound difference in spontaneous long-term functional recovery after defined spinal tract lesions in the rat. Journal of Neurotrauma 23(1): 18-35, 2006