Stem Cells International

Stem Cells in Musculoskeletal Regeneration: From Benchtop to Bedside

Guest Editors: Jiabing Fan, Dong-An Wang, Haifeng Liu, Hongbin Fan, and Fang Yang

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Contents

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Editorial

Stem Cells in Musculoskeletal Regeneration: From Benchtop to Bedside

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The continuing existent challenges of conventional surgical approaches for musculoskeletal reconstruction have led to the favorite of stem cell-mediated treatments over the last decade [1]. However, the in-depth understanding of underlying therapeutic mechanisms, regulatory mechanisms of stem cell differentiation, interaction of stem cells and inductive factors/delivery vehicles, and evaluation criteria *in vitro* and *in vivo* are indispensable to the wide practice of stem cells-based therapeutics for functionally restoring the musculoskeletal tissues from bench to bedside [2]. We are pleased to present a special issue encompassing both basic and translational aspects of stem cells-related research and review work in musculoskeletal regeneration including cartilage, bone, ligament, spinal cord, and meniscus, aiming to blossom in the effective use of stem cells for clinical application.

The proper cell source is one of the determinant elements to the success of stem cells-based tissue regeneration [3]. Mesenchymal stem cells (MSCs) hold the high accessibility as well as capacity of self-renewal and multidifferentiation, representing an attractive cell source for musculoskeletal tissue engineering [3]. In this special issue, S. Morikawa et al. presented a comprehensive review work recapitulating fundamental biology of MSCs and highlighting that neural crest may be a new cell pool attributable to the harvest of MSCs in favor of craniofacial bone repair. And also S. Liu et al. reviewed a large number of literatures to discuss recent advances in meniscus tissue engineering using cell-based strategy, suggesting that scaffold-free cell self-assembly method is a potential approach to manufacture a functional meniscus graft with robust mechanical properties. Another review work conducted by Z. Deng et al. concluded that currently matrix-assisted autologous chondrocyte transplantation is an optimal approach to the repair of cartilage defects after comparing the efficacy and safety of various tissue engineering approaches through a systemic review and meta-analyses. This review also indicated that there are no enough studies collected to verify the efficacy of MSC-based treatment in cartilage repair.

Natural, synthetic, or nanoscale scaffolds, as delivery vehicles, have been widely employed to carrier stem cells or inductive factors (gene, protein, and DNA) for tissue repair [4]. However, it necessitates the further enhancement of scaffold in both conductivity and inductivity [4]. Q. Li et al. conducted a comparative assessment of how two calcium phosphate/collagen composite materials affect osteogenic differentiation of adipose-derived stem cells (ASCs) which are thought to be a promising cell source in bone tissue engineering, demonstrating the hydroxyapatite/β-tricalcium phosphate composite scaffold is a better stimulator for ASCs proliferation and osteogenesis. To overcome the worrisome complications likely caused by exogenous FDA-approved

bone morphogenetic protein 2 (BMP-2) in bone repair, Q. Xie et al. developed a core-shell PEI (polyethylenimine)/pBMP2-(plasmid BMP2-) PLGA (poly(lactic-co-glycolic acid)) electrospun scaffold using both gene modification and coaxial electrospinning techniques, which was revealed to be capable of sustaining expression of BMP-2 as well as enhancing the osteogenic differentiation of periodontal ligament stem cells.

The ultimate goal of fundamental studies involving stem cells and musculoskeletal regeneration is to translate stem cells to clinical application [5]. In a research article, K. Yaghoobi et al. discovered that herbal drug, *Lavandula angustifolia*, significantly promoted human umbilical mesenchymal Wharton's jelly stem cells in the treatment of spinal cord injury created in Wistar rats. Additionally, Z. Kakabadze et al. who are working in several well-known research institutes around world carried out a pioneering phase 1 clinical trial using autologous human bone marrow stem cell transplantation to treat patients with spinal cord injury. Through evaluation of 18 patients after treatment, the therapeutic effects are encouraging in the recovery of spinal cord injury, but further improvements such as establishment of a standard of *in vivo* evaluation are still needed in future investigation. Together, these findings may provide an alternative approach to the use of stem cell-mediated therapy for the restoration of spinal cord injury that is still a challenge in the current clinical treatment.

Furthermore, L. Sun et al. performed a profound work in exploring the effect of mechanical stretch on proliferation and matrix formation of BMSCs and anterior cruciate ligament fibroblasts, two types of cell identified as major seed cells serving ligament reconstruction. The outcomes of this study are also reminiscent of mechanical property that may be emphasized in the following study of stem cells-mediated tissue engineering. Lastly, R. J. F. C. Amaral et al. reported that human blood collected with an anticoagulant of sodium citrate may yield higher amount of human platelet-rich plasma (PRP) and exert higher proliferation of MSCs. The interesting findings suggest PRP as a potential supplement to promote MSC proliferation and differentiation.

In summary, the cutting-edge review and research articles presented by experts in the field of orthopedic surgery, stem cells, and tissue engineering were collected to be published in this special issue, prospectively being a cornerstone to spur stem cell therapy to be applied to musculoskeletal regeneration in clinic.

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Fang Yang

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Research Article

Effects of Mechanical Stretch on Cell Proliferation and Matrix Formation of Mesenchymal Stem Cell and Anterior Cruciate Ligament Fibroblast

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Mesenchymal stem cells (MSCs) and fibroblasts are two major seed cells for ligament tissue engineering. To understand the effects of mechanical stimulation on these cells and to develop effective approaches for cell therapy, it is necessary to investigate the biological effects of various mechanical loading conditions on cells. In this study, fibroblasts and MSCs were tested and compared under a novel Uniflex/Bioflex culture system that might mimic mechanical strain in ligament tissue. The cells were uniaxially or radially stretched with different strains (5%, 10%, and 15%) at 0.1, 0.5, and 1.0 Hz. The cell proliferation and collagen production were compared to find the optimal parameters. The results indicated that uniaxial stretch (15% at 0.5 Hz; 10% at 1.0 Hz) showed positive effects on fibroblast. The uniaxial strains (5%, 10%, and 15%) at 0.5 Hz and 10% strain at 1.0 Hz were favorable for MSCs. Radial strain did not have significant effect on fibroblast. On the contrary, the radial strains (5%, 10%, and 15%) at 0.1 Hz had positive effects on MSCs. This study suggested that fibroblasts and MSCs had their own appropriate mechanical stimulatory parameters. These specific parameters potentially provide fundamental knowledge for future cell-based ligament regeneration.

1. Introduction

Anterior cruciate ligament (ACL) is an important intra-articular structure to maintain the stability of knee joint. However, it cannot heal spontaneously after severe injury due to poor vascularization [1–3]. Allografts or autografts (hamstring or patella tendon) are now frequently used to reconstruct ACL because of the poor results of synthetic grafts. Although the promising results such as subjective satisfaction and partial stability restoration are acquired by allo/auto graft transplantation, no reliable and functional tissue repair is achieved in long-term follow-up studies. The increased concerns including ligament laxity, donor site morbidity, and pathogen transfer are observed in clinical treatments [4–6]. Recently tissue-engineered ligament provides a new approach to the solution of aforementioned problems.

Tissue-engineered ligament has the potential to provide an alternative graft that could be readily available. However, construction of a viable and biomechanically equivalent ligament requires a fundamental understanding of ACL biology including fibroblast matrix synthesis and remodeling in response to the local mechanical environment [7]. The properties of ligament including structure, function, heal capability, and development are significantly affected by mechanical stimulus. With daily activities, the ACL is subjected to varying amounts of tensile strain, which is crucial for ligament homeostasis. Mechanical loads induce changes in the structure, composition, and function of living tissues. It is now well recognized that mechanical forces play

a fundamental role in the regulation of cell functions, including gene induction, protein synthesis, cell growth, death, and differentiation, which are essential to maintain tissue homeostasis [8]. Another study also showed that mechanical loads affect cellular functions such as cell proliferation and collagen synthesis [9].

To reconstruct a functional tissue-engineered ligament, selection of cell source is of great importance. Due to differences in phenotype and function, different seed cell will greatly influence the properties of tissue-engineered ligament. ACL fibroblasts are load-sensitive cells and their complex structure changes in response to mechanical forces. Furthermore, the collagen produced by fibroblasts is the main component of ligament and has great tensile strength [10]. Theoretically, ACL fibroblast should be the primary choice for potential ligament tissue engineering, because especially they could be easily harvested in diagnostic arthroscopy procedure. In addition to ACL fibroblasts, mesenchymal stem cell (MSC) isolated from bone marrow is another potential cell source for ligament repair due to their multipotent and proliferate capabilities. The scaffold fabricated from woven silk fibers has mechanical properties similar to the native ACL, showing the abilities to enhance MSCs attachment, proliferation, and differentiation [11]. To potentially improve the functionality and structure of tissue-engineered ligament, fibroblasts forming ACL and medial collateral ligament (MCL) tissues were compared with MSCs in previous studies. The proliferation rate and collagen excretion of MSCs were further shown to be higher than ACL and MCL fibroblasts [12]. Although many studies investigated the influence of cyclic mechanical stimulation on graft incorporation, cell morphology, collagen production, and cellular differentiation, few literatures have characterized the optimal parameter of mechanical stimulation [13–15].

In an effort to better understand the effects of mechanical stimulation on different cells and to develop effective approaches for cell therapy, it is necessary to study the biological effects of various mechanical loading conditions on cells. In this study, fibroblasts and MSCs were tested and compared under a novel Uniflex/Bioflex culture system that may mimic mechanical strain in ligament tissue. The objective is to find the optimal parameters (magnitude, frequency, and duration of strain) required for cell proliferation and collagen production, which potentially provides fundamental knowledge for future cell-based ligament regeneration.

2. Materials and Methods

2.1. Isolation and Expansion of MSC and Fibroblast. MSCs and fibroblasts were, respectively, isolated from bone marrow aspirates and ligament tissues of New Zealand White rabbits (12 weeks old, 2.5–3.0 kg) following the methods previously reported [16]. In general, mononuclear cells from bone marrow were separated by centrifugation in a Ficoll-Hypaque gradient (Sigma Co., St. Louis) and suspended in 20 mL of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (HyClone Logan, Utah), l-glutamine (580 mg/L), and penicillin-streptomycin (100 U/mL). Cultures were incubated at 37°C and 5% CO_2. After 72 h, nonadherent cells were removed by changing medium. When reaching 70–80% confluence, adherent cells were freed from the flask with 0.05% trypsin and subcultured. A homogenous MSCs population was obtained after 2 weeks of culture and MSCs (passage 3) were harvested for further use.

For fibroblasts isolation, the collected rabbit ACL was excised under sterile condition. The ligament tissue was minced and washed twice in 1% antibiotic medium for 10 min. The minced ligament tissue was then placed in a solution of 0.25% collagenase at 37°C and agitated overnight for 12–18 h. Fibroblasts were isolated by straining the digest through a 100 μm filter. The cell-containing solution was centrifuged at 300 g for 5 min, the supernatant removed, and the pellet resuspended in 1% antibiotic medium and recentrifuged. The supernatant was removed and the cells suspended in culture medium with 1% antibiotic, 1% glutamine, and 10% fetal bovine serum (FBS) and cultured in T-75 flasks at 37°C, 100% humidity, and 5% CO_2. Confluence was achieved in 2 weeks and subculture was performed. The fibroblasts (passage 3) were collected for further evaluation.

2.2. Cell Culture in Uniflex/Bioflex Plate. Cells were trypsinized by adding 1 mL of 0.25% trypsin solution to a T75 flask with confluent cells followed by 3 min incubation at 37°C with regular gentle shaking. The trypsin reaction was stopped by adding 10 mL of culture medium containing 10% FBS. The cell suspension was then centrifuged at 300 g for 10 min at 20°C. The cell pellet was resuspended in 2 mL of medium (1% antibiotic, 1% glutamine, and 10% FBS) and thoroughly mixed by repeated pipetting. 1×10^6 cells were seeded in each well of the Uniflex/Bioflex culture plates and incubated at 37°C, 100% humidity, and 5% CO_2.

2.3. Mechanical Loading

2.3.1. Uniaxial Strain. The fibroblasts and MSCs were, respectively, loaded in each well of Uniflex culture plates at 37°C, 100% humidity, and 5% CO_2 until it reached confluence. A 0.5 cm gap was made on each side of the cell seeding area for allowing cell migration and proliferation (Figure 1(a)). The cells were uniaxially loaded by placing loading rectangle posts (Flexcell International) beneath each well of the Uniflex culture plates in a gasketed baseplate and applying vacuum to deform the flexible membranes downward. The flexible membrane deformed downward along the long sides of the loading posts thus applying uniaxial strain to loaded cells (Figure 1(b)). The loading regimen was for 5 days, 8 h/day (with 15 min rest every 2 h) at 5, 10, and 15% strain and 0.1, 0.5, and 1 Hz, using a Flexcell Strain Unit (Flexcell International).

