



Adipose tissue derived stromal vascular fraction transplantation can recover spinal cord injury in mice

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Abstract

Introduction: Stem cell therapy is one of the most promising therapies for degenerative diseases and related injuries. Adipose tissue derived stem cells (ADSCs) exhibit some particular properties such as high production of paracrine factors. Indeed, ADSCs have been successfully used to treat diseases, including osteoarthritis, diabetic ulcer, etc.

Methods: In this study, ADSCs were used to treat spinal cord injury (SCI) in a mouse model. Non-expanded ADSCs, from stromal vascular fractions (SVFs) isolated from both autologous and allogeneic adipose tissues, were injected into injured sites of mice at a specified dose. The SCI mouse model were generated by transection of spinal cord at vertebrae T8 - T10. After 1 week of transection, mice exhibiting completed SCI were divided into 4 groups: group 1 was control (mice without any treatment), group 2 was placebo (mice treated with platelet rich plasma (PRP)), group 3 was allogeneic SVF transplantation (mice treated with allogeneic SVFs), and group 4 was autologous SVF transplantation (mice treated with autologous SVFs). For the treatment groups, mice were transplanted with 20 μ L of activated PRP or/and with 10^6 cells of SVF (allogeneic or autologous) into the injured position through laminectomy. The recovery of SCI was evaluated by locomotor test, sensory test and sensory-motor test at 5 weeks after transplantation. The histology of the spinal cord also was checked after 5 weeks.

Results: The results showed that in all groups with PRP injected with or without SVFs, the inflammation was efficiently controlled. The glial scar as well as myelin defragmentation were clearly reduced. However, a significant improvement of BBB score was only recorded in mice transplanted with autologous SVFs. **Conclusion:** The results of our study show that autologous SVF transplantation in combination with PRP can be a promising therapy for SCI.

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Competing interests: The authors declare that no competing interests exist.

Received: 11 October 2016
Accepted: 01 December 2016
Published: 21 December 2016

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Keywords

Adipose derived stem cells, Mesenchymal stem cells, Spinal cord injury, Stem cell therapy, Stromal vascular fraction

Introduction

The spinal cord is an organ within the nervous system with limited ability to regenerate after injury. Mechanical action would affect the internal structure and blood flow interruption could destroy nerve and glial cells. Damaged cells respond by releasing neurotoxic factors, producing cleaved axonal myelin, and inducing greater cell death. The inflammatory response starts to increase and leukocytes are mobilized to the site of damage. Astrocytes and fibroblasts produce glial scars. All of the above impede spinal cord recovery (Blight, 2000; Donnelly and Popovich, 2008).

Many therapies have been administered- some immediately and some long after SCI. There have been the use of nervous growth factors, neuro-protective factors, and scaffolds possessing similar spinal cord structures (Carballo-Molina and Velasco, 2015; Macaya and Spector, 2012; Straley et al., 2010); however, none have to date yielded expected results. Progress in transplantation of neural stem cells (NSC) from pregnant/adult brain or spinal cord, embryonic stem cells (ESCs), and induced pluripotent stem cells (iPSCs) has brought many hopes for patients with SCI.

Adipose-derived stem cells (ADSCs) is a new type of autologous cells with many outstanding features, which include higher attainable cell number, reduced invasive capacity and lower immunogenicity (Nakagami et al., 2006; Safford et al., 2004). Compared to transplantation of bone marrow stem cells (BMSCs), which has been widely used, ADSCs are also advantageous in that they show similar uniform characteristics (in morphology, proliferation, differentiation potential and expression of various surface molecules). Nowadays, several studies have aimed to differentiate ADSCs into Schwann cells, which are important for myelinating axons in the spinal cord. Moreover, ADSC transplantations have been evaluated in a SCI mouse model (Jang et al., 2010; Jiang et al., 2008; Kingham et al., 2007; Zaminy et al., 2013; Zhang et al., 2009).

The aim of this study was to evaluate the effects of stromal vascular fraction (SVF) transplantation on spinal cord regeneration in a mouse model of spinal cord injury.

