Human Adipose-Derived Stem Cells Delay Muscular Atrophy After Peripheral Nerve Injury in Rats

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Research Article

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Abstract

Introduction: Muscle is often accompanied by denervation atrophy after peripheral nerve injury. Human adipose-derived stem cells (hADSCs) and platelet-rich plasma injections into a muscle after peripheral nerve injury were examined.

Methods: The rats were randomly divided into 5 groups. First group rats' sciatic nerve was exposed with untreated, others groups rats' sciatic nerve injury model was created first. PBS, hADSCs, PRP, and a mixture of hADSCs and PRP were injected into the gastrocnemius immediately postoperatively. Quantitation of gross musculature and muscle fiber area, SFI were investigated.

Results: In 4 weeks post-surgery, post hoc Bonferroni tests showed significant differences in wet weight ratios when the hADSC group, the PRP group, and the combine group were compared with the PBS group. Areas of muscle fiber were larger in the hADSC group and the combine group compared with the PBS group at 4 weeks post-surgery.

Discussion: hADSCs injection may delay muscular atrophy after sciatic nerve injury in rats, and PRP injection has little effect on delayed muscular atrophy.

Introduction

Muscle is often accompanied by denervation atrophy after peripheral nerve injury, which makes the treatment of peripheral nerve injury more complicated. For example, atrophy of facial muscles occurred after facial nerve injury, and nerve and muscle transplantation is needed to reconstruct facial expression muscle dynamics[1]. Delaying or even recovering the atrophy of the denervation muscle in clinical practice will shorten the time of peripheral nerve therapy and improve the therapeutic effect. At present, some studies have explored muscular atrophy caused by peripheral nerve injury: Isoflavones reduced apoptosis-dependent signals and significantly alleviated muscle atrophy after denervation in mice[2]; Apigenin appears to inhibit denervation-induced muscle atrophy by inhibiting inflammatory processes in muscles[3]; Appropriate physical stimulation such as electric stimulation and electroacupuncture can alleviate the denervation of muscular atrophy[4, 5]. However, the mechanism of denervation muscular atrophy is very complex, and there are various treatment methods. With the exploration of some new fields, it is expected to adopt new treatment and remission methods for denervation of muscular atrophy.

Adipose-derived stem cells (ADSCs) are widely available and easy to obtain, and the donor age and collection site do not affect the therapeutic effect of the obtained stem cells[6]. ADSCs have the ability to differentiate into a variety of cell lineages and have strong anti-inflammatory, anti-fibrosis, anti-apoptosis and pro-angiogenesis effects in vitro or in vivo. Therefore, ADSCs become the first choice for preclinical studies[7], and they have been widely applied in the field of cell therapy and tissue engineering regeneration and repair[8–10]. Previous studies have shown that transplantation of ADSCs can delay muscular atrophy caused by peripheral nerve injury, suggesting that ADSCs may delay muscular atrophy partly by inhibiting the occurrence of inflammatory responses, but the mechanism remains unclear[11].

Based on the low immunogenicity of stem cells in the process of allotransplantation[12], human adipose-derived stem cells (hADMSCs) have been injected into the brain of adult rats in previous studies[13]. The result of this study showed that the survival rate was also high under heterogeneous conditions, meeting the ideal standard of cell therapy. We expected to inject hADSCs into the gastrocnemius muscle of muscular atrophy following peripheral nerve injury in rats. To verify whether allogeneic adipose stem cells can also delay muscular atrophy caused by peripheral nerve injury, provide a theoretical study for allogeneic ADSCs to treat denervation atrophy, and also lay a foundation for the commercial use of human adipose stem cells.

Methods

Culturing and Identifying of Human Adipose-Derived Stem Cells (hADSC).

We harvested the human subcutaneous fat tissue of healthy donors who had liposuction[14]. The use of human adipose tissue was approved by the Ethics Committee of Plastic Surgery Hospital (No. 2150019022). The fresh fat specimen was washed three times with PBS containing 1% penicillin/streptomycin. The fat tissue was digested with 0.25% collagenase type I (Sigma, USA) at 37℃ for 0.5h and centrifuged at 1000 rpm for 5 min. The cell pellet was resuspended and filtered through a 70-μm filter (Corning, USA). After another centrifugation for 5 min, the cell pellet was resuspended in the culture medium consisting of Human Mesenchymal Stem Cell Medium (MSCM, ScienCell, USA) high glucose. The culture medium was replaced 48h after seeding to remove nonadherent cells; thereafter, the medium was replenished every 2–3 days. The ASCs were passaged 3 times for experiments. The hADSCs immunophenotype was analyzed by flow cytometry using the flow cytometer FACSCalibur (BD, New Jersey, USA). hADSCs cultivated to passage 3 were labeled with Human MSC Analysis Kit (BD, New Jersey, USA). Cell samples were labeled with each antibody separately and after processing, concentrated cell populations were gated and then the percentage of cells labeled with the selected antibodies was identified, as proposed by the International Federation for Adipose Therapeutics and Science (IFATS) and International Cell Therapy Society[15]. The data use isotype as a reference and analysis were performed using the FlowJo7.6.1 Software.