2.3.2. Radial Strain. The fibroblasts and MSCs from T75 flask were trypsinized and cultured in medium (1% antibiotic, 1% glutamine, and 10% FBS) in each well of Bioflex culture plates at 37°C, 100% humidity, and 5% CO_2 until it reached confluence. A 0.5 cm gap was made around the cell seeding area allowing space for cell migration and proliferation (Figure 2(a)). The cells were radially loaded by placing cylindrical loading posts (Flexcell International) beneath each well of the

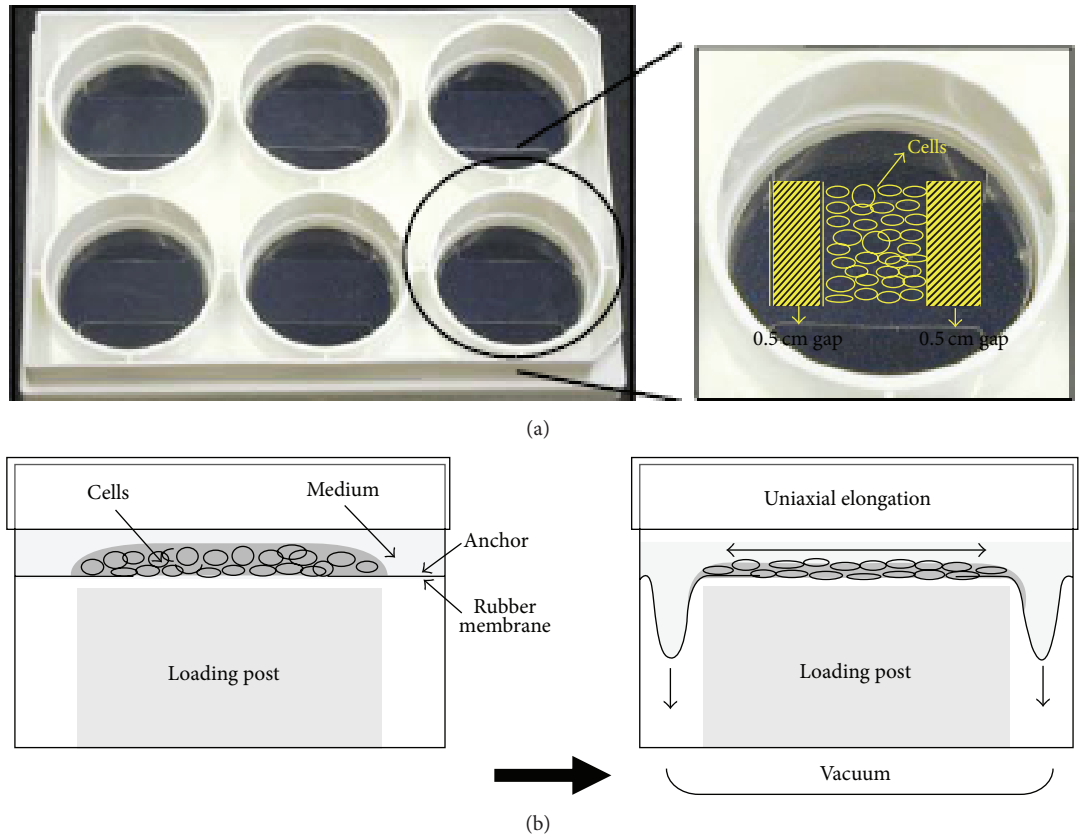

FIGURE 1: (a) Formation of cell sheet construct on Uniflex culture plate; (b) diagram of side view of uniaxial stretch system.

Bioflex culture plates in a gasketed baseplate and applying vacuum to deform the flexible membranes downward. The flexible membrane deformed downward along the circumference of the cylindrical loading posts thus applying radial strain to ACL fibroblast (Figure 2(b)). The loading regimen was for 5 days, 8 h/day (with 15 min rest every 2 h) at 5, 10, or 15% strain and 0.1, 0.5, or 1 Hz, using a Flexcell Strain Unit (Flexcell International).

2.4. Cell Viability/Proliferation. Alamar Blue (AB, Sacramento. CA) was added into the culture media in the 6-well plate at a final concentration of 10% and was incubated for 2 h at 37°C (AB mixture should turn to a purplish/reddish shade). After incubation for 2 h, triplicates of 100 μL AB mixture from each well were transferred and placed in a 96-well plate. Optical density of the AB mixture was measured at 570 and 600 nm with a standard spectrophotometer.

The oxidized form of AB is nonfluorescent and blue (λ_{max} = 600 nm), whereas the reduced form is fluorescent and red (λ_{max} = 570 nm). The proposed mechanism by which the dye detects living cells involves metabolic-based reduction via reactions of the respirator chain. The number of viable cells correlates with the magnitude of dye reduction and is expressed as percentage of AB reduction [17]. The percentage of AB reduction (% AB reduction) was calculated according to the manufacturer's protocol. It was corrected for background values of negative controls containing medium without cells.

2.5. Collagen Production Assay. The culture medium was completely removed from the 6-well plates. The seeded cells were washed twice with PBS solution. The pepsin (0.025%) was then added to the wells and incubated with cells for 2 h to digest all synthesized collagen. The solubilized collagen was neutralized with 1 M NaOH and aliquot to microcentrifuge tubes. 300 μL of Sircol Dye reagent was added to 100 μL of solubilized collagen and was shaken for 30 min. During this period the Sircol Dye will bind to soluble collagen. The dye reagent is designed so that the collagen-dye complex will precipitate out of solution. The microcentrifuge tubes were spun at 10,000 ×g for a 10 min. It is important to firmly pack the insoluble pellet of the collagen-dye complex at the bottom of the tubes, so as to avoid any loss during draining. The unbound dye solution is removed by carefully inverting and draining the tubes. Alkali Reagent (500 μL) was added to each tube and vortexed to release the bound dye into solution. 150 μL aliquots of the released bound dye were transferred into a microtitter plate. The absorbance was read at 540 nm and reference wavelength at 600 nm.

2.6. Statistical Analysis. Unpaired t-test was used for statistical data analysis of the stretching effects on cells at a significance level of 0.05 and sample size of 6.

3. Results

3.1. Uniaxial Stretch for Fibroblasts. After 5%, 10%, and 15% stretching at 0.1 Hz, 8 hrs/day for 5 days, the ACL fibroblast

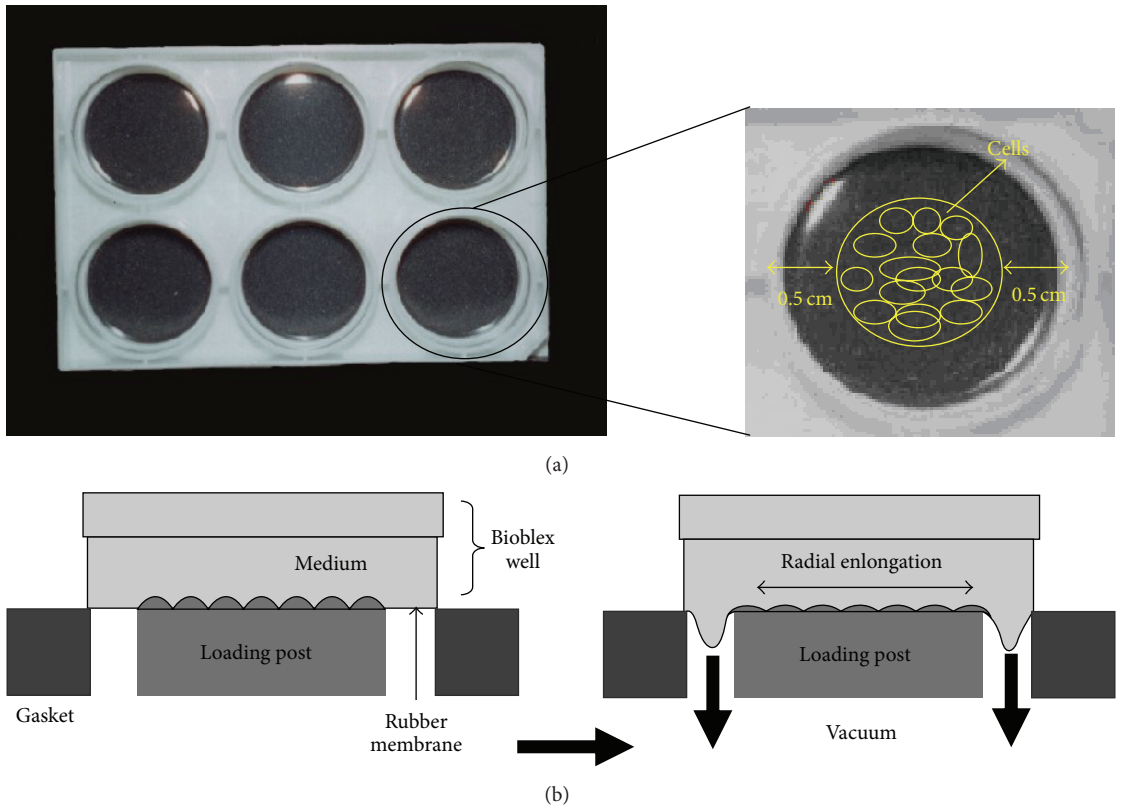

Figure 2: (a) Formation of cell sheet construct on Bioflex culture plate; (b) diagram of side view of radial stretch system.

proliferation decreased significantly by 3.9%, 4.1%, and 13.1%, respectively ($p < 0.05$). The collagen production was decreased significantly by 21%, 14%, and 11.1%, respectively. ($p < 0.05$).

5% and 15% stretching of the ACL fibroblast at 0.5 Hz significantly increased cell proliferation by 6% and 11%, respectively ($p < 0.05$). 10% stretch at 0.5 Hz significantly decreased cell proliferation by 5% ($p < 0.05$). Collagen production was significantly decreased by 15.1% when the cells are stretched at 5% and 0.5 Hz ($p < 0.05$). However, when the cells are stretched at 10% and 15% with the same frequency, collagen production was increased by 3.0% ($p < 0.05$) and 33.9% ($p < 0.05$), respectively.

Cyclic stretching of ACL fibroblast at 1 Hz with magnitude of either 5% or 15% showed a decrease in cell proliferation by 2.5% and 12.0%, respectively ($p < 0.05$). Similarly, the collagen production was decreased by 7.0% and 21.9%, respectively ($p < 0.05$). On the other hand, 10% stretch at 1 Hz increased cell proliferation by 4.0% ($p < 0.05$) and collagen production by 12% ($p < 0.05$) (Figures 3 and 4).

3.2. Uniaxial Stretch for MSCs. The proliferation of MSCs showed similar trend with fibroblasts. After 5%, 10%, and 15% stretching at 0.1 Hz, 8 hrs/day for 5 days, the MSCs proliferation all decreased significantly ($p < 0.05$). However, when stretching at 0.5 Hz with 5%, 10%, and 15% strain, the proliferation all increased by 12%, 14%, and 18% ($p < 0.05$). When the frequency increased to 1 Hz, only 10% strain could enhance proliferation (Figure 5).

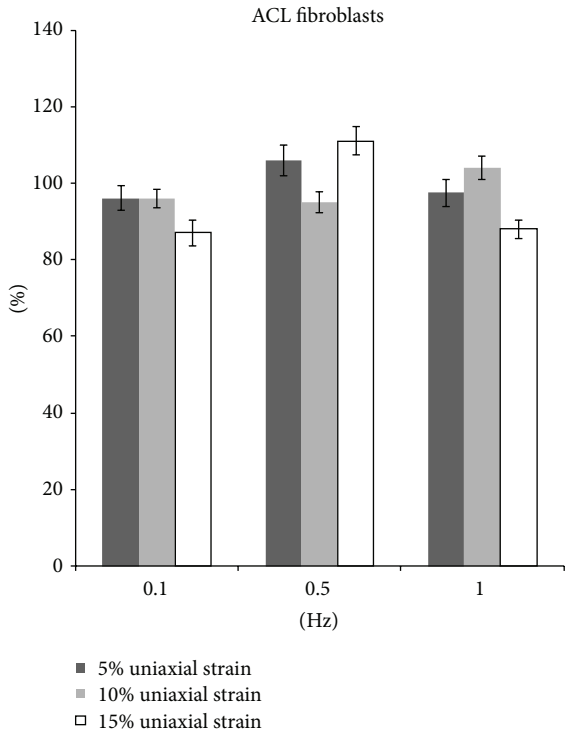

Figure 3: The proliferation of fibroblasts after uniaxial stretch stimulation.

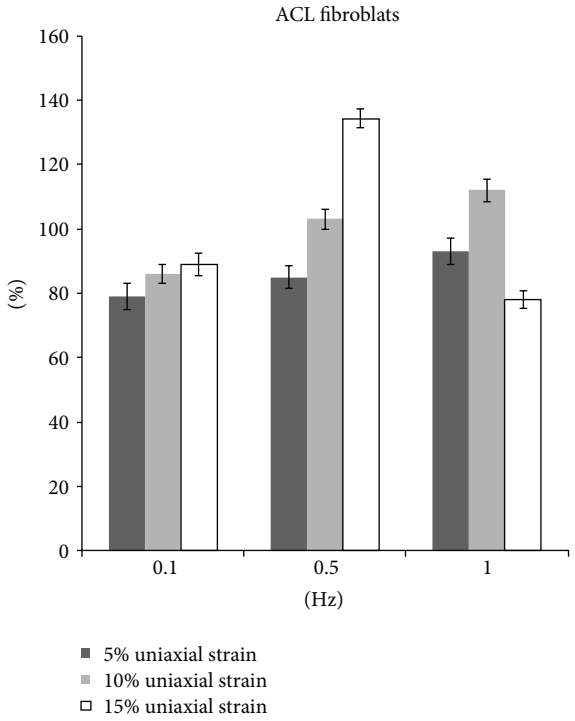

Figure 4: The collagen production of fibroblasts after uniaxial stretch stimulation.