Materials-Methods

Spinal cord injury (SCI) mouse model

Non-disease mice (8-10 weeks of age, 30-40 g in weight) were used in this study. Mice were anesthetized, fixed and underwent laminectomy on the back-lumbar. The SCI mouse model was generated by cutting the spinal cord (transection) at vertebrae T8-T10 to induce damage. Care after surgery included sutures, antiseptics, feeding, and antibiotic injections (penicillin dose of 5mg/kg/mouse/day). All manipulations were approved by the local ethical committee for animal use.

Stromal vascular fraction extraction from adipose tissues

One gram of fat tissue was collected through surgery and washed with PBS supplemented with antibiotics. The SVF was then extracted using a commercial ADSC extraction kit (Geneworld Ltd., HCM, VN). Briefly, the fat tissue was mixed with SuperExtract via gentle mixing for 5 min at room temperature and 50 min at 37°C. After 30 min, the digested fat was centrifuged at 2500 rpm for 5 min to obtain the pellet. Next, the pellet was resuspended and filtered through an 70- μ m filter to collect single cells. The single-cell suspension was diluted with platelet-rich plasma (PRP) to obtain a density of 10^8 cells/mL. The PRP was prepared from human umbilical cord blood according to a previously published protocol (Van Pham et al., 2013).

The SVFs were evaluated for presence of ADSCs and cell survival rate. The presence and percentage of ADSCs in SVFs was evaluated by flow cytometry based on expression of these markers: CD44, CD73 and CD90. Cell survival rate was evaluated based on 7-AAD dye staining and analyzed by flow cytometry. Non-expanded ADSCs, from SVFs isolated from both autologous and allogeneic adipose tissues, were used for subsequent transplantation studies.

Transplantation and recovery assessment

One week after spinal cord damage induction, mice with complete SCI were divided into 4 treatment groups (15 mice per group): Group 1 (control, i.e. untreated mice), group 2 (placebo, i.e. mice treated with PRP), group 3 (mice receiving 20 μ L of activated PRP containing 10^6 allogeneic SVFs at the injury position via laminectomy), and group 4 (mice receiving 20 μ L of activated PRP containing 10^6 autologous SVFs at the injury position via laminectomy).

To evaluate the damage level and recovery of the spinal cord injury in our mouse model, the following tests were conducted at 5 weeks post transplantation: locomotor test according to the BBB scale (Barros Filho and Molina, 2008; Basso et al., 1995), sensory test (Gale et al., 1985), and a sensory-motor test (Behrmann et al., 1992). All tests were conducted each week, for 4-5 weeks.

According to several recent studies (Ankeny et al., 2009; Donnelly and Popovich, 2008), changes in leukocytes in the mouse models have greatly affected their ability to recover. Therefore, changes in the percentage of total leukocytes were assessed each week. The structure/histology of the spinal cord was also assessed, via HE staining, at 5 weeks after transplantation.

Existence of transplanted cells

Male SVF cells were isolated and transplanted into female recipient mice. The existence of grafted cell can be evaluated by using PCR method for spinal cord DNA extraction with SRY specific primers that only exist in the male cells.

Statistic analysis

Data were analyzed by GraphPad Prism 5 software, using 2 way ANOVA analysis followed by Bonferroni post-tests to assess differences between experiment groups; $p < 0.05$ was considered statistically significant.

Results

Adipose tissue derived cells

Both autologous and allogeneic adipose tissues were used to isolate SVF cells. The results showed that there were $(1.31 \pm 0.08) * 10^6$ SVF cells per 1 gram of fat with $90.87 \pm 1.01\%$ live cells. To determine the existence of mesenchymal stem cells in SVFs, total cells were analyzed for expression of CD44, CD73 and CD90. The results showed that approximately $63.26 \pm 2.02\%$ of the cells stained positive for both CD44, CD73 and CD90 markers.

Survival rate

In all the groups, more than 50% of mice died after 5 wks. The group with the highest survival rate was the group which received autologous SVF transplantation; the group with the lowest survival rate the placebo group (**Fig. 1**).