Preparation of Platelet-rich Plasma

Rats were placed under general anesthesia by intraperitoneal injection of sodium pentobarbital (50 mg/kg), and whole blood was drawn through cardiac puncture and transferred into a 15 ml centrifuge tube including 1.5ml sodium citrate. After centrifuging at 2500 rpm for 10 min, the whole blood was separated into three layers including plasma (upper layer), buffy coat (intermediate layer), and red blood cell (bottom layer) (Fig. 1A). The upper layer and intermediate thin layer were transferred to an empty sterile tube and again centrifuged at 1000 rpm for 10 min at room temperature (Fig. 1B). The top two-thirds of the supernatant which consisted of platelet poor plasma (PPP) was removed. The remaining

layer (1/3) was considered as PRP[14]. The platelet number of PRP was counted, and it was about three times the concentration of platelets in the whole blood of rats.

Sciatic Nerve Injury Model Construction and Injection treatment.

All animal experiments conducted in this study were reviewed and approved by the Local Animal Ethics Committee (No. 202003003). Surgeries were performed by an experienced surgeon under a neurosurgical microscope (M400-E, Leica, Germany). Rats were placed under general anesthesia by intraperitoneal injection of sodium pentobarbital (50 mg/kg). At about 0.5 cm below the midpoint of the femur in rats, the skin was cut parallel to the femur, the biceps femoris muscle was bluntly separated to expose the sciatic nerve trunk which was cut with straight micro-scissors. It can be seen that the two severed ends retracted and a gap of about 0.5cm appeared (Fig. 2A and B). The surgical wound was closed with 4 − 0 nylon sutures, and rats were returned to their cages. The whole process is carried out under aseptic conditions.

Rats were randomly divided into the following 5 groups (n = 10 each): injury without ischiatic nerve (A: Non-operated group); ischiatic nerve was transected, and gastrocnemius muscle was injected with phosphate buffered saline (PBS)(PBS group)(Fig. 2C); ischiatic nerve was transected, and gastrocnemius muscle was injected with 0.5ml of 10^6/ml hADSCs in PBS (hADSCs group); ischiatic nerve was transected, and gastrocnemius muscle was injected with 0.5ml PRP (PRP group); ischiatic nerve was transected, and gastrocnemius muscle was injected with a mixture of 0.25ml PRP and 0.25ml hADSCs (Combine group). Before experimentation, injections were practiced on rats, where methylene blue was injected in this manner, and complete muscular dissection revealed diffuse injection throughout the gastrocnemius muscle (Fig. 2D). The injection group was injected 3 days after the operation, once a week. All experiments were approved by the Local Animal Ethics Committee.

Walking footprint analysis

Before the ischiatic nerve was transected, 1, 2, 3, and 4 weeks post-operation, walking track assessments of all animals that had not been sacrificed at each time point were performed. Briefly, the walking footprint was analyzed using the Digigait System (Mouse Specifics, Framingham, MA, USA). Rats were trained at a speed of 25 cm/s before recording and then tested at a speed of 25 cm/s. For each test, at least 2 seconds of continuous walking were recorded. Footprints were captured and analyzed using Digigait analysis software (Digigait 12.4). The sciatic functional index (SFI) for each time point was calculated using previous formulas: $SFI = -51.2 \times [(EPL - NPL)/NPL] + 118.9 \times [(ETS - NTS)/NTS] - 7.5$, where EPL is the experimental print length, NPL is the normal print length, ETS is experimental toe spread, and NTS is normal toe spread[16].

Gastrocnemius muscle wet weight and Masson’s trichrome staining

2 and 4 weeks after surgery, rats were randomly selected and killed by cervical dislocation in each group. The double lower limbs were cut open and the intact gastrocnemius was cut off. The bilateral gastrocnemius muscles were blotted with absorbent paper, and evaluated for wet muscle weight, and recorded. The remaining rate of wet muscle weight (WMW) is defined as: WMW in the operation side/WMW in the healthy side × 100%[17].

Next, muscles were fixed in 4% paraformaldehyde, dehydrated with a serial gradient of ethanol, and cleared in xylene. Afterward, specimens were embedded in paraffin and sectioned into 5-μm slices, which were dyed with Masson's trichrome (Solibor, Beijing, China) and observed under a microscope (Leica). Digital images were captured and muscle fiber areas were calculated using Image-pro plus 6.0 software, 50 muscle fibers were counted in each sample, the area was expressed as mean ± SD per group.

Statistical analysis

All data were presented as the means ± standard error of the mean (SEM). GraphPad Prism 6.0 (USA) was used for statistical analysis and draft graphs. The data were analyzed by two-way ANOVA with factors of treatment and time. Intergroup differences were analyzed by performing post hoc Bonferroni tests. P values ≤ 0.05 were considered statistically significant (### or ***, P ≤ 0.001; **, P ≤ 0.01; *, P ≤ 0.05).

Results

Culturing and Identifying of Human Adipose-Derived Stem Cells (hADSC).

hADSCs were successfully isolated from the fat tissue. Cells adhered to the plastic culture flask and were identified as hADSCs at passage 3. In passage 0, hADSCs grew adherent and showed a fibroblast-like cell morphology, while the rest of the circular suspension cells were other cells or inactivated adipose stem cells. During sub-culturing up to passage 3, no morphologic or growth pattern changes were observed (Fig. 3A). The stem cell markers CD73, CD90, and CD105 were highly expressed (> 90%) in cells, and the cells had negative expression (≤ 4%) of the negative cocktail markers (CD11b, CD19, CD34, CD45, HLA-DR) (Fig. 3B). The result of flow cytometry showed cells were stem cells[15]. In conclusion, the cells we harvested were hADSCs.