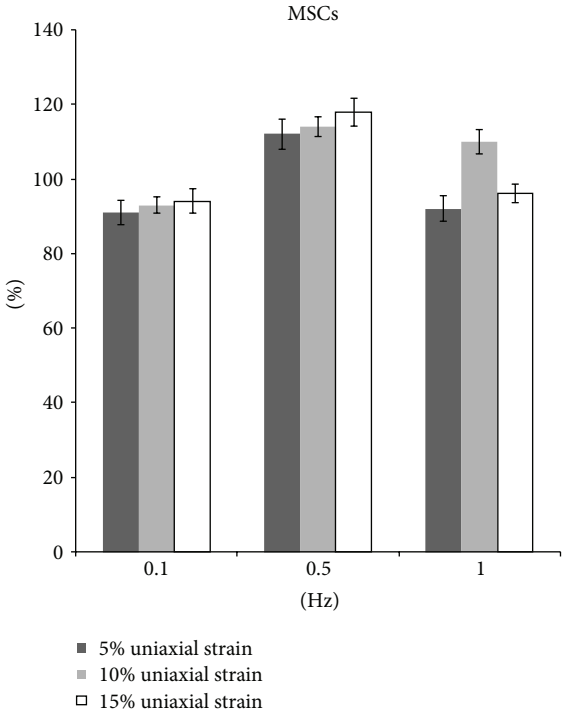

Figure 5: The proliferation of MSCs after uniaxial stretch stimulation.

MSCs showed the decreased collagen production at 0.1 Hz with magnitude of either 5%, 10%, or 15% ($p < 0.05$). On the contrary, the collagen production increased by 21%, 18%, and 30%, respectively, at 0.5 Hz with 5%, 10%, and 15% strain ($p < 0.05$). At 1 Hz, only 10% stretch increased collagen production by 15% ($p < 0.05$) (Figure 6).

3.3. Radial Stretch for Fibroblasts. After 15% stretching at 0.1 Hz, 8 hrs/day for 5 days, the ACL fibroblast proliferation increased by 4% ($p < 0.05$). No significant difference was detected in cells with 5% and 10% stretching as compared to unstretched cells (Figure 7). However, there was a significantly increased collagen production by 39.3%, 28.1%, and 4.0% in 5%, 10%, and 15% radial strain groups, respectively (Figure 8).

At 0.5 Hz, 10% stretch group showed a decrease in proliferation by 6.0% ($p < 0.05$) and collagen production by 17.0% ($p < 0.05$). In 5% stretch group, an increase in collagen production by 37.2% ($p < 0.05$) was observed although the cell proliferation showed no significant difference compared with nonstretch group. No significant change was observed in cell proliferation and collagen production in 15% stretch group (Figures 7 and 8).

Cyclic stretching of ACL fibroblast at 1 Hz with magnitude of 5% and 10% showed an increase cell proliferation by 7.1% and 6.1% ($p < 0.05$), respectively. However, at 15% stretch cell proliferation decreased by 7.0% ($p < 0.05$). There was no significant change in collagen production at 5%, 10%, and 15% stretch group (Figures 7 and 8).

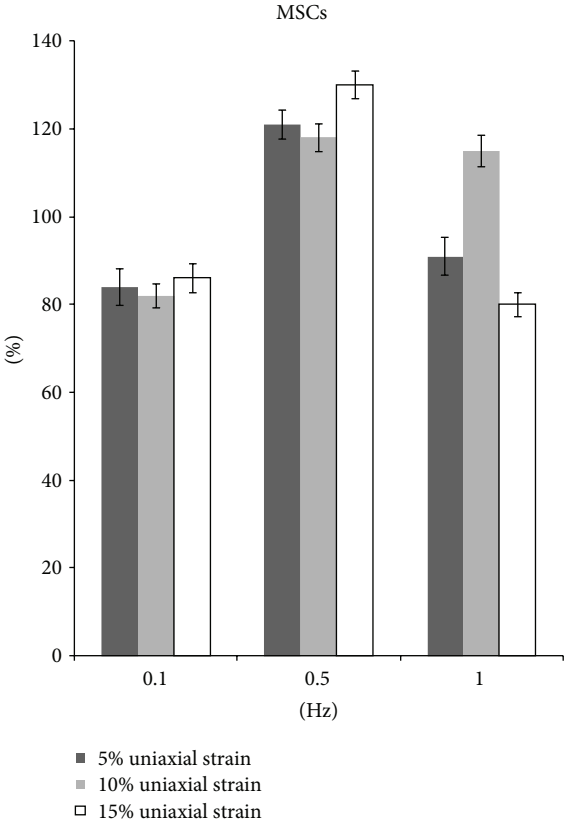

Figure 6: The collagen production of MSCs after uniaxial stretch stimulation.

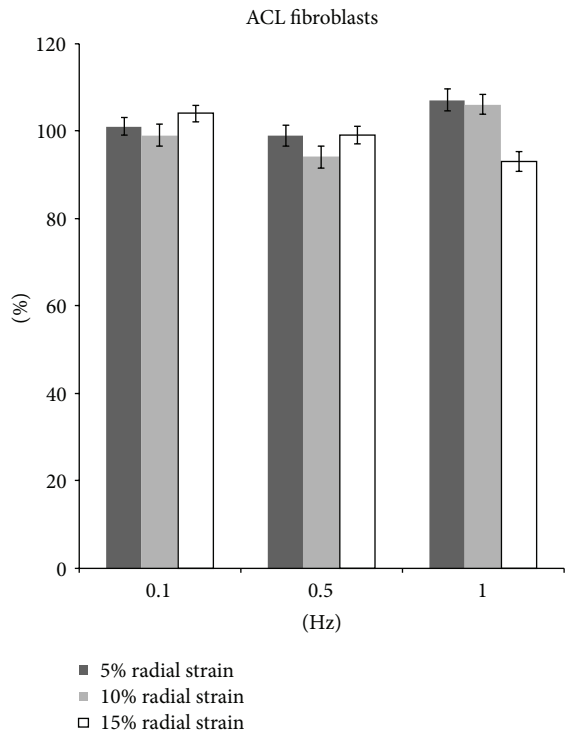

Figure 7: The proliferation of fibroblasts after radial stretch stimulation.

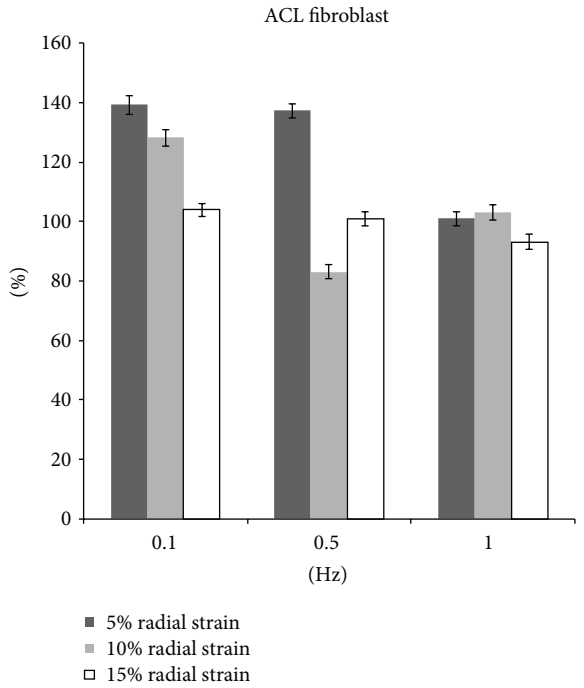

Figure 8: The collagen production of fibroblasts after radial stretch stimulation.

3.4. Radial Stretch for MSCs. In comparison with non-stretched group, the MSCs proliferation increased significantly by 6%, 8%, and 9% in 5%, 10%, and 15% radial strain groups, respectively, at 0.1 Hz, 8 hrs/day for 5 days ($p < 0.05$). The amounts of collagen production in all stretching groups were significantly higher than those of control group (Figures 9 and 10).

At 0.5 Hz, the proliferation decreased significantly by 7.0%, 6%, and 9% in 5%, 10%, and 15% strain groups, respectively ($p < 0.05$). Correspondingly, the collagen production also decreased by 15.0%, 16.9%, and 14.0% ($p < 0.05$) (Figures 9 and 10).

At 1.0 Hz, the cell proliferation and collagen production showed no significant difference in 5% stretch and 10% stretch groups. However, at 15% stretch the cell proliferation decreased by 6.0% and collagen production decreased by 6.9% ($p < 0.05$) (Figures 9 and 10).

4. Discussion

Ligament is a strong, dense structure made of connective tissue. It connects bone to bone across the joint to keep the dynamic and stable movement. The ACL is one of the most important four strong ligaments connecting the bones of knee joint. The function of ACL is to provide stability to knee and minimize stress across the knee joint. However, it has a poor self-regenerative capacity due to ligament's low cellularity and vascularity. Therefore, it is important to determine the effects of mechanical loading on ACL fibroblast in order to better understand ACL mechanobiology as well as pathophysiology. In addition, the tissue-engineered ligament has been extensively studied in recent years as an alternative graft in preclinical study. Mesenchymal stem cells (MSCs) are among the most promising and suitable stem cell types for ligament tissue engineering. The microenvironment of ACL not only contains biochemical factors but also exerts hemodynamic forces, such as shear stress and cyclic strain, which may influence the differentiation of MSCs [18]. Although many studies investigated the influence of cyclic mechanical stimulation on graft incorporation and cellular differentiation, few literatures have characterized the optimal parameter. In current study, using an in vitro system (Flexcell) that can control the magnitude and frequency of the stretching, the proliferation and collagen production of fibroblast and MSCs were compared to explore the optimal strain condition.

Appropriate mechanical loads at physiological levels would positively influence the expression of ECM and therefore the mechanisms of tendon regeneration. However, while excessive mechanical loading caused anabolic changes in tendons, it also induced differentiation of tendon stem cells into nontenocytes, which may lead to the development of degenerative tendinopathy frequently seen in clinical settings [19]. The mechanical strain used in current study ranged from 5% to 15% elongation, which was within the physiological range experienced by human tendons, given that tendons can elongate by 12–15% [20]. When fibroblasts were uniaxially stretched, the optimal frequency for proliferation and collagen production was 0.5 Hz (Figures 3 and 4). ACL fibroblasts showed an increase in either proliferation or

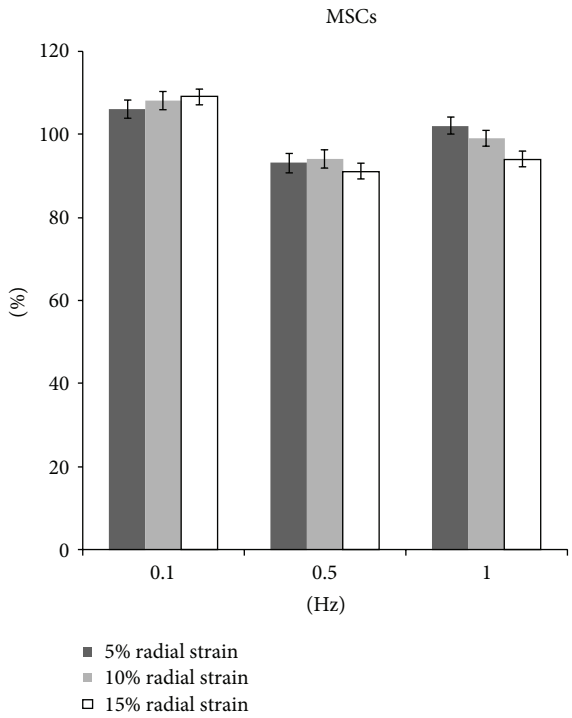

Figure 9: The proliferation of MSCs after radial stretch stimulation.

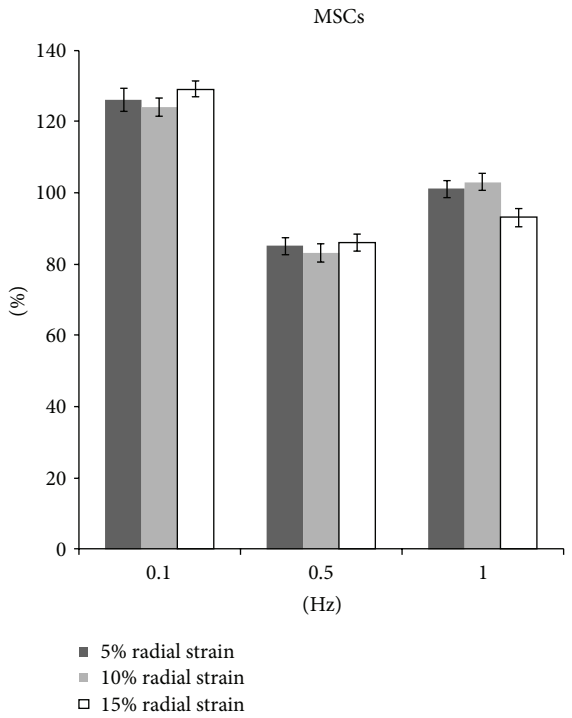

Figure 10: The collagen production of MSCs after radial stretch stimulation.

collagen production when they are stretched at different strains (5%, 10%, and 15%).

15% uniaxial strain at 0.5 Hz and 10% uniaxial strain at 1 Hz both stimulated fibroblast proliferation and collagen production. The results indicated that as the frequency increased, lower magnitude of stretch is more favorable for cell proliferation and collagen production. Collagen type I, collagen type III, decorin, and tenascin-C are fundamental proteins in the ECM of tendons [21]. Lohberger et al. [22] stimulated human rotator cuff fibroblast using Flexcell tension system with 10% elongation and a frequency of 0.5 Hz. The total soluble collagen was measured in cell culture supernatants. Cyclic strain significantly increased the collagen production on days 7 and 14. The expression of tenascin-C and scleraxis increased significantly in the mechanically stimulated groups at both time points. There results were correlated with our findings in current study. Uniaxial strain at 0.1 Hz is the least favorable for fibroblast proliferation and collagen production. The cells showed a decrease proliferation and collagen production when they are stretched at 0.1 Hz at different strains (5%, 10%, and 15%) (Table 1).

