Abstract. – OBJECTIVE: To investigate the synergistic effects of quercetin (Qu) administration and transplantation of human umbilical cord mesenchymal stromal cells (HUMSCs) following spinal cord injury (SCI).

MATERIALS AND METHODS: HUMSCs were isolated, cultured and certificated via flow cytometry. Sixty Sprague-Dawley (SD) female rats were used and SCI models were made. All rats were divided into five experimental groups: culture medium treated group (n=28); HUSMCs + quercetin-treated group (n = 28); HUMSCs treated group (n=28); quercetin-treated group (n = 28); sham group (n = 20). Basso, Beattie, and Bresnahan (BBB) were used to assess neurological function recovery. Axons at the injury epicenter of the injury were checked by immunohistochemical analysis. Cystic cavity was measured and rat cytokine Luminex custom 8-plex kits (for interleukin (IL)-4, IL-1β, IL-6, IL-10, interferon (IFN)-γ, tumor necrosis factor (TNF)-β1) were checked.

RESULTS: The combination treatment with Qu and delayed transplantation of HUMSCs after rat SCI improved neurological functional recovery, increased axonal preservation, promoted macrophage polarization, decreased the size of the cystic cavity, reduced the proinflammatory cytokines, including IL-1β and IL-6. Also, it increased anti-inflammatory cytokines, including IL-4, IL-10, and transforming growth factor (TGF)-β1.

CONCLUSIONS: We showed that HUMSCs transplantation in combination with Qu was a potential strategy for reducing secondary damage and promoting functional recovery following SCI.

Key Words: Spinal cord injury, Quercetin, Mesenchymal stromal cells, Inflammation, Regeneration.

Introduction

Spinal cord injury (SCI) can result in severe neurological damage, which requires hugely expensive, long-term care. Reported traumatic SCI annual incidence rates yielded values ranging from 12.1 to 57.8 cases per million1-3. Experimental studies and clinical observations showed that SCI are greatly enlarged more often by secondary injury than by primary neuronal damage4,5. Secondary injury is caused by a complex of pathological processes, including oxidative stress inflammatory processes, edema, ischemia, apoptosis and tissue necrosis6.

To date, there is no effective treatment to promote functional recovery except for routine medical intervention and care. Thus, the development of improved treatment modalities would be of enormous clinical and economic benefit.

Transplantation of stem cells is one of the most promising strategies to promote neuroregeneration7. It can produce some cytokines or neurotrophic factors that facilitate the regeneration of the injured tissue, resulting in either replacement of the missing cells or rescue of the damaged cells in the injured spinal cord8,9. Among various candidate cells, human umbilical cord mesenchymal stromal cells (HUMSCs) have shown notable potential.

Quercetin (Qu), a natural flavonoid found in high quantities in fruits and vegetables, has been reported to possess potential antioxidant and free radical scavenger10,11. Neuroprotective effects have been demonstrated in established ischemia/reperfusion, intracerebral hemorrhage, and SCI...
animal models\textsuperscript{12-14}. Previous studies have shown that administration of Qu contributes significantly to the inhibition of inflammatory process and promote functional recovery in the early phase after acute traumatic SCI.

Since many different pathogenic processes are involved in SCI, it is unlikely that treatment with a single agent will result in maximum recovery. We used Qu to inhibit inflammatory microenvironment in the early injury stage, and observed the repair of SCI by combination with HUSMCs in rat models of spinal cord crush injury. We provide an experimental basis for new methods of treatment of clinical SCI.

\textbf{Materials and Methods}

\textbf{Cell Culture and Identification}

All parts of this research, especially the isolation of the human umbilical cord, were performed according to the Declaration of Helsinki. Ethical approval was obtained from the Zhujiang Hospital, Southern Medical University (Guangzhou, China), and written informed consent was obtained from donors of umbilical cord (UC). The isolation and culture of HUMSCs were carried out according to the methods previously described\textsuperscript{15}. In brief, each human umbilical cord was collected from full-term Caesarian section births and processed within 3-6 hours. Umbilical arteries and veins were removed, and the remaining tissue was transferred to a sterile container in Dulbecco's modified essential media/nutrient mixture F-12 (DMEM/F12; HyClone, South Logan, UT, USA) and diced into small fragments. The explants were transferred to 50-mL culture flasks containing the DMEM/F12 along with 10\% fetal bovine serum (FBS; PAA, Linz, Austria). They were left undisturbed for 4-6 days to allow migration of cells from the explants, at which point the media were replaced. Cultures were maintained at 37°C in an incubator containing 5\% CO\textsubscript{2}. They were re-fed and passaged as necessary.