Walking footprint analysis

To compare the ischiatic nerve functional recoveries, we performed a walking track test. The SFI was detected through Digigait every week. Regarding the different treatments of the gastrocnemius after ischiatic nerve injury, two-way ANOVA showed no significant interactions between different treatments and times for SFI ($F(12, 32) = 0.2193$; $p > 0.05$). Post hoc Bonferroni tests indicated significant differences when compared with the non-operated group for SFI ($p < 0.0001$). There were no significant differences in SFI between the different treatments groups ($p > 0.05$) (Fig. 4).

Gastrocnemius muscle wet weight and Masson’s trichrome staining

Recovery of the gastrocnemius muscle from atrophy was assessed by the wet weight of gastrocnemius muscles ratio. Recovery was also evaluated by calculating the muscle fiber area of sections with Masson’s trichrome staining. According to the macroscopic appearance of gastrocnemius muscles (Fig. 5A) and Masson’s trichrome-stained sections (Fig. 6A) of 2 and 4 weeks post-surgery, different extents of atrophy existed in the four treatments groups. 2 weeks post-surgery, gastrocnemius muscle wet weight ratios were notably declined in four nerve injury treatments groups ($P < 0.0001$). However, there were no significant differences between the four nerve injury treatments groups. 4 weeks post-surgery, post hoc Bonferroni tests showed significant differences in wet weight ratios when the hADSC group (37.01 ± 1.88, $p < 0.0001$), the PRP group (33.45 ± 1.61, $p < 0.01$), and the combine group (38.590 ± 0.61, $p < 0.0001$) were compared with the PBS group (25.20 ± 0.36) (Fig. 5B). Sections with Masson’s trichrome staining showed that gastrocnemius muscle fiber area reduction of the four nerve injury treatments groups ($p < 0.0001$). Areas of muscle fiber were larger in the hADSC group ($936.83 \pm 56.74 \mu m^2$, $p < 0.05$) and the combine group ($912.01 \pm 45.07 \mu m^2$, $p < 0.05$) compared with the PBS group ($642.26 \pm 4.31 \mu m^2$) at 4 weeks post-surgery (Fig. 6B).

Discussion

Denervation of muscle results in a rapid and programmed loss of muscle size and function known as muscular atrophy. It is generally considered that muscle function is irretrievable after prolonged denervation (6 to 12 months) despite re-acquisition of innervation. This prolonged denervation results in a decrease in the number of muscle stem cells, which is detrimental to restoring muscle regeneration after innervation. Previous studies suggested that muscular atrophy is the result of protein homeostasis deficiency, and some studies have suggested that this process is related to the apoptosis of muscle cells. The mechanism of apoptosis is still unclear, and the molecular mechanism controlling the imbalance of protein synthesis and degradation pathways in denervation muscle atrophy remains to be explored. Many studies have explored the inhibition of muscle atrophy: resveratrol has anti-aging effects and can relieve metabolic disorders, and it can significantly prevent muscle atrophy after denervation in mice[18]; NF-κB targeted drugs have been used in the treatment of denervation muscular atrophy to delay muscle atrophy, inhibiting apoptosis by inhibiting the classical NF-κB signaling pathway[19]; In the study of Schilling et al., denervated muscular atrophy treated by injection of the allogenic ADSC, showed a decrease in inflammatory factors and achieve delay muscular atrophy[11]. The treatment we have chosen is also intended to delay muscle atrophy by promoting the regeneration of muscle stem cells and acting anti-inflammation.

Human adipose stem cells have low immunoantigenicity, due to their great commercial value, hADSC has been applied in animal experiments to expand their clinical adaptability[20, 21]. We selected hADSC for injection and further explored it based on Schilling's study[11]. After analyzed by two-way ANOVA, it was

found that the hADSC group delayed muscle atrophy was manifested in muscle wet weight and muscle fiber area in 4 weeks after surgery. This may suggest that hADSC needs a certain period time to play a role in the muscle, which is consistent with Schilling's view that ADSC needs to overcome the trauma of injection and take a period of time to show its regenerative effect[11]. The results of our study also showed that the effect of the hADSC group and the combined group was similar, which also suggested that hADSC might be the key factor in the treatment, while PRP injection had little effect on the results.

Platelet-rich plasma (PRP) is a useful treatment method used in orthopedics, oral surgery, plastic surgery, dermatology and other medical fields[22–25]. PRP is an autoserum containing a high concentration of platelets and growth factors[25]. α particles in platelets are responsible for promoting stem cell regeneration and soft tissue remodeling. PRP particles contain many basic growth factors, such as platelet-derived growth factor, vascular endothelial growth factor, epithelial growth factor, transforming growth factor, insulin-like growth factor, etc.[26, 27]. These growth factors are also thought to induce cell proliferation, angiogenesis, and chemotaxis, and also contain serotonin, dopamine, histamine, adenosine, and calcium, all of which increase cell membrane permeability[28]. Studies have shown that platelet-rich plasma can promote the recovery of arthritis models induced by pro-inflammatory cytokines with the assistance of collagen protein, showing certain anti-inflammatory ability[29]. A study by Bendinelli et al. showed that platelet-rich plasma may exert an anti-inflammatory effect on human chondrocytes through the mechanism of NF-κB inhibition by HGF[29]. Previous studies have found that ADSCs can inhibit denervation muscular atrophy by inhibiting inflammatory response[11]. Therefore, we wanted to inhibit denervation atrophy by injecting PRP, and play the role of PRP in inhibiting inflammation and promoting cell regeneration. Meanwhile, PRP has a variety of growth factors, which can induce the proliferation of stem cells. We also hope that PRP can delay muscle atrophy by inducing the proliferation of muscle stem cells. In our results, the walking analysis showed that sciatic nerve function had not been restored, eliminating the impact of nerve regeneration on muscle. The wet weight of the gastrocnemius muscle in the PRP injection group increased 4 weeks after operation compared with that in the PBS group and the difference was statistically significant. However, there was no increase in the area of single muscle fiber, which may indicate that PRP injection may not delay the atrophy of muscle fiber to achieve weight increase, but may increase the weight of other tissues, or some substances may not be fully metabolized after PRP injection. But the specific reasons may need further research.