In contrast to uniaxial strain, 0.5 Hz was least favorable for cell proliferation. Radial strains (5% and 15%) at 0.5 Hz did not have significant effect on cell proliferation. The 10% radial strain showed negative effect and decreased cell proliferation. The strains (5% and 10%) at 1 Hz and 15% strain at 0.1 Hz all stimulated cell proliferation. Interestingly, the collagen production under these conditions showed no significant difference compared to that of nonstretched group. Although the strains (5% and 10%) at 0.1 Hz and 5% strain at 0.5 Hz had no effect on cell proliferation, the cells under these conditions showed significantly increased collagen production (Table 1).

For MSCs under uniaxial stretch condition, 0.5 Hz is favorable for cell proliferation and collagen production. Different strains (5%, 10%, and 15%) all showed positive effects. In addition, 10% strain at 1.0 Hz also upregulated cell proliferation and collagen synthesis. Interestingly, for radial stretch groups, MSCs showed an increase in both proliferation and collagen production when they are stretched at 0.1 Hz at different strains (5%, 10%, and 15%) (Table 2).

In summary, uniaxial stretch (15% at 0.5 Hz; 10% at 1.0 Hz) showed positive effects on fibroblast. The uniaxial strains (5%, 10%, and 15%) at 0.5 Hz and 10% strain at 1.0 Hz showed positive effects on MSCs. Radial strain did not have significant effect on fibroblast. On the contrary, all radial strains (5%, 10%, and 15%) at 0.1 Hz had positive effects on MSCs.

5. Conclusion

This study suggested that exposing fibroblasts and MSCs to uniaxial or radial strains promoted cell proliferation and collagen production. The fibroblasts and MSCs had their own appropriate mechanical stimulatory parameters. These specific parameters had great parental application in cell expansion to fabricate tissue engineering products.

Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Table 1: Effects of strains at various frequencies on fibroblasts.

Function	Uniaxial stretch									Radial stretch								
	0.1 Hz			0.5 Hz			1.0 Hz			0.1 Hz			0.5 Hz			1.0 Hz		
	5%	10% (strain)	15%	5%	10% (strain)	15%	5%	10% (strain)	15%	5%	10% (strain)	15%	5%	10% (strain)	15%	5%	10% (strain)	15%
Proliferation	↓	↓	↓	↑	↓	↑	↓	↑	↓	—	—	↑	—	↓	—	↑	↑	↓
Collagen	↓	↓	↓	↓	↑	↑	↓	↑	↓	↑	↑	—	↑	↓	—	—	—	↓

"↑": increase; "↓": decrease; "—": no difference ($p < 0.05$).

Table 2: Effects of strains at various frequencies on MSCs.

Function	Uniaxial stretch									Radial stretch								
	0.1 Hz			0.5 Hz			1.0 Hz			0.1 Hz			0.5 Hz			1.0 Hz		
	5%	10% (strain)	15%	5%	10% (strain)	15%	5%	10% (strain)	15%	5%	10% (strain)	15%	5%	10% (strain)	15%	5%	10% (strain)	15%
Proliferation	↓	↓	↓	↑	↑	↑	↓	↑	↓	↑	↑	↑	↓	↓	↓	—	—	↓
Collagen	↓	↓	↓	↑	↑	↑	↓	↑	↓	↑	↑	↑	↓	↓	↓	—	—	↓

"↑": increase; "↓": decrease; "—": no difference ($p < 0.05$).

Authors' Contributions

Liguo Sun, Ling Qu, and Rui Zhu contributed equally to this work and were regarded as co-first authors.

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Clinical Study

Phase 1 Trial of Autologous Bone Marrow Stem Cell Transplantation in Patients with Spinal Cord Injury

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Introduction. A total of 18 patients, with complete motor deficits and paraplegia caused by thoracic and lumbar spine trauma without muscle atrophy or psychiatric problems, were included into this study. *Materials and Methods.* The bone marrow was aspirated from the anterior iliac crest under local anesthesia and the mononuclear fraction was isolated by density gradient method. At least 750 million mononuclear-enriched cells, suspended in 2 mL of saline, were infused intrathecally. *Results and Discussion.* The study reports demonstrated improvement of motor and sensory functions of various degrees observed in 9 of the 18 (50%) cases after bone marrow stem cell transplantation. Measured by the American Spinal Injury Association (ASIA) scale, 7 (78%) out of the 9 patients observed an improvement by one grade, while two cases (22%) saw an improvement by two grades. However, there were no cases in which the condition was improved by three grades. *Conclusions.* Analysis of subsequent treatment results indicated that the transplantation of mononuclear-enriched autologous BMSCs is a feasible and safe technique. However, successful application of the BMSCs in the clinical practice is associated with the necessity of executing more detailed examinations to evaluate the effect of BMSCs on the patients with spinal cord injury.

1. Introduction

Spinal cord injury is a disorder that often causes severe disability, such as incomplete or complete tetraplegia or paraplegia. The economic burden relative to the estimated lifetime costs for treatment and healthcare of one patient could be as high as 4.5 million USD. According to the National Spinal Cord Injury Statistical Center, the annual incidence of spinal cord injury in the USA is 40 cases per million, including those who have survived the accident. Annually the crude incidence is 12,500 new cases [1]. There is neither a cure for the disorder nor any effective treatment for patients with injured spinal cords. The main surgical procedure is a decompression of the spinal cord in addition to a high dose of methylprednisolone [2].

Though early decompression could have a neuroprotective effect, less than 1% of patients showed complete neurological recovery at hospital discharge. Many patients remain in a wheelchair. Pharmacological agents such as methylprednisolone [3, 4], naloxone, monosialotetrahexosylganglioside (GM-1), or TRH were studied in clinical trials with no treatment demonstrating strong evidence for clinical

benefits [5]. Autologous stem cells could help the regeneration of an injured spinal cord. Bone marrow mesenchymal and hematopoietic stem cells have differentiation potential. There are findings that BMSCs differentiate into mature neurons or glial cells under experimental conditions [6, 7]. It was demonstrated that mesenchymal stem cells could differentiate into neuronal-like cells *in vivo*, which express the neural cell marker. Preclinical studies have shown that such differentiated cells were able to improve or restore damaged spinal cord function. Replacement, dedifferentiation, or paracrine effects were suggested. These findings demonstrated that the use of BMSCs has a therapeutic potential in patients with neurological diseases.

Different cell types were used in preclinical studies for SCI treatment. NSCs, MSCs, ESCs, OECs, SCs, and iPSCs have all proved that they have regenerative potential [8]. MSCs, specifically, have low immunogenicity and possess anti-inflammatory and immunosuppresive effects [9]. Bone marrow-derived MSCs are the most widely used stem cells for SCI experiments. They differentiate into neurons and glia cells [10]. Some authors suggest that cell fusion and transdifferentiation are the main mechanisms [11–13]. In animal models BM MSCs were injected into spinal cord injury area [14] or intrathecally with some effects [15]. Experiments in nonhuman primates and pigs demonstrated successful mean improvement of locomotor function [16, 17]. The secretion of growth factors and anti-inflammatory cytokines has been proposed as the main mechanism in cell transplantation [18–20]. Clinical trials showed safety and feasibility of BM MSCs transplantation for SCI patients. No serious complications were reported and patients showed improvement of motor and sensory functions [21–23]. The objective of this study was to assess the safety and efficacy of transplanting of autologous bone marrow stem cell in patients with spinal cord injury.

2. Materials and Methods

2.1. Patients. Starting from March of 2012 until December of 2014, a total of 18 patients, with complete motor deficits and paraplegia caused by thoracic and lumbar spine trauma without muscle atrophy or psychiatric problems, were included into this study. All patients signed a written informed consent. The study protocol was confirmed according to ethical guidelines of the 1975 Declaration of Helsinki and was approved by Archangel St. Michael Multi Profile Clinical Hospital, Tbilisi, Georgia. After Ethics Committee approval, patients between the ages of 18 and 65 years and of either gender were preliminary candidates. Inclusion criteria of subjects were as follows: subjects have chronic spinal cord injury (>6 months after initial spinal cord injury surgery) who have stable neurological symptoms for at least 6 months; subjects have current neurological status of ASIA score A; the location of neurological injury of the patient is between C5 and T11; the injured site of the spinal cord is within three vertebral levels; subjects must be able to read, understand, and complete the VAS; and subjects have voluntarily signed and dated an informed consent form prior to any study procedures. Those screened were excluded on the basis of the following criteria: anatomical transection of the spinal cord; spinal cord lesion by sharp objects; ongoing infections; terminal, neurodegenerative, or primary hematological diseases; osteopathy which might increase the risk of spinal cord puncture; coagulopathies; severe hepatic, renal, or heart failure; and pregnancy or lactation.

2.2. Bone Marrow Cell Therapy. 100–120 mL of bone marrow was aspirated from the anterior iliac crest under local anesthesia and placed in sterile tubes containing heparin. The aspirates were diluted 1 : 2 with PBS. The mononuclear fraction was isolated by density gradient centrifugation at 400 ×g for 30 min at room temperature using Ficoll Paque Plus or Ficoll Paque Premium solution (GE Healthcare, USA). At least 750 million mononuclear-enriched cells, suspended in 2 mL of saline, were infused intrathecally.

2.3. Flow Cytometry and Viability Testing. 0.4 mL of the final cell product was subjected to trypan blue dye exclusion test and flow cytometric analysis. Viability test was performed by 0.4% trypan blue solution (Sigma, USA) according to standard protocol. For cell immunophenotyping cell suspensions were incubated with anti-human CD34, anti-human CD45, and antihuman CD 271 (all from Miltenyi Biotec, Germany) and anti-human-STRO-1 antibodies (Santa-Cruz Biotechnology, USA) in 0.5% BSA/PBS (Sigma, USA) buffer in accordance with manufacturer's instructions. Flow cytometry analysis was carried out on BD FACSCalibur flow cytometer (Becton Dickinson, USA).

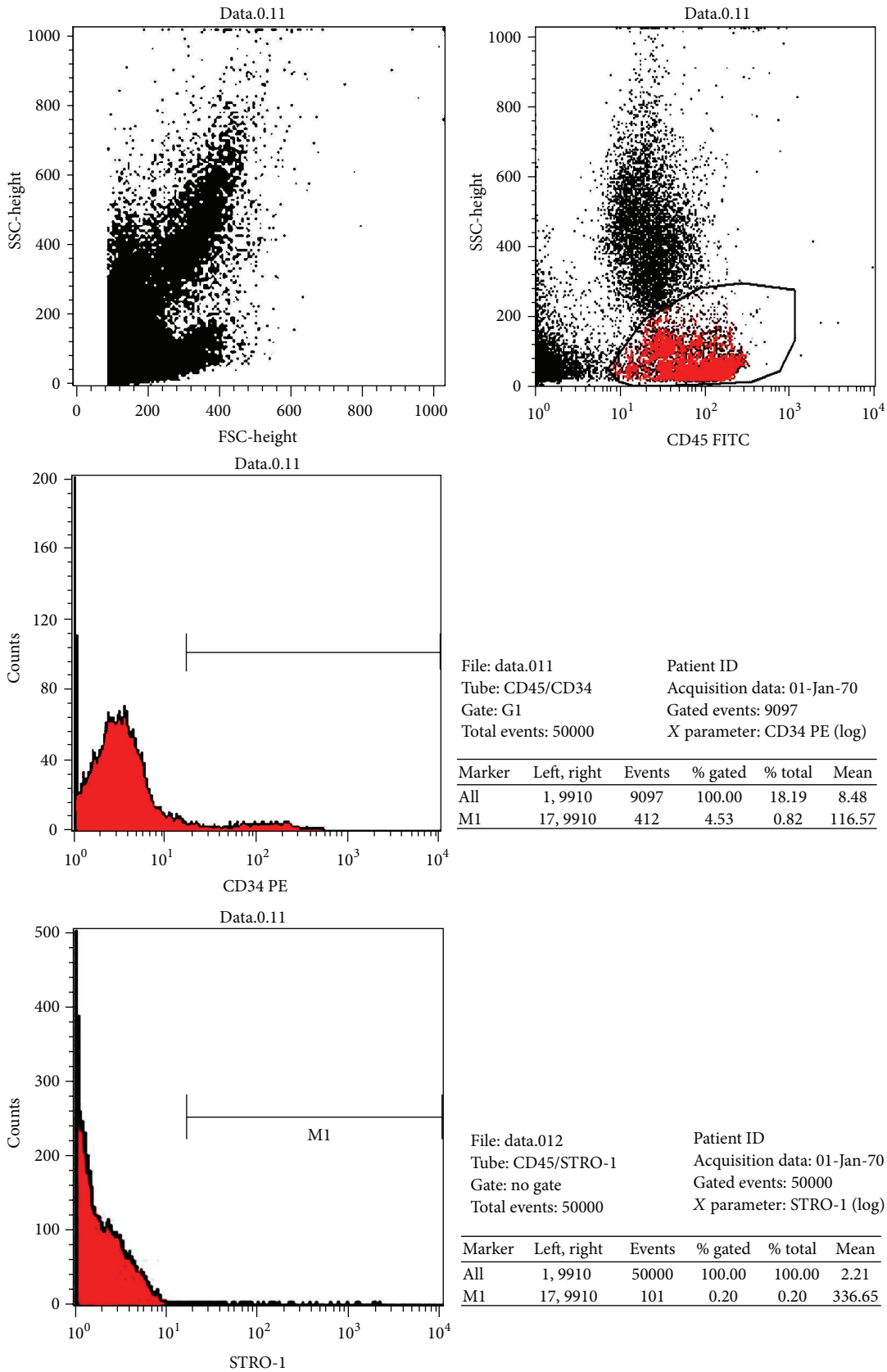
Mononuclear CD45−/CD34−/CD271+/STRO-1+ cells were defined as BM MSCs and their percentage and absolute count were enumerated. Bone marrow hematopoietic stem cells were determined in CD45+/CD34+ mononuclear cell population and their percentage and absolute counts were enumerated (Figure 1). The total amount of autologous bone marrow and the detailed number of final bone marrow stem cell products are shown in Table 1.