Body weight

The results presented in Figure 2 show that treatment of the SCI strongly affects mean body weight. After inducing complete SCI in mice, mouse body weight in all the mouse groups significantly declined after the first week. However, afterwards mice in the control, placebo and allogeneic SVF transplantation groups showed similar mean body weights (no significant difference among these groups at 2, 3, 4 and 5 wks post treatment). However, mice in the autologous SVF transplantation group showed a rapid weight decline after 1 wk of inducing SCI, and continuously decreased from wk 2 to wk 5. In total, mice in

this group reduced 10 grams after 5 wks, compared to before SCI induction and treatment (Fig. 2).

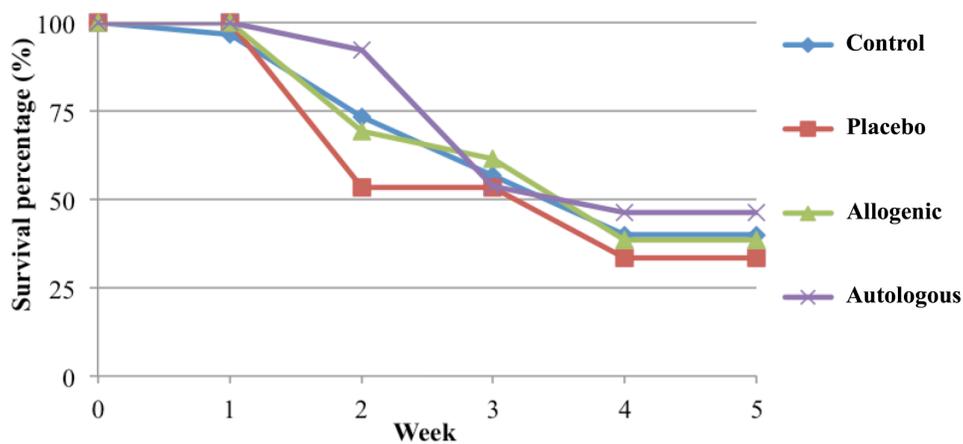


Figure 1. Survival rate of mice with SCI after various treatments. The survival rate of mice in the autologous SVF transplantation group was lowest. Survival rates of the control, placebo and allogenic SVF transplantation groups were not significantly different.

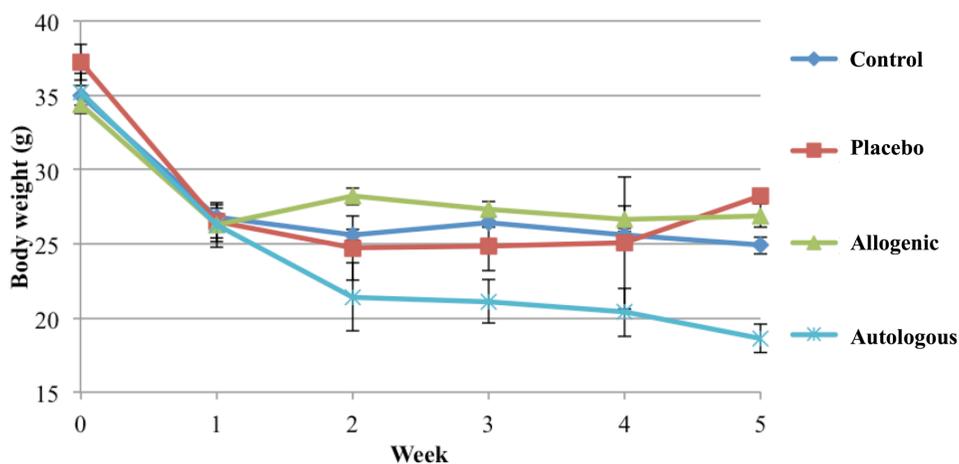


Figure 2. Mean body weight of mice after SCI induction and various treatments.

Mice in the autologous SVF transplantation group showed a rapid reduction in body weight over the course of 5 weeks, while mice in other groups stayed steady from week 1 to 5.