Our study has several limitations. First, we just observed four weeks, the long-term prognosis is unclear. If we want to understand the long-term outcome, the trial period can be extended. Finally, we only carried out animal experiments and did not explore the mechanism further. Future studies are needed to unravel these limitations.

In conclusion, hADSCs injection may delay muscular atrophy after sciatic nerve injury in rats, and PRP injection has little effect on delaying muscular atrophy after sciatic nerve injury in rats.

Declarations

Funding and Acknowledgements:

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Conflict of interest:

The authors declare no conflict of interest.

Ethics approval and consent to participate

All experiments conducted in this study were reviewed and approved by the Local Animal Ethics Committee (No. 202003003). The use of human adipose tissue was approved by the Ethics Committee of Plastic Surgery Hospital (No. 2150019022). All methods in this study were carried out in accordance with relevant guidelines and regulations. All methods are reported in this study in accordance with ARRIVE guidelines for the reporting of animal experiments.

Availability of data and materials:

All data generated or analysed during this study are included in this published article and its supplementary information files.

Author's Contributions:

QSW performed the experiments, analyzed the data, and wrote the manuscript. MN, WWx, Cs, WQ, LYq designed the research, analyzed the data, and contributed to the writing of the manuscript. YZ supervised the study.

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Figures

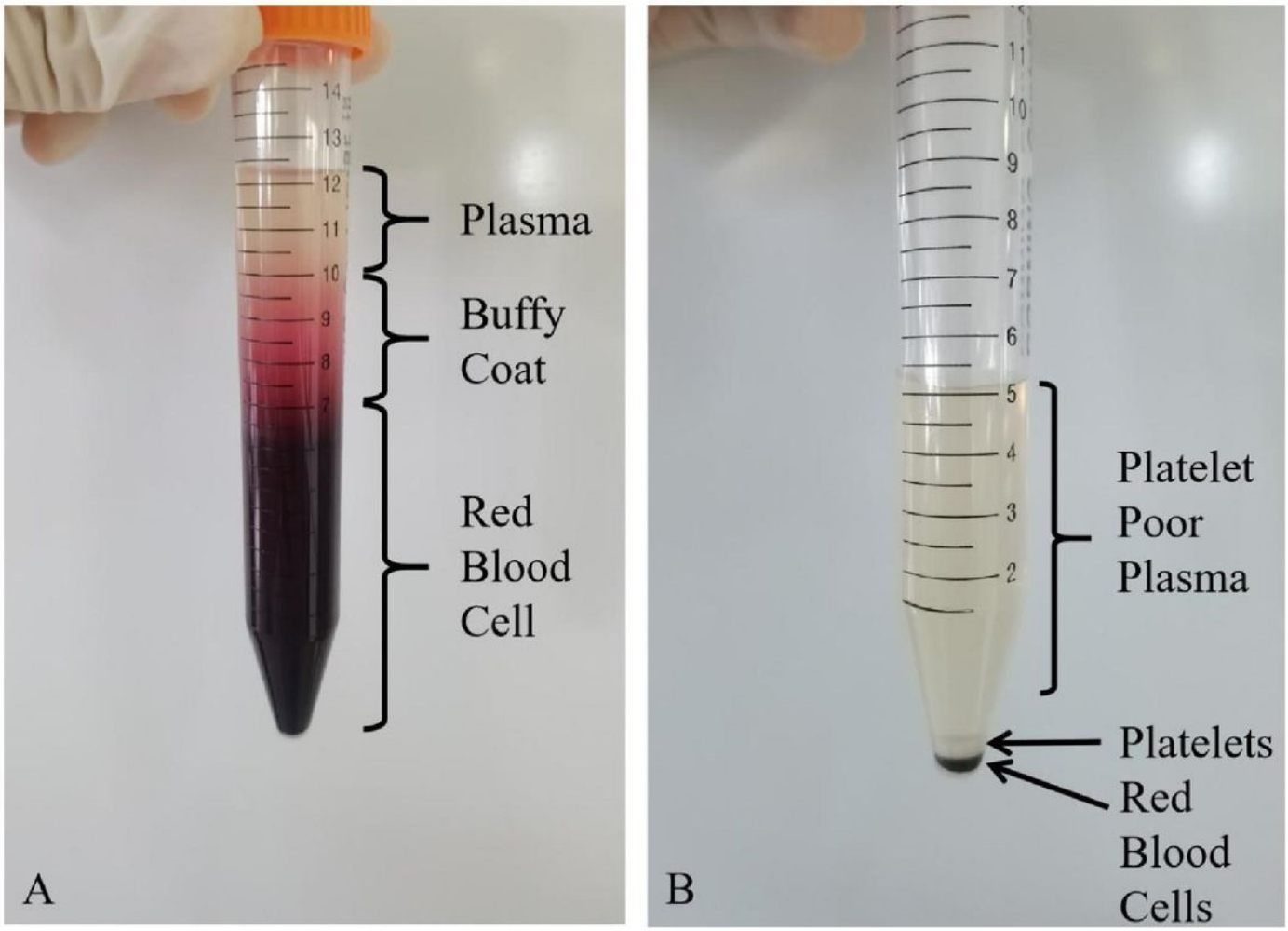

Figure 1

(A) After the first centrifugation, the blood sample was divided into 3 layers. The upper layer was plasma, the middle was the white blood cell layer, and the lower layer was red blood cell. (B) After the second centrifugation, the bottom layer was the red blood cells, the white layer was accumulated platelets, and the supernatant was platelet-poor plasma. The top two-thirds of the supernatant which consisted of platelet poor plasma (PPP) was removed. The remaining layer (1/3) was considered as PRP.

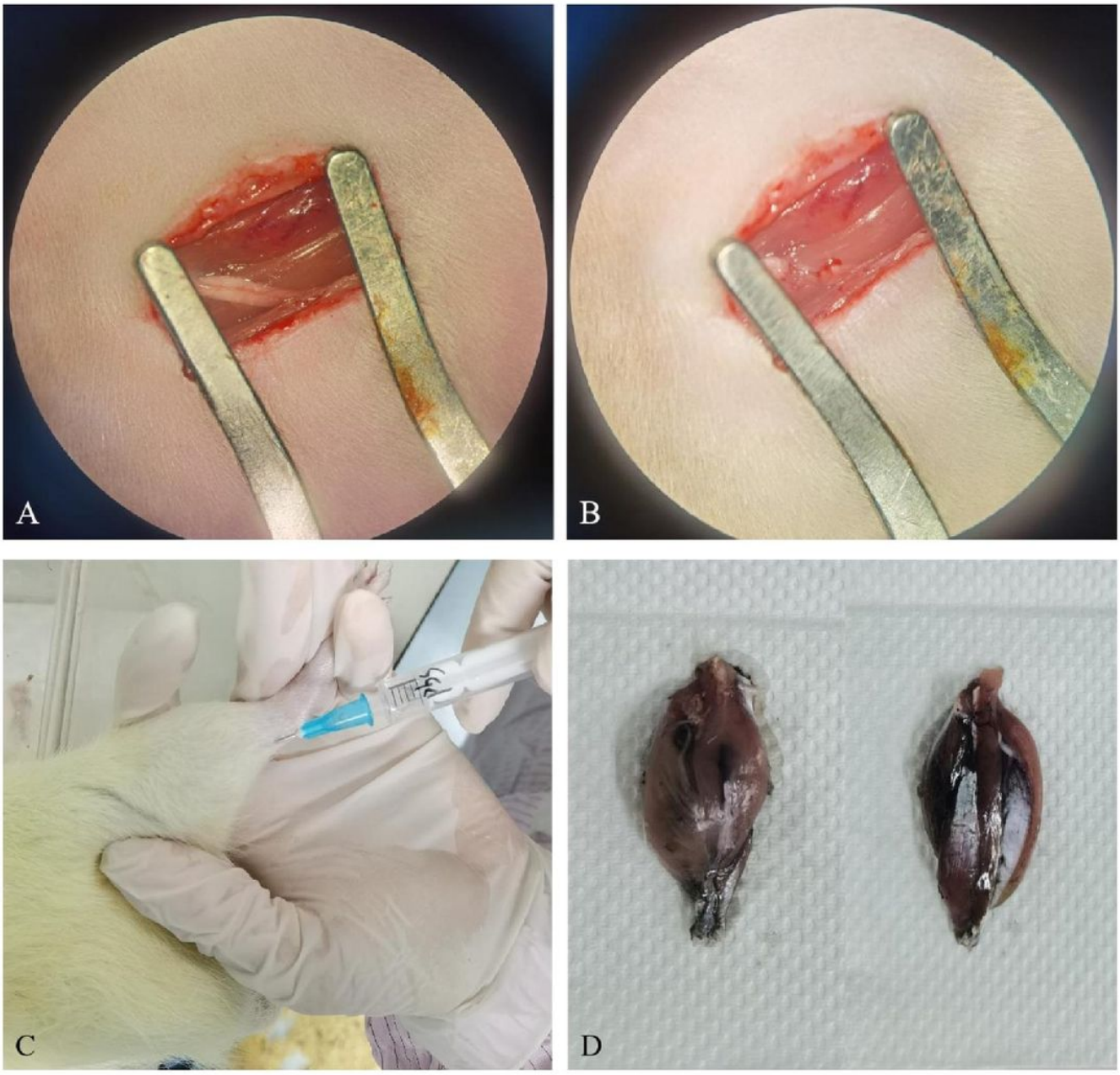

Figure 2

(A) The sciatic nerve trunk was exposed. (B) Two severed ends of the sciatic nerve retracted and a gap of about 0.5cm appeared. (C) The gastrocnemius muscle was injected with phosphate buffered saline. (D) The pros and cons of complete muscular dissection revealed diffuse injection throughout the gastrocnemius muscle.