2.4. Follow-Up Period. The follow-up visits were scheduled for 12 months after transplantation. During every follow-up visit, preoperatively, and after 3 and 6 months after surgery, the results were evaluated by assessing ASIA impairment scale, measuring electrophysiological parameters, including electroneuromyography and enhanced MRI.

2.5. Statistical Analysis. Statistical analysis was performed by using SPSS Statistics v20 software. A paired sample t-test was used for determining whether or not there is a statistically significant difference between the results acquired before and after the treatment. Initial data (before treatment) was compared to that acquired at 6 and 12 months after treatment was performed. A significance level of 0.05 was chosen.

3. Results and Discussion

Transplantation of bone marrow stem cells was performed on 18 patients, among which 13 were male (72%) and 5 were female patients (28%), aged 22 to 65. Patients' profiles are shown in Table 2. There were 12 (67%) patients with injury of the thoracic spine and 6 (33%) patients with lumbar spine.



Marker	Left, right	Events	% gated	% total	Mean
All	1, 9910	9097	100.00	18.19	8.48
M1	17, 9910	412	4.53	0.82	116.57

Marker	Left, right	Events	% gated	% total	Mean
All	1, 9910	50000	100.00	100.00	2.21
M1	17, 9910	101	0.20	0.20	336.65

Figure 1: Representative picture of flow cytometric analysis. Mononuclear CD45−/CD34−/CD271+/STRO-1+ cells defined as bone marrow mesenchymal stem cell and their enumerated percentage and absolute count. Bone marrow hematopoietic stem cells determined in CD45+/CD34+ mononuclear cell population and their enumerated percentage and absolute count.

Table 1: The total amount of autologous bone marrow and the detailed number of final bone marrow stem cell products.

Patients	Bone marrow (mL)	Total cells ($\times 10^6$)	CD45		Mononuclear cells		Mesenchymal cells STRO-1+/CD271+		CD34	
			%	Number of cells ($\times 10^6$)	%	Number of cells ($\times 10^6$)	%	Number of cells ($\times 10^3$)	%	Number of cells ($\times 10^6$)
1	120	536	50	268	28	150	0.04	214	1.5	7.0
2	100	516	53	284	34	176	0.02	103	1.4	7.2
3	100	531	51	271	31	165	0.03	159	1.4	7.4
4	120	542	54	293	38	206	0.04	217	1.5	8.1
5	100	405	55	223	45	181	0.05	713	0.9	3.6
6	100	964	55	530	40	386	0.04	384	1.5	14.5
7	120	620	50	310	37	229	0.03	186	0.8	5.0
8	100	903	58	524	41	375	0.02	181	0.9	8.1
9	100	749	61	457	35	262	0.01	75	1.5	12.0
10	120	902	55	491	29	261	0.02	181	2.5	22.6
11	100	748	61	456	34	261	0.01	74	1.5	11.2
12	100	526	51	267	27	151	0.04	214	1.5	7.9
13	120	512	52	283	33	175	0.02	103	1.4	7.2
14	110	559	81	451	33	187	0.04	210	7.5	41.9
15	100	690	48	330	36	245	0.04	284	2.4	16.6
16	120	542	52	280	27	145	0.05	260	0.9	4.9
17	120	628	49	310	34	214	0.03	210	2.7	17.0
18	100	696	42	294	44	308	0.02	148	2.6	18.1

Table 2: Patient demographics.

Patients	Sex	Age	Cause of injury	Injury site	Duration between injury and surgery (months)	ASIA grade
1	M	44	Car accidents	L3-4	20	A
2	M	51	Fall injury	T12–L4	5	A
3	M	24	Gunshot wound	T11-12	10	B
4	F	61	Fall injury	T8	8	A
5	M	38	Car accidents	S3	16	A
6	M	42	Car accidents	T2	7	A
7	M	22	Car accidents	L3-4	12	B
8	M	28	Car accidents	T8	9	B
9	F	54	Fall injury	T12–L3	10	A
10	F	31	Car accidents	L2-3	6	A
11	M	59	Car accidents	T10–12	14	C
12	M	44	Car accidents	L3	19	C
13	M	22	Gunshot wound	L2-3	11	A
14	F	31	Car accidents	T4	10	B
15	F	46	Car accidents	T5-6	17	C
16	M	20	Car accidents	T10–12	20	B
17	M	32	Fall injury	T8	14	A
18	M	19	Gunshot wound	T11-12	10	A

The period of time that has passed since the SCI was from 5 to 20 months. According to the ASIA classification of SCI, there were 10 cases (56%) with ASIA A score, 7 of which were males and 3 were females; five cases (28%) had B score, 4 of these were males and 1 was female; and there were 3 (17%) cases with ASIA C score, out of which 2 were males and 1 was female.

Out of the 18 total patients, the reasons of SCI in 11 (61%) of the cases were car accidents, 4 (22%) cases due to falling and 3 (17%) cases due to a gunshot wound. All procedures were performed without any specific side effects or complications except for mild pain in the anterior iliac crest region at the sites of bone marrow puncture. Headaches were observed in 9% of the patients and temperature increased up to 37.5°C in 6% of patients, which lasted for two days. No other complications or specific side effects related to the infusion procedure were reported.

Continuous patient monitoring was carried out during the first 24 hours after transplantation. Following clinical observation patients were discharged. In this study, our attention was mainly focused on assessing the safety of this method. The ASIA score and nerve conduction study reports demonstrated improvement of motor and sensory functions of various degrees observed in 9 of the 18 cases (50%) after bone marrow stem cell transplantation. Measured by the ASIA scale, 7 (78%) out of the 9 patients observed an improvement by one grade. While two cases (22%) saw an improvement by two grades. However, there were no cases in which the condition was improved by three grades. Of the 18 total patients with SCI, the significant damage of the small and large tibial nerves using ENMG did not occur in 8 (44%) patients, in the form of insertional positive sharp waves and end plate potential, where the bioelectric activity is registered in the lower limb muscles. After cell transplantation, 5 (42%) of the 12 patients suffering from urinary tract dysfunction saw improvements in urinary function of varying degrees. Additionally, 7 (78%) of the 9 patients suffering from intestinal dysfunction demonstrated improved function in various degrees. CT changes were observed in four patients in postoperative months ranging from eight to twelve.

Spinal cord injuries are accompanied by a number of complications, causing death of neurons, degeneration of nerve fibers, hemorrhage, and eventually the absence of complete regeneration in areas of injury. In most of the cases, traditional methods of treatment are very rarely able to restore the lost functions of tissues. However, the use of stem cells in such patients gives hope for the opportunity to achieve functional improvements. Currently, human OPCs, Schwann cells, bone marrow stromal cells, nasal olfactory ensheathing cells, and others are being used for stem cell therapy during spinal cord injuries. Preclinical studies of the human OPCs application have shown that the effects of transplantation included robust white and gray matter sparing at the injury epicenter and, in particular, preservation of motor neurons that correlated with movement recovery [18]. One critical aspect of successful cell-based SCI therapy is the time of injection following injury [19]. The authors note that they injected the majority of transplanted OPCs at two clinically relevant times when most damage occurs to the surrounding tissues, 3 and 24 hours following injury.

The derived OPCs expressed oligodendrocyte markers, including 2′,3′-cyclicnucleotide 3′-phosphodiesterase, galactocerebroside, oligodendrocyte transcription factor (Olig1), and oligodendrocyte markers (O4 and O1). Moreover, OPCs survived when injected at the center of injury and migrated away from the injection sites after one week. Other authors think that human embryonic stem cell-derived OPCs can be transplanted sooner than conventionally accepted. Transplantation of Schwann cells can also be a promising

therapeutic strategy for spinal cord repair. They are one of the most widely studied cell types for repairing the spinal cord. Unlike oligodendrocytes and their precursors, Schwann cells possess many of the characteristics that are desirable for transplantation in spinal cord lesions. They can be easily collected from peripheral nerve and easily purified and grown in culture in large quantities. Due to their ability to dedifferentiate, migrate, proliferate, and express growth promoting factors and myelinate regenerating axons, Schwann cells play a crucial role in endogenous repair of peripheral nerves [20, 21]. The transplantation of Schwann cells in the injured spinal cord boosts the regeneration of axons, myelinates or ensheathes regenerated axons in a normal way, reduces cyst formation in the injured tissue, and reduces secondary damage of the tissues around the initial injury site [22]. In order to improve the clinical condition of the patients with SCI, the cultured and purified autologous Schwann cells, which were previously isolated from the sural nerve, were transplanted [23].

As it is noted by authors, there were some signs of improvement in the autonomic, motor, and sensory function of all patients. Authors report that they have assessed the safety and feasibility of a combination of bone marrow mesenchymal stromal cells and Schwann cells for the treatment of patients with chronic spinal cord injury [24]. However, when transplanting Schwann cells, the regeneration and myelination occur only where the graft is located. The inhibitory nature of the glial scar surrounding the injury axons does not allow for the regeneration of cells beyond the graft [25]. Thus, despite the fact that the Schwann cells having great potential for repairing the injured spinal cord, in order to successfully use them, the researches continue to address issues such as encouraging the survival and growth of damaged axons using neurotrophins, which can help with the establishment of appropriate connections between regenerating axons and target neurons and, thus, provide functional recovery [26], by neutralizing inhibitory molecules associated with the failure of axonal regeneration [27] and many others.

Another promising candidate for cell transplantation in SCI cases may be the olfactory ensheathing cell, considered to be a result of the unique capabilities of these cells as they are being continually replaced throughout one's lifetime, and also the rate of neurogenesis can be regulated by manipulating the system in order to abbreviate or prolong the average life of a sensory neuron [28].

A phase 1, single-blinded clinical trial has shown that, up to one year after implantation, the transplantation of autologous olfactory ensheathing cells into the injured spinal cord is feasible and safe [29]. However, there are contradictory opinions about the effectiveness of transplantation of olfactory ensheathing cells for spinal cord injury. Thus, some authors report that the interfaces of Schwann cells and olfactory ensheathing cells form a 3-dimensional matrix providing a permissive microenvironment for successful axon regeneration in the adult mammalian central nervous system [30]. Others report that the transplantation of Schwann cells or olfactory ensheathing glia, or their combination in the adult Fischer rat thoracic (T9) spinal cord, after 1 week from a moderate contusion, is more effective in promoting axonal sparing/regeneration rather than the combination of Schwann cells with olfactory ensheathing glia or olfactory ensheathing glia graft [31]. Special interest is given to bone marrow hematopoietic and mesenchymal cells. In a phase I/II open-label nonrandomized study, patients were transplanted with BMSCs in acute (within 14 days after injury), subacute (2–8 weeks), and chronic patients (more than 8 weeks). Control group patients were treated with conventional decompression and fusion surgery without BMSC transplantation. At 4 months, the MRI presented spinal cord enlargement without any hemorrhage, new cysts, or infections. The ASIA grade increased up to 30–33% of the acute and subacute treated patients, respectively, (ASIA A to B or C), whereas no significant improvement was observed in the chronic treatment group [32]. In 2007 Sykova and Jendelova published a study on 20 complete spinal cord injury patients. Patients were transplanted 10 to 467 days after injury. Intra-arterial bone marrow cell (via catheterization of a. vertebralis) transplantation was successful in the acute patient group (10–30 days after injury). Patients were evaluated with the ASIA protocol, Frankel score, MRI evaluation, and electrophysiology (MEPs and SEPs). Motor and sensory function were improved in most patients within 3 months. No complications were observed [33]. In 2008 Geffner et al. reported eight cases of treatment of SCI (four acute, four chronic) with bone marrow stem cells. Cells were injected via multiple routes: directly into the spinal cord, intrathecally, and intravenously. For neurological evaluation ASIA, Frankel, and Ashworth scales were used. Comprehensive evaluations demonstrated improvements in ASIA, Barthel (quality of life), Frankel, and Ashworth scoring. ASIA Motor Score/Sensory Light Touch Score/Sensory Pin Prick Score were improved in all 8 patients as well as Barthel Index Score and Bladder Function Score and showed stable improvement even after 2 years after treatment [17].

The similar results were obtained by the authors that transplanted mesenchymal bone marrow stem cells in 40 patients with SCI. The cells were transplanted in the area surrounding the injury. During the whole period of observation there were significant improvements in the patients that had no serious complications [34]. Other authors report that they obtained satisfying results using the transplantation of autologous bone marrow-derived cells in addition to physical therapy in patients with chronic cervical and thoracic SCI; the duration of the injury in these patients was at least 12 months. The injection of stem cells was conducted with intrathecal injection [35]. Others obtained satisfying results when treating the patients with SCI using hematopoietic progenitor stem cells [36]. According to our preliminary research, the prognosis for SCI patients may depend on various reasons, including the etiology of SCI, the time that elapsed since the injury, the age of the patient, the type of stem cells that are more suitable for transplantation in patients with SCI, the amount of cells, the ways to deliver the cells into the lesion, and various other covariates. The further successful application of stem cell therapy in the patients with SCI depends largely on solving the above-mentioned issues.