To certificate the cultured cells, passage 3 cells (1×10\textsuperscript{5}) were suspended in 50 mL phosphate-buffered saline (PBS) for analysis. Cell fluorescence was evaluated by flow cytometry in a FACSCalibur instrument (Becton Dickinson, Franklin Lakes, NJ, USA). Data acquisition from at least 10,000 events was performed using the Cell-Quest\textsuperscript{™} software (BD Biosciences, Franklin Lakes, NJ, USA) and the Summit MoFlo software (Dako Cytomation, Glostrup, Denmark).

\textbf{PKH-26 Labeling of HUMSCs Before Transplantation}

Cells were labeled with PKH-26 (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. Briefly, a total of 2×10\textsuperscript{7} human Wharton's jelly cells (passage 2) were washed and suspended with serum-free Dulbecco's Modified Eagle Medium (DMEM). After centrifuging at 400 g for 5 min, supernatant was discarded. Cells were resuspended and completely dissolved in 1 mL solution C. Shake should be avoided. 2×10\textsuperscript{6} M PKH-26 staining reagent (diluted in solution C) was prepared prior to labeling. Cells were mixed with PKH26 reagent immediately. The mixture was incubated at 25°C for 2-5 min, and was gently mixed by rocking the tube forward and backward during the incubation. The staining action was blocked by adding the same volume of serum for 1 min of incubation. Then, cells were centrifuged at 400 g for 10 min at 25°C. The supernatant was removed and cells were transferred into a new tube. After three times washing, 10 mL of complete culture medium were applied, and cells were centrifuged. Next, cells were adjusted to an appropriate density, and were observed under fluorescent microscope.

\textbf{Spinal Cord Injury}

Sixty Sprague-Dawley (SD) female rats (8-10 weeks old, weight 174-236 g) were used in the present work with the approval from the Animal Committee of General Military Hospital of Beijing. All animal experiments were performed in accordance with the Guidelines for Animal Experiments of National Institutes of Health (NIH) that were laid down in compliance with the international Regulations for Animal Welfare. All efforts were made to minimize the number of animals and their suffering throughout the experiments. Rats were anesthetized by intraperitoneal injection (0.3 mL) of a mixture of nembutal (pentobarbiturate sodium, Sigma-Aldrich, St. Louis, MO, USA) and atropine (1:1) (Sigma-Aldrich, St. Louis, MO, USA). The body temperature of
the rats was kept at 38°C throughout the surgical procedure. Laminectomy was performed at the vertebra Th9-10 vertebra, and the exposed spinal cord was compressed with a microvascular clip (BS4 61-0196) for 30 s of spinal segment L1. Following removal the clip, the muscles and skin were sutured in layers. After surgery, 5-10 mL physiological medium were injected subcutaneously to prevent dehydration of the rat. The animals were placed in warm cages overnight. Food and water were given ad libitum. Manual bladder expression was performed twice a day until the recovery of the bladder reflex. Ektacin was given if signs of urinary infections were seen.

**Treatments and Animal Groups**

For the different treatments, the rats were divided into five experimental groups: (A) culture medium treated group \((n=28)\); (B) HUSMCs + quercetin-treated group \((n=28)\); (C) HUMSCs treated group \((n=28)\); (D) quercetin-treated group \((n=28)\); (E) Sham group \((n=20)\).

For quercetin \((50 \mu\text{mol/kg})\) or saline treatment, it was dissolved in 0.1% dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO, USA) and administered 1 h after SCI, and then every 12 h until day 3 post-injury.

A total of PKH-26 labeled \(4\times10^5\) cells, divided into two dosages, were transplanted into the injured spinal cord after 3 days after SCI. The injections were made at 2 mm rostral and 4 mm caudal to the epicenter of the lesion at a depth of 1.2 mm. At each site, 2 mL of a cells suspension containing \(2\times10^5\) cells or PBS were injected through a glass micropipette with a tip diameter < 60 mm at a rate of 0.4 mL/min.

**Behavioral Assessments**

Each animal was familiarized with the Bassom, Beattie, and Bresnahan (BBB) open field apparatus and pre-trained on the horizontal ladder prior to surgery. Following the spinal cord compression, the animals were assessed in the BBB open field apparatus at day 1 post-injury and then weekly for 4 weeks\(^6\). The BBB score ranged from 0 to 21, where 0 reflects no movement of the hind limbs and 21 implies normal locomotion. If the score was < 8, the rat could only move its hind limb joint without supporting its body weight. Scores from 9 to 13 represent the rat being able to support its body weight without coordination. A score from 14 to 21 means the rat can stabilize its trunk and coordinate the movement. In this work, the assessment of hind limb motor function was strictly based on the objective criteria. Scoring was conducted by 2 independent observers blinded as to the treatment group using the digital recordings, which could be analyzed frame by frame when necessary. Averaged scores were used as the final score for each trial.