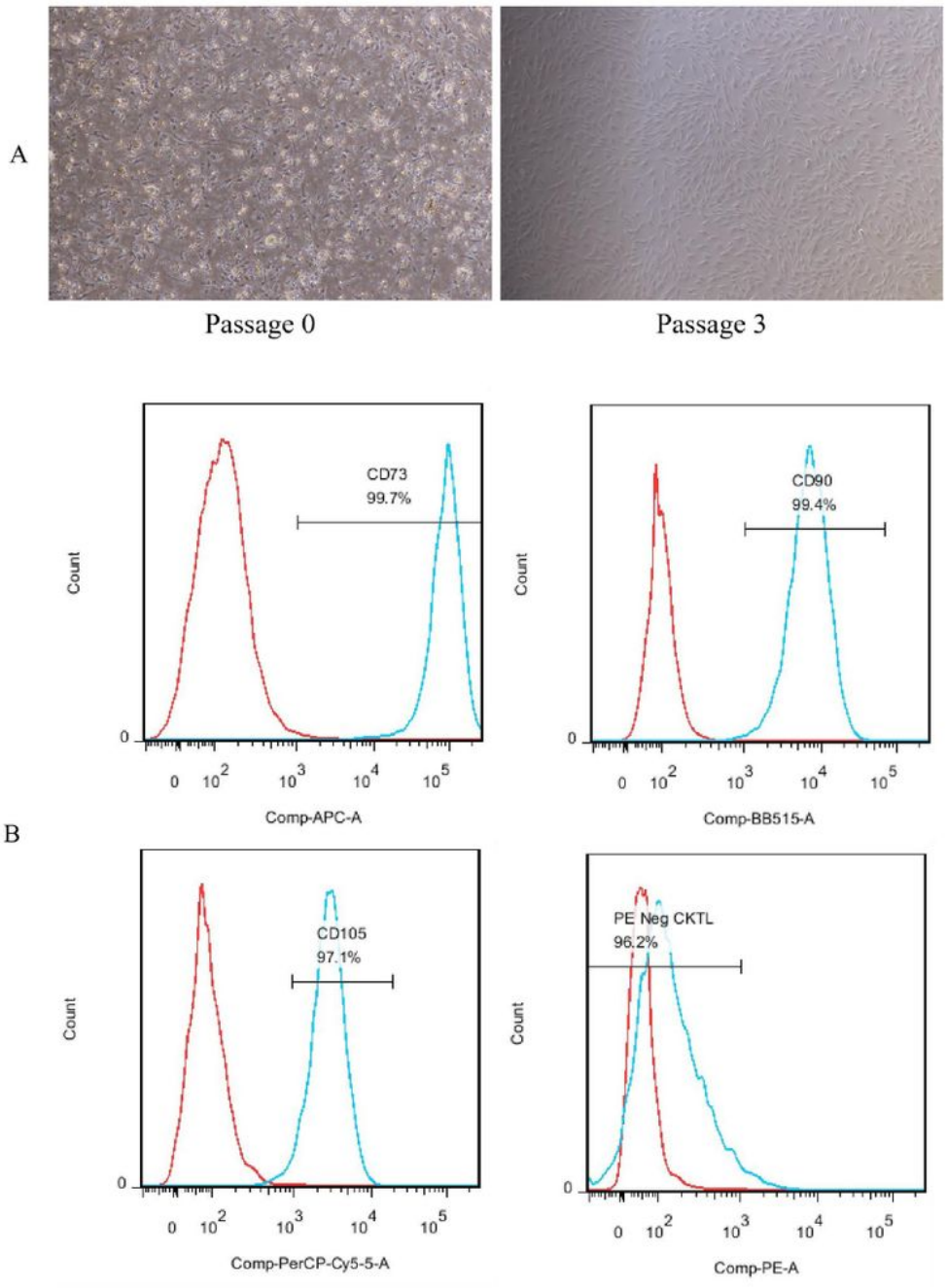

Figure 3

Morphology and immunophenotype characteristics of hADSCs. (A) Representative microscopic illustrations of hADSCs. Scale bar 250 μm. (B) Representative histograms from the flow cytometry analysis showing surface marker expression on hADSCs, monoclonal antibody control (red) and the stained cells (blue).