4. Conclusion

Analysis of subsequent treatment results indicated that the transplantation of mononuclear-enriched autologous BMSCs is a feasible and safe technique. Among the adverse effects, patients noted fever and headache, which disappeared within 24–48 hours without intervention. However, successful application of the BMSCs in the clinical practice is associated with the necessity of executing more detailed examinations to evaluate the effect of BMSCs on the patients with spinal cord injury.

Abbreviations

ASIA: American Spinal Injury Association
BM MSC: Bone marrow mesenchymal stem cell
BMSC: Bone marrow stem cell
ENMG: Electroneuromyography
ESC: Embryonic stem cell
GM-CSF: Granulocyte-macrophage colony-stimulating factor
iPSC: Induced pluripotent stem cell
MEP: Motor evoked potential
MSC: Mesenchymal stem cell
MRI: Magnetic resonance imaging
NSC: Neural stem cell
OEC: Olfactory ensheathing cell
OPC: Oligodendrocyte progenitor cell
SC: Schwann cell
SCI: Spinal cord injury
SEP: Somatosensory evoked potential
SPSS: Statistical Package for the Social Science
TRH: Thyrotropin-releasing hormone
VAS: Visual analogue scale.

Competing Interests

The authors declare that they have no competing interests.

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Research Article

Platelet-Rich Plasma Obtained with Different Anticoagulants and Their Effect on Platelet Numbers and Mesenchymal Stromal Cells Behavior In Vitro

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There are promising results in the use of platelet-rich plasma (PRP) for musculoskeletal tissue repair. However, the variability in the methodology for its obtaining may cause different and opposing findings in the literature. Particularly, the choice of the anticoagulant is the first definition to be made. In this work, blood was collected with sodium citrate (SC), ethylenediaminetetraacetic acid (EDTA), or anticoagulant citrate dextrose (ACD) solution A, as anticoagulants, prior to PRP obtaining. Hematological analysis and growth factors release quantification were performed, and the effects on mesenchymal stromal cell (MSC) culture, such as cytotoxicity and cell proliferation (evaluated by MTT method) and gene expression, were evaluated. The use of EDTA resulted in higher platelet yield in whole blood; however, it induced an increase in the mean platelet volume (MPV) following the blood centrifugation steps for PRP obtaining. The use of SC and ACD resulted in higher induction of MSC proliferation. On the other hand, PRP obtained in SC presented the higher platelet recovery after the blood first centrifugation step and a minimal change in MSC gene expression. Therefore, we suggest the use of SC as the anticoagulant for PRP obtaining.

1. Introduction

Platelet-rich plasma (PRP) is a blood-derived product in which platelets are concentrated at least five times in plasma above the baseline of that in the whole blood [1]. PRP is being investigated as an autologous product to improve tissue repair in different conditions and lesions, especially for musculoskeletal tissues, such as chondral lesions [2–4], tendinopathies [5–7], muscle strains [8, 9], and bone repair [10, 11]. Besides its clinical application, PRP may be an efficient substitute to fetal bovine serum in cell culture [12–15]. Its therapeutic potential is based mainly on the growth factors present in platelet's alpha granules [16], such as transforming growth factor beta (TGF-β) [17], vascular endothelial growth factor (VEGF) [18], and platelet-derived growth factor (PDGF) [19], which have already been demonstrated to play important roles in tissue repair. When platelets are concentrated and activated, it is expected that the concentration of the factors released reaches three to five times of that found in the plasma [16].

The general methodology to obtain PRP involves the collection of whole blood with anticoagulants, followed by one or two centrifugation steps. After a first low-speed centrifugation, erythrocyte-free platelet concentrated plasma is recovered and submitted to high-speed centrifugation. Platelet-poor plasma is then discarded and the remaining platelet

pellet is homogenized into what is regarded as PRP [16]. Several aspects on this method are still under debate, such as number of centrifugations, presence of leukocytes, and use and type of platelet activator and anticoagulants [20]. When no anticoagulant is used, a blood clot will form, and serum can be obtained but without increase in platelet concentration [21]. In the case of PRP obtaining, coagulation is not intended to occur prior to platelet concentration; hence, blood must be collected in the presence of anticoagulants.

For transfusion purposes, blood is usually collected in bags containing citrate phosphate dextrose adenine (CPDA-1) solution, as anticoagulant [22, 23], from which a platelet concentrate is obtained by double centrifugation of the whole blood or apheresis. Platelets concentrates obtained by such methodologies may also be used for promoting tissue repair [24]. On the other hand, recent PRP formulations for autologous applications are usually prepared in collection tubes containing citrate solutions, in the form of sodium citrate [25–29] or ACD-A [30–32]. This last, ACD, is present in the majority of available commercial kits for PRP production [33, 34]. In other cases, heparin [35, 36] or EDTA [37] can be used. For clinical investigations, EDTA is commonly used in hematology tests, SC in coagulation tests, and ACD in plasma levels measurement of platelet-derived components [38]. Therefore, our goal was to analyze how the choice of anticoagulant for blood collection would modulate PRP characteristics as well as its effects on mesenchymal stromal cell culture.

2. Materials and Methods

2.1. Ethics Statement. All of the experimental procedures were approved by the Ethics Research Committee of the Pró-Cardíaco Hospital, Rio de Janeiro (CAAE: 14878813.4.0000.5533), and all donors signed an informed consent.

2.2. PRP Obtaining. PRP was obtained as previously described, with minor modifications [39]. Peripheral blood was collected from nine volunteer donors (6 men and 3 women) using blood collection tubes containing sodium citrate (SC) (Vacutainer®, Ref: 369714; BD Biosciences, San Jose, CA), ethylenediaminetetraacetic acid (EDTA) (Vacutainer, Ref: 367861; BD Biosciences), or anticoagulant citrate dextrose (ACD) solution A (Vacutainer, Ref: 364606; BD Biosciences) solution. The blood collected in one ACD tube was maintained in the same tube or divided into three polypropylene tubes containing no anticoagulant (Falcon™, Ref: 352063; BD Biosciences), termed as ACD-2.

Tubes were centrifuged at 300 g for 5 minutes (Megafuge® 40, Thermo Fisher Scientific, Waltham, MA). Supernatant containing plasma and platelets, termed as platelet-rich plasma 1 (PRP1), was collected from each tube and transferred to new polypropylene tubes containing no anticoagulant. In the case of ACD and ACD-2, after platelet counting, PRP1 from the same donors was mixed for the next experiments. Then, PRP1 was centrifuged at 700 g for 17 minutes. Supernatant was collected, namely, platelet-poor plasma (PPP). Part of the PPP from each tube was used to resuspend the platelet pellet, forming the platelet-rich plasma 2 (PRP2), in order to achieve the expected concentration of 10^6 platelets/μL. The platelets in PRP2 were activated by adding 1 M $CaCl_2$ (final concentration of 20 mM) and incubated at 37°C for 1 hour. After clot formation, tubes were maintained at 4°C during 16 hours to allow clot contraction. Finally, tubes were centrifuged at 3000 g for 20 minutes and the supernatant was collected, termed as platelet-rich plasma releasate (PRPr). The PRPr was freezed at −80°C until thawing for experimental use.

2.3. Hematological Analysis. Counting of platelets, red blood cells, white blood cells, and analysis of mean platelet volume (MPV) were determined in whole blood, PRP1, PRP2, and PPP fractions. Those analyses were performed with a hematological analyzer (Mindray BC 2800, Perdizes, SP, Brazil). Platelet recovery after the first centrifugation step, expressed as a percentage, was calculated by dividing the total number of platelets in PRP1 by the total number of platelets in whole blood.

2.4. Quantification of Growth Factors. PRPr-derived TGF-β1 and VEGF were quantified using ELISA kits (Ref: KAC1688 and Ref: KHG0111; Invitrogen™, Thermo Fisher Scientific) according to manufacturer's instructions. Absorbance was determined using a microplate reader (Multiskan GO, Thermo Fisher Scientific).

2.5. Bone Marrow-Derived Mesenchymal Stromal Cell (BM-MSC) Isolation and Culture. 120 mL of bone marrow was obtained after donation with informed consent from two donors (a 41-year-old woman, whose cells were used for cell viability assays, and a 60-year-old man, whose cells were used for gene expression analysis). Mononuclear cells were separated using the Sepax system (Biosafe, Eysins, Switzerland), according to manufacturer's instructions, and plated at 4×10^5 cells/cm^2 in Minimum Essential Medium Eagle Alpha Modification (alpha MEM) (Cultilab, Campinas, SP, Brazil) supplemented with 10% fetal bovine serum (FBS) (Gibco) in T 150 cm^2 culture flasks (Corning Incorporated, Corning, NY) and maintained in a 5% CO_2 incubator at 37°C. After 5 days, medium was changed and nonadherent cells were discharged. Medium was changed every two days. This was termed as "primary culture." After approximately 10 days, 70–80% confluence, cells were detached from culture flasks using 0.05% trypsin solution (Gibco®, Thermo Fisher Scientific) and replated onto new culture flasks at a density of 8×10^3 cells/cm^2. After first trypsinization, culture was termed as at "passage #1." Experiments were performed until "passage #5."

2.6. Cell Viability Assay. The analysis of cell viability was performed by incorporation with thiazolyl blue tetrazolium bromide (MTT assay) (Sigma Aldrich, São Paulo, SP, Brazil). Cells (passage #3) were plated at a density of 5×10^3 cells/cm^2 in duplicate in 48-well plates (Corning Incorporated) in alpha MEM (Cultilab) supplemented with 10% FBS (Gibco), 1% PRPr, 2.5% PRPr, or 5% PRPr. Four different PRPr donors were used. In another group, the four samples were pooled with equal proportions of each donor, namely, PRPr MIX.

After 8 days of culture, 0.5 mg/mL MTT was added. Medium was removed after 4 hours of incubation, and 400 μL/well of DMSO was added to dissolve the reduced formazan product. The volume in each of the 48 wells was split into two wells in a 96-well plate (Corning Incorporated). Finally, the plate was read in a microplate reader (Multiskan GO, Thermo Fisher Scientific) at 570 nm. Cell culture medium was not changed during this experiment.

2.7. Gene Expression Evaluation. Cells (passage #5) were cultured in alpha MEM (Cultilab) supplemented with 10% FBS (Gibco), 1% PRPr, 2.5% PRPr, or 5% PRPr. The PRPr was used as a pool of four different donors. After five days of culture, total RNA was extracted using TRIzol® (Ambion®, Thermo Fisher Scientific). RNA concentration was determined using a Nanodrop 2000 UV-Vis spectrophotometer (Thermo Fisher Scientific) and 2 μg was reverse-transcripted into complementary DNA (cDNA) using SuperScript® First-Strand Synthesis System for RT-PCR (Invitrogen, #11904-018) in a total reaction volume of 20 μL, following manufacturer's protocol. Oligonucleotides and probes for qPCR were purchased from Applied Biosystems (TaqMan Gene Expression Assay, #4331182): HPRT1 (Hs02800695_m1), which was analyzed as the housekeeping gene, SOX9 (Hs00165814_m1), RUNX2 (Hs00231692_m1), PPARG (Hs01115513_m1), and POU5F1 (Oct-4) (Hs0099634_9H). qPCR reactions were performed in an Applied Biosystems 7500 Standard Time PCR System in a 20 μL reaction volume using TaqMan® Universal Master Mix II, with UNG (Applied Biosystems, #4440038), according to manufacturer's instructions. Analysis was performed using the $\Delta\Delta$Ct method [40].

2.8. Statistical Analysis. Data were analyzed using a two-tailed paired t-test for the hematological analysis, where a group of the same donors were analyzed with different anticoagulants. In the case of growth factor quantification and cell culture experiments, where pairing of samples did not necessarily occur, a two-tailed unpaired t-test was performed. Statistical significance was considered when $p < 0.05$.

3. Results

3.1. Effect of Different Anticoagulants on Initial Platelet Counting and Recovery. Blood samples were collected from five different donors in tubes containing EDTA, SC, or ACD, and platelets were counted in an automated system. Blood samples collected with EDTA yielded higher numbers of platelets, followed by SC and ACD (Figure 1(a)). In average, platelet counting in SC was 16.28% lower than that in EDTA, while that in ACD was 23.01% lower than in EDTA and 7.94% lower than in SC. However, platelet recovery, regarding the total number of platelets obtained after the first centrifugation step, was higher in the presence of SC compared to EDTA and ACD. The average of platelet recovery in EDTA and SC was 76.15% and 81.21%, respectively. Strikingly, platelet recovery in samples collected with ACD was 45.71%, almost half of those when using EDTA or SC. All three anticoagulants tested herein were purchased in commercially distributed tubes. ACD containing tube was bigger—taller and larger—compared to EDTA and SC.

In order to verify if the lower platelet recovery was related to the tube format, PRP was obtained from blood samples anticoagulated in ACD using tubes of similar size compared to EDTA and SC (ACD-2). Platelet recovery improved (49.82%) but remained much lower than those recovered when using EDTA (76.15%) and SC (81.21%). Values from ACD-2 were statistically different from those obtained using SC ($p < 0.05$) but not when compared to EDTA ($p > 0.05$) (Figure 1(b)). If analyzed separately, it was possible to observe that platelet recovery has increased in three of the five donors when using ACD-2 instead of ACD, especially in donor 2, with an increase of 63.74%, while it has decreased in two donors, especially in donor 1, with a decrease of 25.48% after the distribution of blood into the smaller tubes (Figure 1(c)). In average, platelet concentration in PRP2 was $1{,}009 \pm 57 \times 10^3/\mu$L in EDTA samples, $582 \pm 108 \times 10^3/\mu$L in SC samples, $726 \pm 200 \times 10^3/\mu$L in ACD samples, and $664 \pm 170 \times 10^3/\mu$L in ACD-2 samples. All values were statistically similar between each other ($p > 0.05$), except between EDTA and SC ($p < 0.05$) (data not shown).