**Immunohistochemistry**

Rats were sacrificed 8 weeks after injury, and were deeply anesthetized by an intraperitoneal injection of Nembutal (100 mg/kg), and perfused intracardially with 50 mL PBS, followed by 200 mL of a fixative containing 2% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA) in 0.1 M phosphate buffer, pH 7.4. Specimens were processed in a standard procedure for embedding in optimal cutting temperature (OCT) compound, and cut coronally or horizontally into 15-μm-thick frozen sections with a cryostat (CM1510S; Leica, München, Germany). Frozen sections were mounted on a coated glass slides.

According to previous reported protocols for immunohistochemistry\(^3\), the sections, after been washing three times with PBS and blocking with a 0.1% bovine serum albumin (BSA) solution containing 0.1% Tween 20 (Beyotime, Beijing, China) in PBS for 30 min, were incubated overnight with a solution containing primary antibodies as follows: rabbit anti-glial fibrillary acidic protein (GFAP) antibody \((1:400)\); Sigma-Aldrich, St. Louis, MO, USA) for astrocytes, rabbit anti-β-tubulin III antibody \((1:500)\); Chemicon, Temecula, CA, USA) for neurons, rabbit anti-GalC antibody \((1:600)\); Chemicon, Temecula, CA, USA) for oligodendrocytes, rabbit anti-NF-200 antibody \((1:100)\); Sigma-Aldrich, St. Louis, MO, USA) for axons, and mouse anti-serotonin antibody \((5-HT)\) \((1:1000)\); ImmunoStar, Hudson, WI, USA) for the detection of raphe spinal fibers, rabbit anti-Arginase-1 antibody \((1:100)\); Abcam, Cambridge, MA, USA) for M2, and mouse anti-iNOS antibody for M1. Secondary antibodies were Alexa Fluor 488 goat anti-mouse IgG \((1:200)\) and Alexa Fluor 594 goat anti-rabbit IgG \((1:200)\); both Molecular Probes, Eugene, OR, USA). Nuclei were counterstained with 4’,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, St. Louis, MO, USA). Cells were defined as any non-autofluorescent object expressing PKH-26, with typical cell morphology and clearly delineated cell borders.

**Measurement of Cystic Cavity**

To measure the area of the cystic cavity, every sixth section \((120 \text{ mm apart})\) of the central por-
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Quantitative Analyses of Stained Tissue Sections

Cytokines

Statistical Analysis

Isolation and Characterization of HUMSCs

Results

Isolation and Characterization of HUMSCs

HUMSCs displayed a fibroblastic morphology after three days in culture. After three to four passages, cells developed an elongated or spindle-shaped morphology and became relatively homogeneous in appearance (Figure 1A). The loaded PKH26 dye was distributed homogeneously on cell surface. Clear cell outline with red fluorescence was observed under phase-contrast fluorescent microscope (Figure 1B). HUMSCs were characterized by the expression of MSC markers using flow cytometry (Figure 1C), including CD44 and CD90, but not the hematopoietic lineage markers CD34 and CD45.
**HUMSCs + Qu Improved Neurological Function**

The locomotor function (Figure 2) of hind-limbs in the sham group recovered to a score of 21 after one week after injury. In comparison, the BBB scores of the other groups, including control group, Qu group, HUMSCs and HUMSCs + Qu groups, were < 8 by the end of one week after injury. Throughout the observation period, there was no significant difference between HUMSCs-treated and saline-treated groups. Functional recovery took place and, starting from day 14 post-injury, HUMSCs + Qu-treated rats showed significantly greater improvement of neurological function compared with rats in the control- (p<0.01), HUMSCs- (p<0.01) and Qu (p<0.01)-treated groups. Both HUMSCs + Qu- and Qu-treated rats showed significant higher motor functional recovery than those in the control group from 21-28 days post-injury. In comparison, the HUMSCs + Qu-treated group showed better functional recovery than those in the Qu group (p<0.05) from 14 days to the end of experiment.