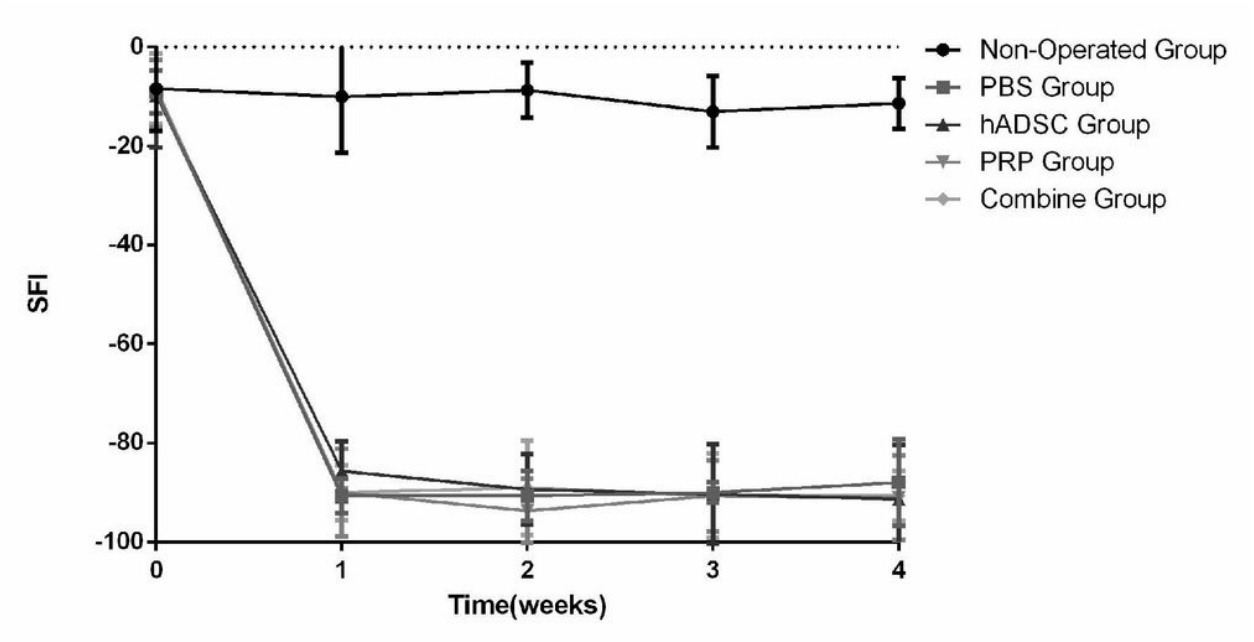

Figure 4

Analysis of the recovery of motor function by the sciatic nerve functional index curve.

Data are expressed as the mean ± SD (number of footprints from each group = 5; two-way ANOVA analysis followed post hoc multiple comparisons with a Bonferroni correction). SFI: sciatic function index.

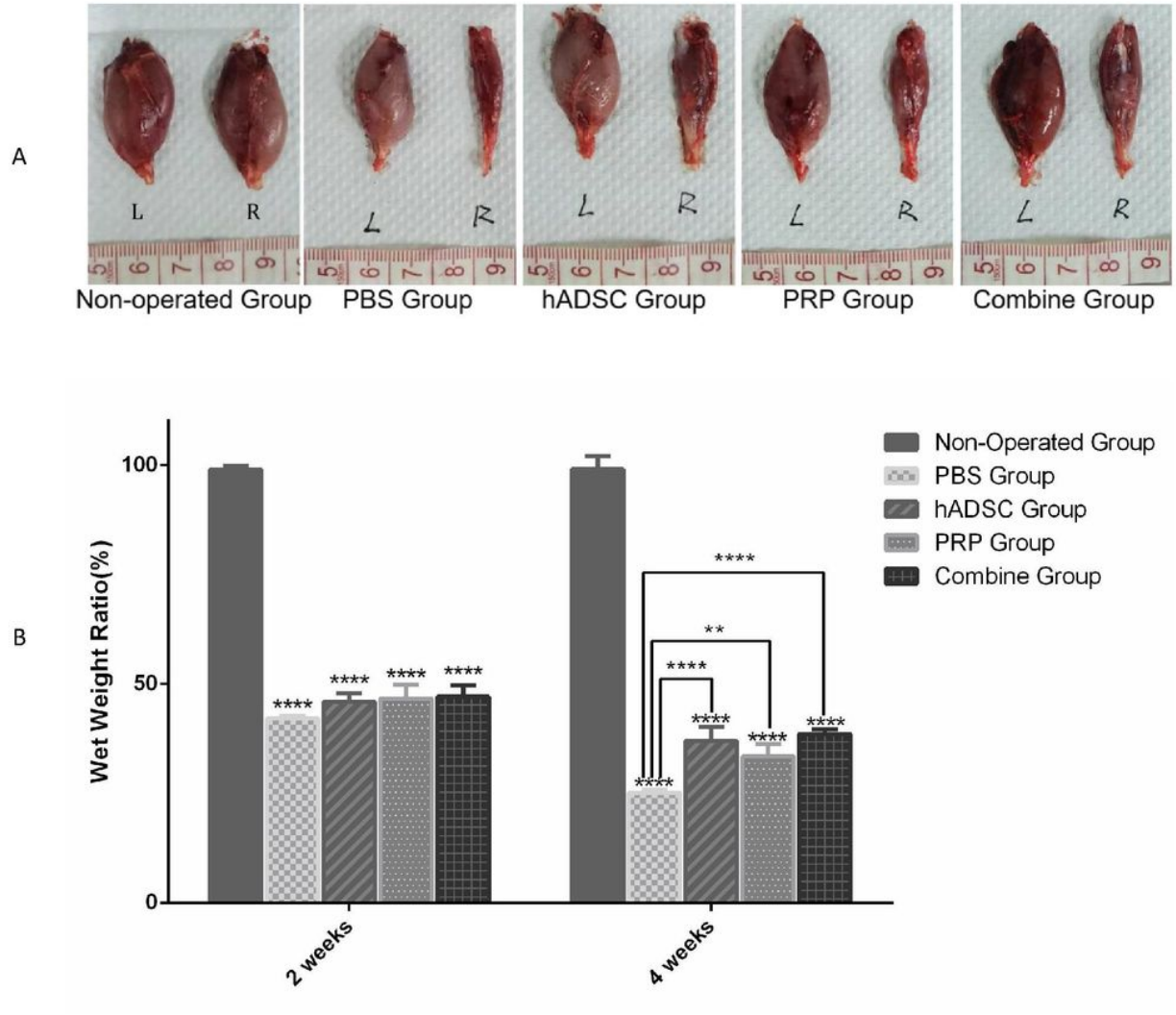

Figure 5

General observation of gastrocnemius muscle at 4 weeks post-surgery, and assessment of the wet weight ratio of gastrocnemius muscle at 2 and 4 weeks post-surgery.