Although the mean platelet volume (MPV), which is related to platelet size and indicates its degree of activation, was similar when whole blood (WB) samples were anticoagulated in all three anticoagulants tested, it increased progressively following the two centrifugation steps in EDTA group in all donors (in average an increase of 11.60% after the first centrifugation step and an additional increase of 2.84% after the second centrifugation step, totaling 14.44% increase compared to whole blood). This was not observed when WB was anticoagulated in SC and ACD (Figure 2).

3.2. TGF-β1 and VEGF Release from Platelet-Rich Plasma in Different Anticoagulants. Up to this point, it was clear that the anticoagulant has an impact on platelet recovery after blood centrifugation. However, we questioned if it would also change growth factors release from recovered platelets. For that, we quantified TGF-β1 and VEGF levels in an ELISA assay. Growth factors concentrations were similar between anticoagulant groups ($p > 0.05$) for both TGF-β1 and VEGF. TGF-β1 concentration was 18,146.99 ± 2,370.33 pg/mL in EDTA; 48,559.10 ± 12,839.86 pg/mL in SC; and 30,786.15 ± 6,654.49 pg/mL in ACD (Figure 3(a)). VEGF concentration was 278.88 ± 71.78 pg/mL in EDTA, 143.65 ± 71.63 pg/mL in SC, and 362.70 ± 77.95 pg/mL in ACD (Figure 3(b)).

3.3. Bone Marrow-Derived Mesenchymal Stromal Cell Culture. In order to show the effects of factors released from platelets obtained using different anticoagulants on modulating cell expansion in vitro, we used the MTT cell viability assay to analyze BM-MSC proliferation in the presence of different concentrations of PRPr. FBS-supplemented culture medium was used as reference (Figure 4). PRPrs were tested separately and mixed (MIX). All concentrations of PRPr tested from all donors were able to stimulate cell proliferation in vitro. As expected, the higher concentration tested (5%) stimulated the higher proliferative rate in vitro, regardless of the anticoagulant used. However, for this concentration, in average,

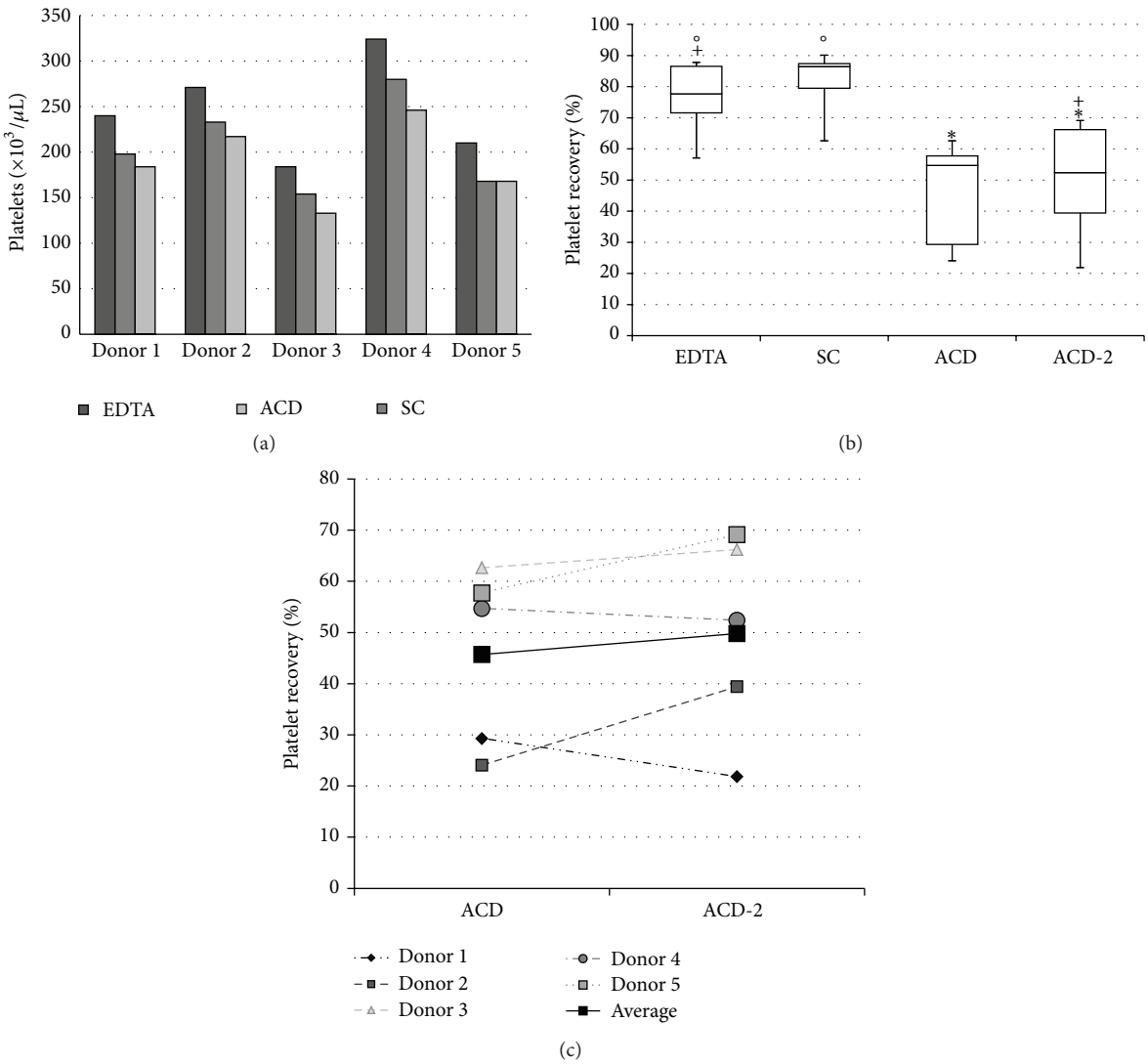

Figure 1: Platelet yield and recovery in blood collected with different anticoagulants. Blood was collected in EDTA, SC, and ACD in five different donors and platelet concentration was quantified (a) as well as platelet recovery after the first centrifugation step (b). An individual analysis between ACD and ACD-2 of platelet recovery was also performed (c). Data are expressed as bar (a), box (b), and dot (c) plots. Similar symbols in (b) correspond to statistic similarity among groups ($p > 0.05$).

cell proliferation was lower in the presence of EDTA derived PRPr compared to SC and ACD (Figure 4). In addition, cells maintained their fibroblast-like morphology regardless of the anticoagulant type (Figure 5).

3.4. Modulation of Bone Marrow-Derived Mesenchymal Stromal Cell Gene Expression by Platelet-Rich Plasma Culture. We also analyzed gene expression of cells expanded in vitro (Figure 6). Only cells cultured in 5% PRPr were tested. Using 10% FBS as reference, RUNX2 was slightly upregulated in EDTA group and downregulated in SC group. PPARγ2 was slightly upregulated in EDTA group and downregulated in SC and ACD groups. SOX9 was downregulated in all groups. Oct-4 was upregulated in EDTA and ACD groups and downregulated in SC group. Although, in general, the gene expression was similar between the PRPr groups, especially when observing the maximum and minimum relative quantification of gene expression, the SC group presented the smallest variation compared to the control group, by analyzing the average relative expression of the four genes in the PRPr groups compared to the FBS group. In average, SC relative gene expression was 24.73% different from the control group, while EDTA was 46.79% and ACD was 29.74% different.

4. Discussion

Platelet-rich plasma (PRP) is currently one of the main strategies to promote musculoskeletal tissues repair. There are several reports in the literature evidencing its potential in clinical trials [2–11] as well as in vitro analysis [12–15]. As a cost-effective source of autologous growth factors that can affect stem cells proliferation and differentiation, it is being

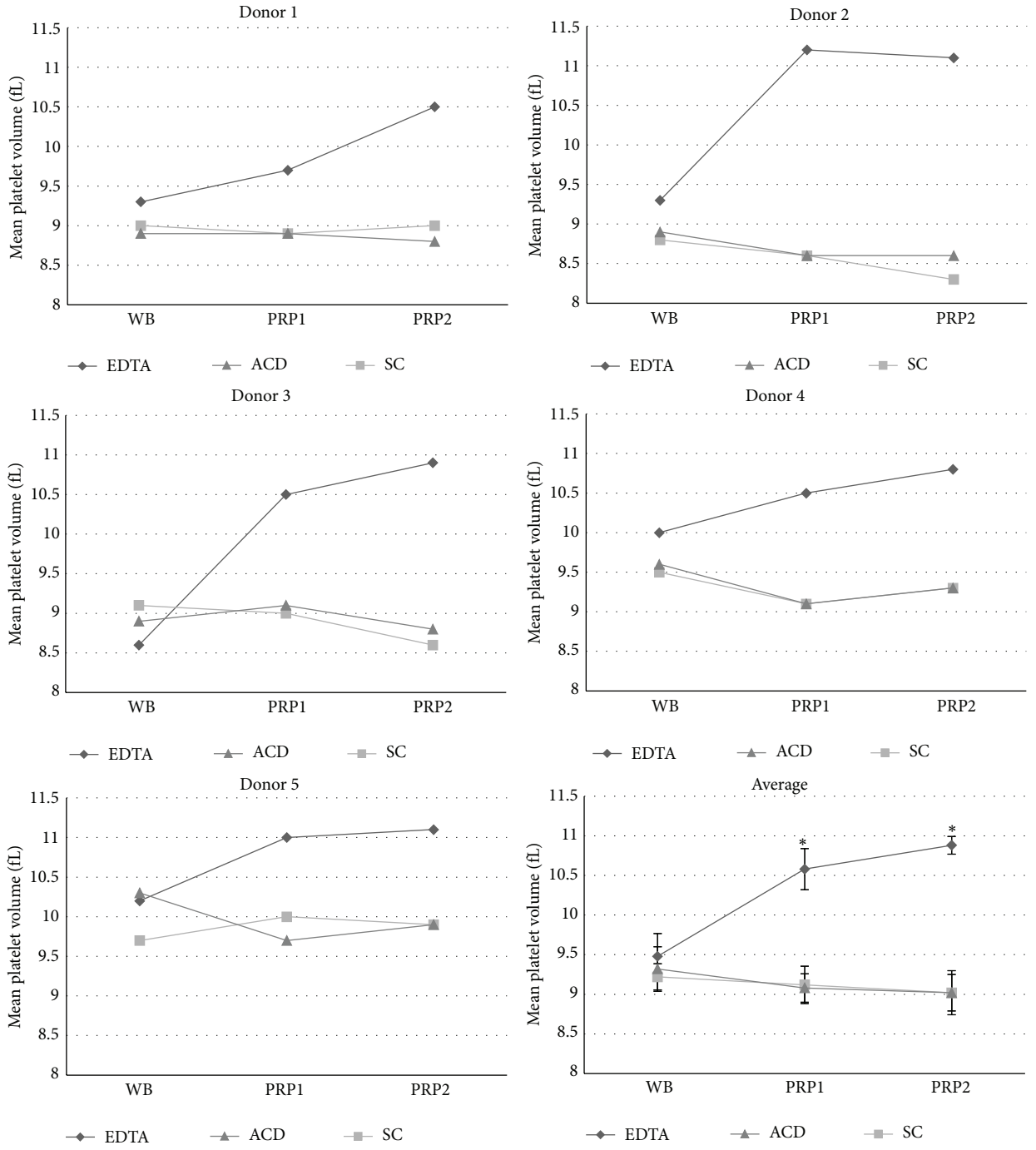

Figure 2: Mean platelet value quantification of samples containing different anticoagulants. Mean platelet value was quantified in five different donors in whole blood (WB), PRP1, and PRP2, in tubes containing EDTA, SC, or ACD solution. The average values of the five different donors are also represented in the figure. "$*$" corresponds to statistical difference between EDTA and SC groups as well as EDTA and ACD groups ($p < 0.05$).

increasingly investigated as a supplement, adjuvant, carrier, or scaffold for stem cells-based therapeutics [41–45]. However, the lack of standardization between the methodology to obtain and use PRP among different groups may hamper the development of this technology [20]. The use of anticoagulant to collect blood is a major issue. The present work aimed to verify PRP obtaining with three types of commercially available blood collection tubes containing EDTA, SC, or ACD as anticoagulants.