**HUMSCs + Qu Reduced the Size of the Cystic Cavity**

To elucidate the efficacy of Qu treatment and HUMSCs transplantation for tissue protection or tissue sparing after SCI, we measured the area of the cystic cavity with cresyl violet staining 4 weeks after transplantation. The average area of the cystic cavity was significantly smaller in

![Figure 1](image)

*Figure 1.* The morphology and identification of HUMSCs. The appearance of HUMSCs in primary A, and B, four passages. C, Immunophenotypic analysis of cultured HUMSCs.
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The HUMSCs + Qu group than other groups (vs. control group: \( p < 0.01 \); vs. Qu group: \( p < 0.05 \); vs. HUMSCs group: \( p < 0.05 \)) (Figure 3). Both the HUMSCs (\( p < 0.05 \)) and Qu (\( p < 0.05 \)) groups showed significantly decreased areas than control group.

**Fate of Grafted HUMSCs**

Histological examination revealed that the HUCMSCs survived for 4 weeks after transplantation. To evaluate the neural differentiation potential of the HUCMSCs in the spinal cord environment, the expression of neuronal and glial marker proteins was analyzed using immunohistochemistry. The results showed that, whether they were within or outside of the lesion zone, the HUCMSCs failed to express the early neuronal cell marker \( \beta \)-tubulin III or the glial proteins GFAP and GalC. Therefore, the HUCMSCs did not differentiate into neurons, astrocytes or oligodendrocytes after transplantation into the acutely injured spinal cord (Figure 4).

**Axonal Immunoreactivity**

To examine whether treatment with HUCMSCs + Qu affected the preservation of neurofilaments, we performed immunohistochemical analysis with an anti-neurofilament (NF-200) 4 weeks post-injury (Figure 5A). Compared to the control group, the HUCMSCs + Qu, HUMSCs and Qu-treated group exhibited greater preservation of NF-200° axons at the injury epicenter of the injury. The maximum preservation of NF-200° axons was observed in the HUMSCs + Qu group. The descending serotonergic raphe spinal axons are critical for their recovery of hind-limb locomotion.
Figure 4. The fate of HUCMSCs after transplantation into the injured rat spinal cord. The immunoreactive staining of given neural marker (A, β-tubulin III; B, GFAP; C, GalC) positive cell in HUMSCs and HUMSCs + Qu group after 28 days post transplantation. Scales bar showed in A, A-D = 100 μm.

locomotor function in rat SCI. We, therefore, evaluated whether the staining of these axons was enhanced in the caudal of the lesion in the HUCMSCs + Qu group. The serotonergic raphe spinal axons were immunohistochemically analyzed by antibody that specifically reacts with 5-hydroxytryptamine (5-HT), which is synthesized within the brainstem. On the injury epicenter, the area
of 5-HT+ fibers was significantly greater in the HUMSCs + Qu group than in the other 3 groups (vs. control: \( p<0.05 \); vs. HUMSCs group: \( p<0.05 \); vs. Qu group: \( p<0.05 \)) (Figure 5B).

**Cytokine Expression**

To test whether the combination therapy with HUMSCs + Qu could modulate the inflammatory process by regulating the secretion of pro-and anti-inflammatory cytokines, we analyzed the spinal cord tissue (T11 spinal segment) levels of IL-4, IL-6, TGF-β1, TNF-α, IFN-γ, IL-1β, and IL-10 (Figure 6). Levels of the proinflammatory cytokines, including IL-6 and IL-1β at 4 and 7 days after SCI were all significantly decreased in the HUMSCs + Qu-, Qu- or HUMSCs treated rats groups compared with the control group. In contrast, the production of the anti-inflammatory cytokines, including IL-4, IL-10, and TGF-β1 at 1, 4, 7 days after SCI in the HUMSCs + Qu-, Qu- or HUMSCs groups were markedly higher than those in the control group. The significant upregulation of the levels of IFN-γ in the HUMSCs + Qu-, Qu- or HUMSCs groups than control groups were only observed at 7 days post-injury. When compared among treated groups, the levels of proinflammatory cytokines were significantly attenuated (IL-1β and IL-6) and the levels of anti-inflammatory cytokines (IL-4, IL-10, and TGF-β1) in the HUMSCs + Qu group were significantly higher than those in the Qu groups at 1, 4, and 7 days post-injury.

**Macrophage Polarization**

To determine whether HUMSCs + Qu affect macrophage polarization, we quantified the populations of M1 and M2 phenotypes. iNOS (M1
phenotype) and Arginase (M2 phenotype) immunostaining in coronal sections at 3 and 7 days post-injury identified relatively high numbers of positive cells gathered in the injury epicenter, and extending proximally and distally from the injury epicenter in control group. The iNOS-immunopositive products were located mainly in the membrane of the stained cells (Figure 7A). Quantification analysis showed that the HUMSCs + Qu-, Qu- or HUMSCs groups showed decreased numbers of iNOS-expressing macrophages when compared with the control group. These differences were significant from days 7 and 14 post-injury (Figure 7B). The arginase-immunopositive products were located mainly in the cytoplasm of the stained cells (Figure 7C). Quantification analysis showed that the HUMSCs + Qu-, Qu- or HUMSCs groups showed increased numbers of Arginase-expressing macrophages when compared with the control group. These differences were significant from days 7 and 14 post-injury (Figure 7D). In comparison, HUMSCs + Qu showed decreased numbers of iNOS and increased numbers of Arginase-immunoreactivity than HUMSCs or Qu group from days 7 and 14 post-injury.