Locomotor test

Locomotor testing was conducted every week for 4 weeks according to the Basso, Beattie and Bresnahan (BBB) locomotor scale method (i.e. BBB score) (Basso et al., 1995; Basso et al., 1996). After 4 weeks, all mice in the control (untreated) group were completely paralyzed with score 0 on the BBB scale. The slight improvement of BBB scale was observed in the placebo and allogeneic SVF groups (Fig. 3). A significant improvement of locomotor activity was observed in the autologous SVF transplantation group compared to control and placebo ($p < 0.05$).

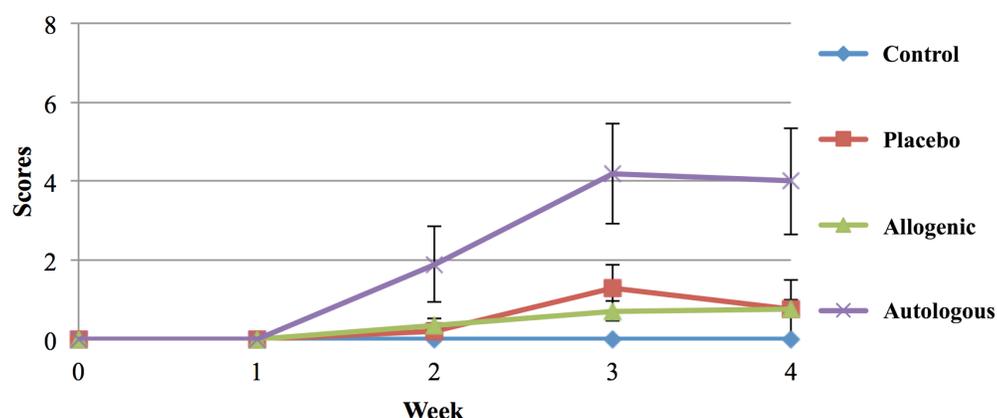


Figure 3. Locomotor score of mice with SCI after various treatments. Locomotor activity in allogeneic SVF-treated mice showed a significant improvement compared to the autologous SVF, control (untreated) and placebo (RPP only) treatment groups.

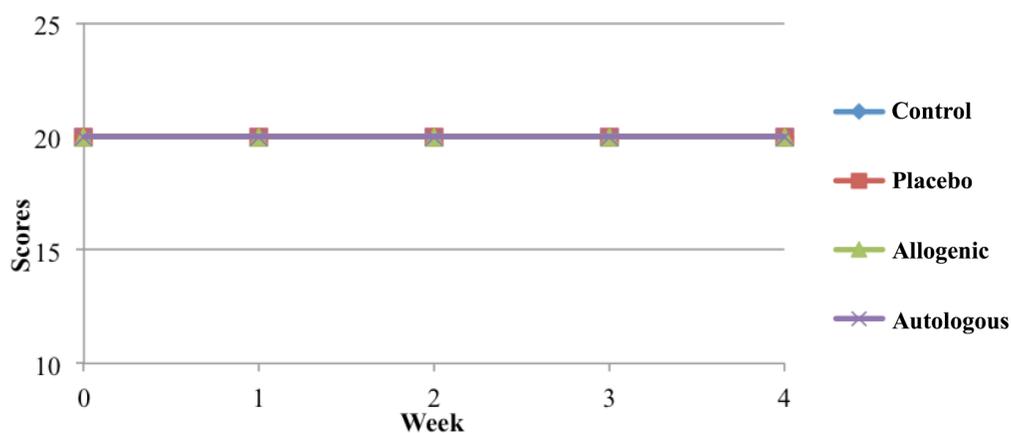


Figure 4. The results of sensory-motor testing of mice with SCI after various treatments. Mice in all groups could not improve their sensory-motor testing scores.

Sensory-motor testing

To assess neural control of hind limb movement, mice were evaluated further by going on a grid. The low improvement of BBB scores suggested a low recovery of nervous control. Normally, only mice with BBB scores of 14 or more can control movement (toe stretching) (Basso et al., 1995; Santos et al., 2011). Similar to what the locomotor scores reflected in the previous results, mice in all the groups were not able to significantly recover their sensory-motor skills (Fig. 4).