(A) General observation at 4 weeks after surgery. (B) Wet weight ratio were mearsured to evaluate atrophy of the gastrocnemius muscle. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, vs. Non-operated group. Data are expressed as the mean ± SD (n = 3; two-way ANOVA analysis followed post hoc multiple comparisons with a Bonferroni correction).

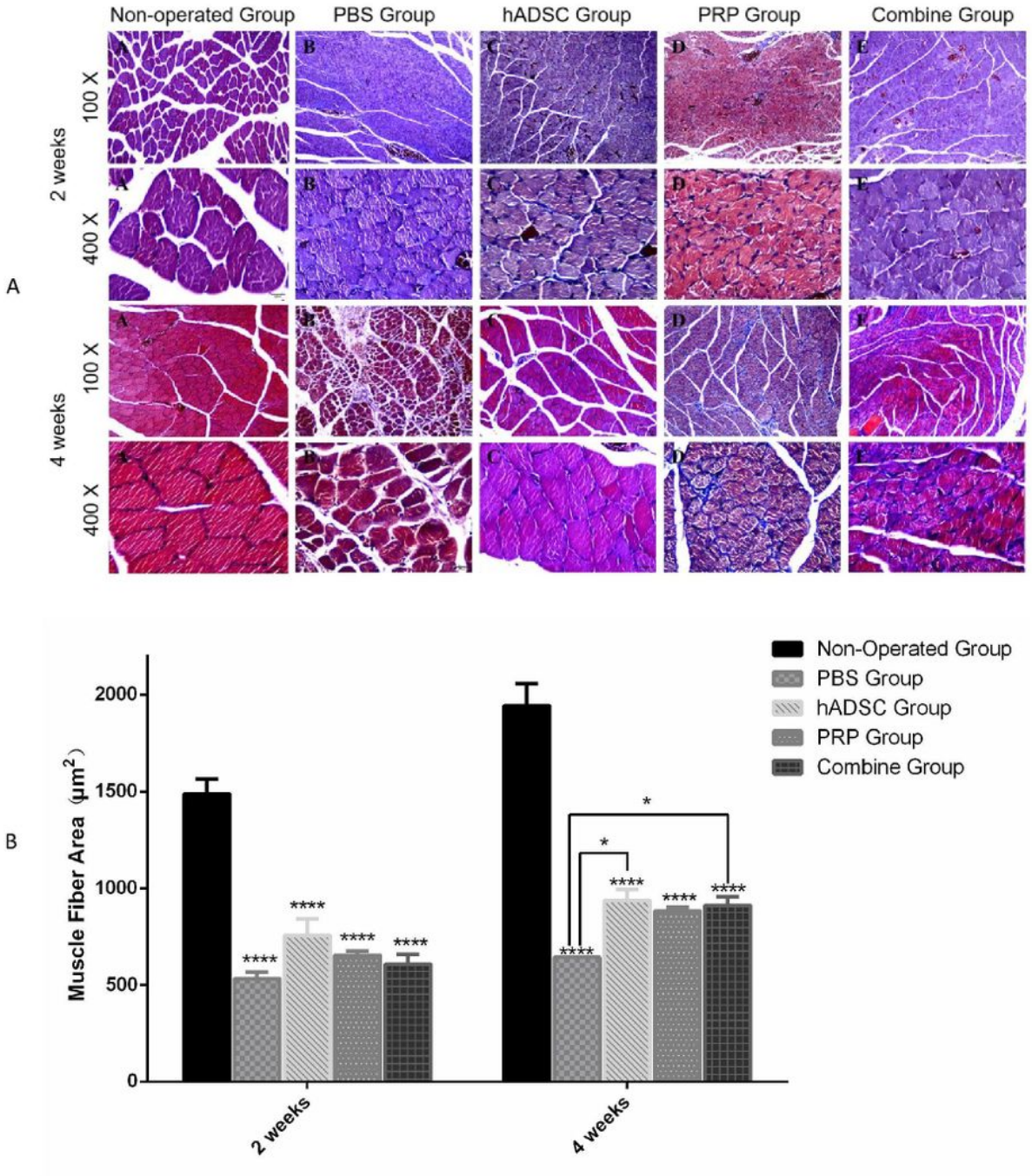

Figure 6

Masson's trichrome staining of gastrocnemius muscle from the experimental side at 2 and 4 weeks post-surgery, and assessment of the muscle fiber area at 2 and 4 weeks post-surgery.

(A) Cross-sections with Masson's trichrome staining at 2 and 4 weeks after surgery. 100X, Scale bar: 100 μm. 400X, Scale bar: 25 μm. (B) Gastrocnemius muscle fiber areas were measured to evaluate atrophy of

the gastrocnemius muscle. *P < 0.05, ****P < 0.0001, vs. Non-operated group.

Data are expressed as the mean ± SD (n = 3; two-way ANOVA analysis followed post hoc multiple comparisons with a Bonferroni correction).