**Discussion**

We demonstrate that the combined therapeutic treatment of Qu infusion with HUCMSCs transplantation has greater therapeutic effects than the use of Qu or HUCMSCs alone following SCI. First, as measured by BBB scores, the rats in the combinatory treatment group showed better functional recovery compared to the other groups. It was found that significant functional recovery began 2 weeks post-injury and increased gradually. Secondly, morphological analysis using HE staining showed that the lesion cavities of the combinatory treatment group were smaller than those of the control, Qu, or HUCMSCs rats. Finally, immunohistochemical analysis showed increased axonal preservation in the HUMSCs + Qu group when compared with other groups. In addition, HUMSCs + Qu modified the inflammatory environment by decreased proinflammatory cytokines and shifting the macrophage phenotype from M1 to M2 in the injury site. HUMSCs grafted alone into SCI models demonstrated variable degrees of functional improvement in different established animal models. Some researches showed that direct injection of HUMSCs into injury site promotes functional recovery at 3-8 weeks after graft in established contusive injury model. Allogeneic rat HUMSCs transplantation improves motor function in hemisected spinal cords in rats. Interestingly, our assessments of behavior and histology following HUMSCs transplant-alone did not detect significantly different locomotor recovery compared with the control group. The reason for the poor response was the site of the transplantation. We injected HUMSCs into rostral and caudal sites to facilitate migration of HUMSCs and remyelination of regenerative fibers. However, histological examination found that resid-

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**Figure 6.** Anti-inflammatory effects of HUMSCs + Qu group. The levels of IL-4, IL-6, TGF-β1, IFN-γ, IL-1β and IL-10 were analyzed. n = 6, *p<0.05, **p<0.01 and ***p<0.001 compared with the control group at the same day; †p<0.05, ‡p<0.01 and ‡‡p<0.001 compared between HUMSCs + Qu groups and Qu group.
ual HUMSCs were scarce and that they did not migrate as we had expected they would. Thus, transplanting HUMSCs directly into the site of injury might yield better outcomes. As shown by our double immunofluorescence analysis, we found no evidence that HUCMSCs differentiated into neuronal or glial cells after transplantation into the injured rat spinal cord. Although some previous studies showed that HUMSCs could transform into neural-like cells in vivo (expressing the surface marker of neuron and glial), very few cells survived following transplantation. In view of the rapidity of recovery, cell replacement unlikely to explain the improvement in functional outcome. Furthermore, they most likely do not involve transdifferentiation with cell replacement.

To make the transplantation of HUMSCs more effective, we combined HUMSCs transplantation with infusion of Qu. There is a pronounced inflammatory response after SCI, accompanied with activation of resident microglia, leukocyte influx into the brain, and production of inflammatory mediators. The anti-inflammatory and immunosuppressive effects of Qu after SCI have been demonstrated widely in the experimental studies. In some cases with cerebral ischemia, HUMSCs were transplanted to reduce the local inflammation. However, we found that HUMSCs

Figure 7. The effect of HUMSCs + Qu on macrophage polarization after SCI. iNOS A, (M1 phenotype) and Arginase C, (M2 phenotype) immunostaining showed relatively high numbers of positive cells gathered in the injury epicenter. Quantification analysis the numbers of iNOS B, and Arginase D, expressing macrophages. Scales bar showed in A, A = 100 μm, n=4, *p<0.05, **p<0.01, ***p<0.001.
Combined therapy showed better locomotor recovery compared with controls, and histological studies of cavity volume as well as macrophage polarization, anti-inflammatory responses and serotonergic fiber counts confirmed the locomotor results. Future studies are required to identify the effect of combined Qu infusion and HUMSCs transplantation to clarify any potential clinical application to damaged spinal cords in humans.

Conclusions

Combined therapy showed better locomotor recovery compared with controls, and histological studies of cavity volume as well as macrophage polarization, anti-inflammatory responses and serotonergic fiber counts confirmed the locomotor results. Future studies are required to identify the effect of combined Qu infusion and HUMSCs transplantation to clarify any potential clinical application to damaged spinal cords in humans.

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Conflict of Interest

The Authors declare that they have no conflict of interest.

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