Total leukocytes

In the control group, the inflammatory response normally occurs after injury of the spinal cord. Indeed, the total leukocyte count significantly increased after 3 wks. Meanwhile, for the placebo (PRP only), autologous SVF transplantation and allogeneic SVF transplantation groups, the mice showed a significant reduction of total leukocytes. From weeks 3 and 4, the total leukocyte count in the placebo and allogeneic SVF transplantation groups increased (Fig. 5), as the number of surviving mice greatly reduced (Fig. 1). This correlation suggests perhaps that one mechanism by which mouse vitality is affected is through the immune system (Fig. 5).

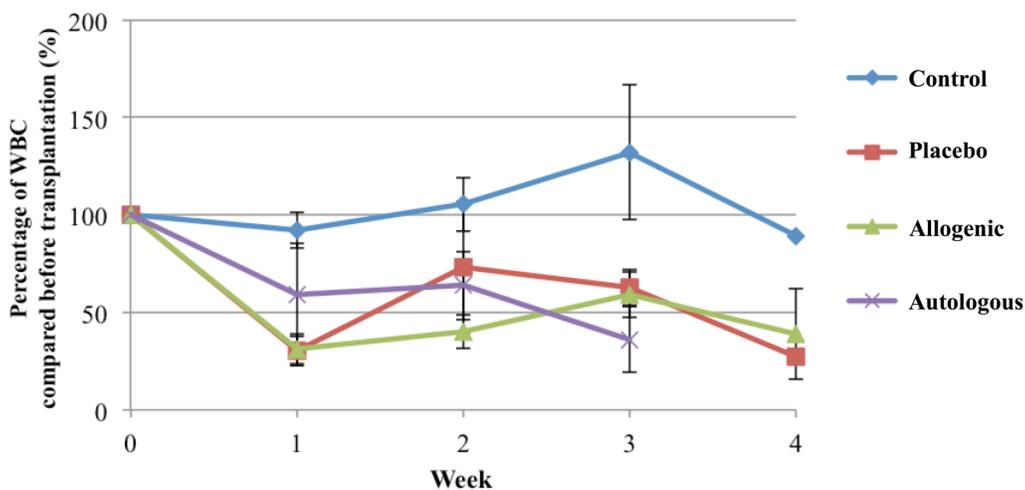


Figure 5. Change in total leukocyte count of mice with SCI after various treatments. The results suggest that strong inflammation is occurring in mice of the control group; however, the inflammatory process appears partly inhibited in mice of the placebo (PRP only), autologous SVF transplantation and allogeneic SVF transplantation groups.

Histological analysis

The formation of glial scars stimulated by astrocytes has been shown to negatively affect recovery. Therefore, the inhibition of glial cell proliferation after injury can significantly improve SCI recovery in mice, both in movement and sensation. Histological analysis showed glial scar formation in almost all the mice, and that inflammation niches appeared after SCI induction and the various treatments (Fig. 6). However, for the autologous SVF transplantation mouse group, the spinal cord exhibited greater integral structure and reduced demyelination (Fig. 6). These factors can cause mice to recover partial hind limb function, but not fully.

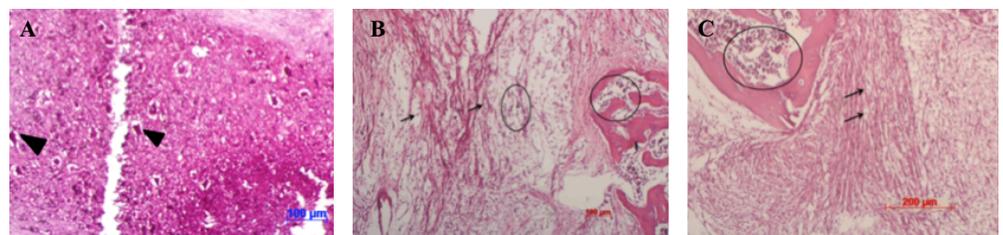


Figure 6. Damaged spinal cord tissue structures of mice with SCI after various treatments. The cross-sections of spinal cord tissues were analyzed in mice of the following groups: control (untreated) (A), placebo (PRP only) (B), autologous SVF transplantation (C). Arrowheads: neurons; arrows: fragments of myelin; circle: inflammation site.

Detection of transplanted cells

To determine the presence of ADSCs in grafted mice, allogeneic SVF cells from male mice were collected and transplanted into female SCI mice. The detection of the SRY gene in grafted spinal cord tissue was evaluated by PCR. The results showed that grafted spinal cord tissue did not express the SRY gene.

Discussion

SCI is an extreme injury with low regeneration potential. Therefore, almost all cases of SCI develop into complete paralysis. The primary aim of this study was to evaluate the effects of SVF transplantation in a mouse model of SCI.

In the first experiment, SVFs from both autologous and allogeneic adipose tissues were extracted according to the manufacturer's recommendations. The results showed that SVFs could be successfully extracted from adipose tissues

with a high percentage of viable cells. More importantly, the ratio of ADSCs in the SVFs was high (approximately $63.26 \pm 2.02\%$).

The SVFs were evaluated as treatment for mice with SCI. The results showed that after 5 weeks of treatment, there was a significant improvement in the autologous SVF transplantation group, in combination with PRP. The results appeared to show that PRP could cause anti-inflammation in the placebo, autologous SVF transplantation and allogeneic SVF transplantation groups. In fact, the role of PRP in inflammation control has been observed in some previous studies (Bendinelli et al., 2010; Osterman et al., 2015; Rios et al., 2015). In human chondrocytes, PRP induced anti-inflammation by NF- κ B inhibition via HGF (Bendinelli et al., 2010), and via decreased expression of TIMP-1 and ADAMTS-5 in cartilage (Osterman et al., 2015).

It seems that anti-inflammation is very important to regenerate the injured spinal cord. Phil Popovich et al. (1999) injected clodronate liposomes into SCI mice and obtained the following results: a reduction in leukocyte migration to the lesion location and the hollow cavity in the cord tissue, an increase in score according to the BBB scale, a reduction in gray matter degeneration, and an increase in the number of axons and myelinated axons (Popovich et al., 1999). Decrease of leukocyte number could be a reason for the recovery.

Indeed, the secondary loss of neurons, axons and myelin were also reduced after inhibiting monocytes and neutrophils (Bao et al., 2004; Bao et al., 2005; Blight, 1994; Blight et al., 1995; Giulian and Robertson, 1990; Gris et al., 2004). Daniel et al. (2009) suggested that antibody secretion of B cells can also influence the pathology of spinal cord injury. Inflammation can also trigger glial scar formation. In fact, the formation of glial scar is stimulated by astrocytes and has been shown to negatively affect recovery (Block et al., 2007; Silver and Miller, 2004). A recent study inhibited the proliferation of glial cells after injury and showed a significant improvement of recovery in mice- both in movement and sensation (Lin et al., 2014).

In our study, total leukocyte number decreased in the three treatment groups where PRP was given (placebo, allogeneic SVF transplantation, and autologous SVF transplantation). The combination of PRP with SVFs could elicit a reduction in inflammation in mice. Moreover, the combination triggered spinal cord regeneration.

Indeed, histological analysis demonstrated that healing of the injured cord took place in mice treated with PRP. Moreover, de-methylation also decreased in those mice. Although there was no overserved complete recovery, based on assessment of mice motility and sensation, PRP had a significant impact on the total leukocyte count and spinal cord tissue structure. The total leukocyte count significantly decreased compared to non-transplanted (untreated, control) mice. Additionally, the spinal cord in PRP-treated mice showed less damage than in the control mice.

Additionally, mice treated with PRP alone or allogeneic SVF cells have slightly improved mouse body weight (though not statistically significant). These mice do not show recovery with regards to movement, feeling or motor control ability. According to some previous studies, PRP may affect peripheral nerve regeneration. The mechanism of action of PRP could be by stimulating the regeneration of new myelination, leading to the recovered neurotransmitter capability of axons (Cho et al., 2010; Ding et al., 2009; Farrag et al., 2007). However, treatment with a single dose did not yield any clear improvement (Sariguney et al., 2008).

Our results are entirely consistent with those aforementioned and other studies. According to some recent studies (Farrag et al., 2007; Landi et al., 2011; Shen et al., 2009; Yu et al., 2011), PRP is typically used to treat a variety of neurological disorders and diseases. PRP is part of the blood system that eliminate cells and PRP contains platelets. When activated by damage, PRP will release growth factors to stimulate angiogenesis, proliferation and cell differentiation, thus inducing the formation of new areas of tissue.

In our study, compared to the other groups, the autologous SVF transplantation group showed a clear improvement in spinal cord regeneration. Spinal cord tissue in this group showed denser structures, more stroma, less glial scarring, and fewer myelin debris. Initially, we thought that immune modulation was caused by PRP as well as ADSCs inside SVFs. However, the spinal cord was clearly regenerated in this group compared to allogeneic SVF transplantation group. Therefore, the observed difference could be from the role of autologous ADSCs.

The greatest disadvantage of autologous SVF transplantation is the adverse effects of fat collection and the strong decrease of body weight. However, autologous SVF transplantation did yield the highest survival rate in mice with SCI and autologous SVFs could persist for a long time in mice. The grafted cells also produced paracrine factors to heal the injured site, as well as differentiate into neuronal cells. Some recent studies have showed the capability of ADSCs to differentiate into functional nervous cells, e.g. Schwann cells- to myelinate axon, both *in vitro* and *in vivo* (Anghileri et al., 2008; Chi et al., 2010; Jiang et al., 2008; Kingham et al., 2007; Krampera et al., 2007; Safford et al., 2004; Safford et al., 2002; Xu et al., 2008; Zhang et al., 2009).

In the allogeneic SVF transplantation, ADSCs only persisted at the grafted sites for a short time, and their effects on injury healing was not obvious. Evaluation of SRY expression supported this thought. After 5 wks, using sensitive PCR to evaluate SRY of male cells in the female recipients, we could not detect the expression of SRY at the grafted sites.

Our results of autologous SVF transplantation for SCI treatment are similar to the results of Sykova et al. (2006) that used bone marrow derived stem cells (Syková et al., 2006). In another study, Zaminy et al. (2013) showed better results when

using Schwann cells; the average BBB score difference was 4 (Zaminy et al., 2013). However, Zhang et al (2009) and Chi et al. (2010) transplanted functional Schwann cells, differentiated from ADSCs, and showed that improvement was low and that there was no statistically significant difference between treatment and control groups (Chi et al., 2010; Zhang et al., 2009). The main reason was suggested to be due to the huge difference of *in vitro* and *in vivo* differentiation of stem cells to Schwann cells.

Conclusion

SCI is an extreme injury with low regeneration. Almost all patients with SCI develop completely paralysis. In this study, we produced a mouse model of SCI and treated them with non-expanded ADSCs (from SVFs). In combination with PRP, autologous SVF transplantation significantly improved the locomotor score in mice compared to allogeneic SVF transplantation using the same dose of SVFs. Transplantation of autologous SVF mixed with PRP decreased the number of total leukocytes, reduced glial scarring, reduced myelin defragmentation, and significant increased the BBB score. Meanwhile, SCI mice treated with PRP alone or with allogeneic SVFs mixed with PRP showed only a slight improvement of their BBB scores. This preliminary study shows that autologous SVF transplantation can be promising for SCI treatment.

Abbreviations

ADSC: Adipose derived stem cells; HGF: Hepatic growth factor; MSCs: Mesenchymal stem cells ; PRP: Platelet rich plasma; SCI: Spinal cord injury; SVF: Stromal vascular fraction.

Author Contribution

HTML: isolated SVF from adipose tissues, transplanted SVFs to mouse models and monitoring treated mice; MNTT, KAB & TTTL: produced the mouse models, measured some total leukocytes, histological analysis; KHTB, NKP & PVP: performed flow cytometry, revised and corrected the manuscript, suggested the ideas for this study.

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