Platelet counting was higher in blood collected in tubes containing EDTA, followed by SC and ACD. Indeed, it has been previously shown that platelet count in EDTA can be

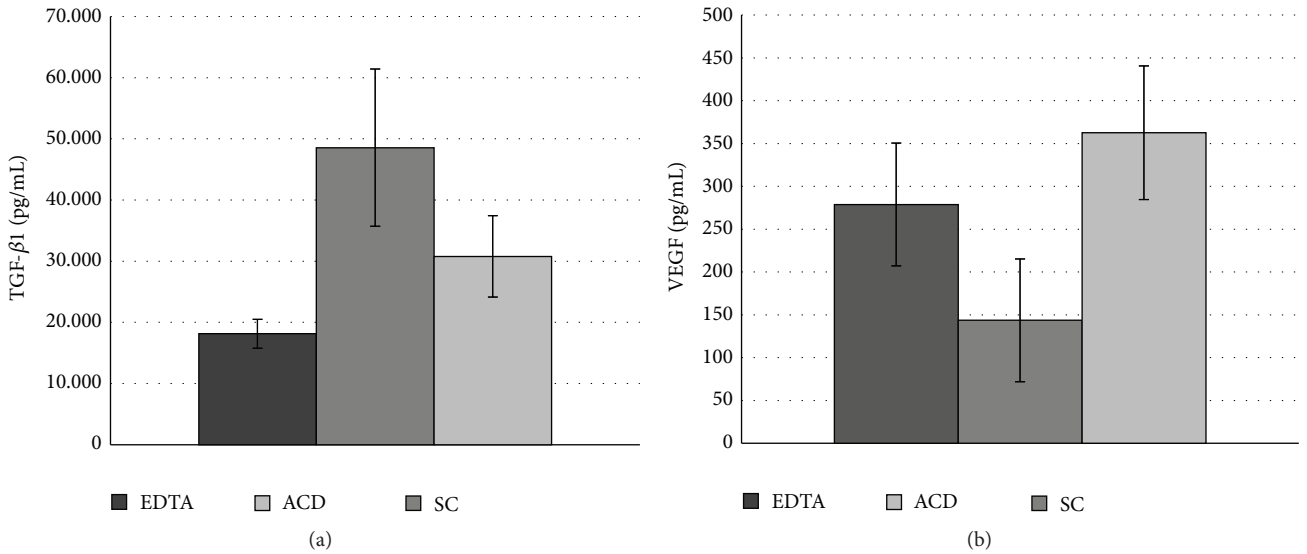

Figure 3: Growth factor quantification in PRPr obtained in different anticoagulant. TGF-β1 (a) and VEGF quantification (b). Data are expressed as mean, and error bars correspond to standard error.

higher than in citrate anticoagulants [46]. Moreover, when EDTA is added to citrated samples, it can enhance platelet count in whole blood [47]. Platelet recovery after the first centrifugation step was diminished in ACD tubes compared to EDTA and SC. Additionally, a higher concentration of PDGF-BB was found in PRP obtained with EDTA compared to ACD [48]. In our case, we tried to enhance platelet recovery in ACD tubes by dividing its content into smaller tubes (12 × 75 mm × 5 mL) with no additional anticoagulant. Although no statistical difference has been detected between those two ACD forms, the splitting of blood in the smaller tubes resulted in a similar platelet recovery compared to EDTA group. Since ACD tube is bigger (16 × 100 mm × 8.5 mL) than EDTA (13 × 75 mm × 4.0 mL) and SC tubes (13 × 75 mm × 4.5 mL), it is possible that the lower platelet recovery is due not only to the type of anticoagulant itself but also to the tube format. Particularly, the tube format may have superior influence on whole blood/plasma than serum centrifugation, in view of its higher viscosity [49]. For the following experiments, we decided to mix ACD and ACD-2 PRP1 from the same donors, since it is comprised of the same type of anticoagulant and since the following centrifugation to prepare PRP2 was performed in the same type of tube for all groups analyzed.

In order to verify if the centrifugation steps influenced platelets morphology, we quantified the mean platelet volume (MPV) in whole blood, PRP1, and PRP2 obtained with different anticoagulants. The MPV in the EDTA group, but not in SC and ACD groups, has increased after the centrifugation steps, which may be an indicator of platelet activation [50, 51]. Indeed, a higher MPV is expected in whole blood collected in EDTA compared to citrated samples [46]. In addition, EDTA may change platelet morphology from a discoid to an irregular spherical shape [52]. Indeed, the use of EDTA faces ethical issues, such as being pointed to as a persistent pollutant in natural environments [53], and impediments of use in certain countries [54]. ACD and citrate-theophylline-adenosine-dipyridamole (CTAD) are also more efficient in maintaining platelet morphology than heparin and SC. In that case, it has also been shown that PRP obtained with ACD and CTAD resulted in higher TGFβ-1 concentration and induction of MSC proliferation [55]. In another previous study, EDTA, SC, and ACD were compared as maintainers of platelet responsiveness to aggregation inducers. ACD was the most capable to maintain intraplatelet signal transduction mechanisms during PRP formulation [56]. The same group, lately, showed that ACD was also capable of maintaining platelet functions for periods of time superior to SC [57]. In our case, we could not find any difference in the capability of platelet activation and clot formation in PRP2 obtained with the three different anticoagulants.

Our next step was to evaluate the effect of PRP obtaining in cell culture. For that, we normalized the platelet concentration to ideally $1000 \times 10^3/\mu$L in all groups, in a way that the results would not correlate to platelet concentration but to the type of the anticoagulant. This platelet concentration in PRP is being pointed to as a therapeutic concentration for in vivo purposes [1]. In bone, for example, lower concentrations are unsatisfactory and higher concentrations are inhibitory to promote tissue repair [11]. The only statistical difference observed in platelet concentration in PRP2 was between EDTA and SC group. The lower value in SC group may be attributed to a difficulty to resuspend platelet pellet. Platelet concentrate bags prepared with citrate samples, in form of ACD, contain more aggregates than bags prepared with EDTA [52]. Moreover, the effects of PRPr on cell culture were always compared to 10% FBS medium supplementation. In spite of being a xenogeneic serum with ethical and scientific issues [58], FBS is still widely used in cell culture [59] and MSC in vitro expansion [60].

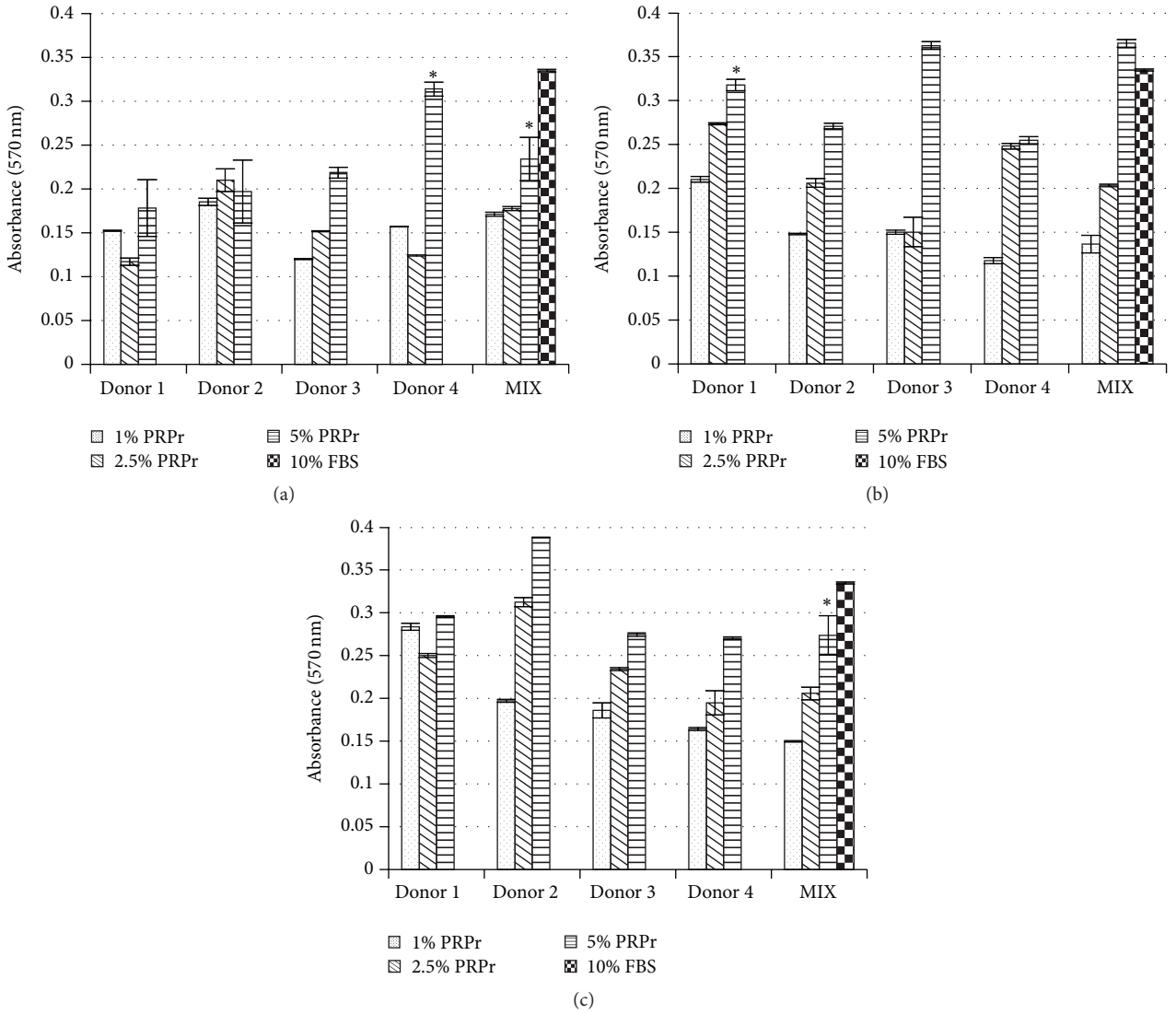

Figure 4: BM-MSC viability in PRPr obtained with different anticoagulant. Absorbance at 570nm was measured after MTT viability assay of cells cultivated in different PRPr concentrations, obtained with EDTA (a), SC (b), and ACD (c), as well as 10% FBS (control). Data are expressed as mean, and error bars correspond to standard error. "$*$" corresponds to statistical similarity with 10% FBS ($p > 0.05$).

In our study, we could not find differences in TGFβ-1 and VEGF concentration among the anticoagulants. It could be possibly due to the similar platelet concentration between groups. In average, TGF-β1 concentration varied from 18.15 ng/mL in EDTA to 48.56 ng/mL in SC. Other literature reports present concentrations superior to our finding: 120 ng/mL [61], 169 ng/mL [62], and 89 ng/mL in a PRP with platelet concentration 4.69 times superior to whole blood baseline but 20 ng/mL in a PRP with platelet concentration 1.99 times superior to whole blood baseline [63]. VEGF concentration ranged from 143.65 pg/mL in SC to 362.70 pg/mL in ACD. Other reports present concentrations varying from 50 pg/mL [64] to 155 ng/mL [61]. Nevertheless, our results were superior to some commercially available kits [65].

As expected, PRPr induced cell proliferation. Although there were variations comparing the influence on cell proliferation between donors, possibly due to an inherent difference on growth factors and other molecules content among each platelet granule, an evident pattern emerged: the concentration of 5% PRPr in cell culture medium was sufficient to induce cell proliferation in a similar level to 10% FBS. Additionally, SC and ACD-derived PRPr presented greater effects over cell proliferation compared to EDTA group. Cell morphology was not changed among groups. BM-MSC maintained their fibroblastic morphology regardless of the anticoagulant. Although there are still controversies in the literature regarding PRP effects on BM-MSC differentiation, there is a consensus on its effect as an inducer of proliferation [66].

As a final analysis of PRPr effects on BM-MSC, we observed slight modulations in the expression of the master genes for the osteogenic (RUNX2), adipogenic (PPARγ2), and chondrogenic (SOX9) lineages [67], as well as Oct-4, a gene related to maintenance of stemness [68, 69]. Particularly, SOX9 expression was downregulated. Indeed, there is a

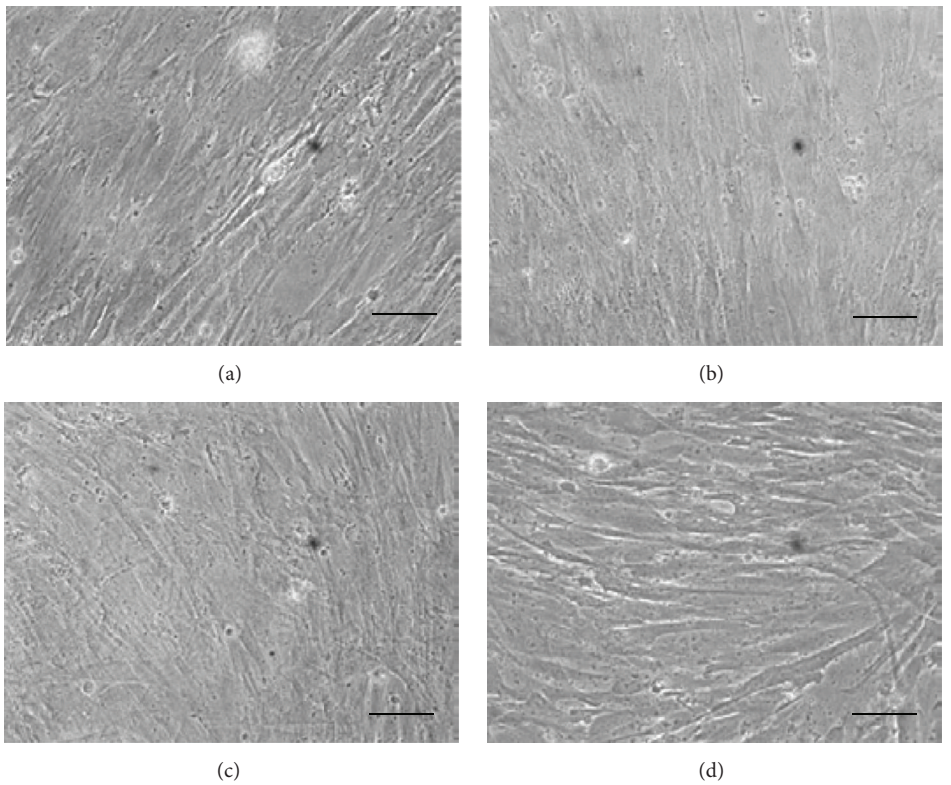

Figure 5: Photomicrography of BM-MSC. Cells cultivated for eight days in medium supplemented with 10% FBS (a) or a pool from four donors of 5% PRPr obtained from collection tubes containing EDTA (b), SC (c), or ACD (d). Phase contrast, 200x magnification, and scale bars: 50 μm.

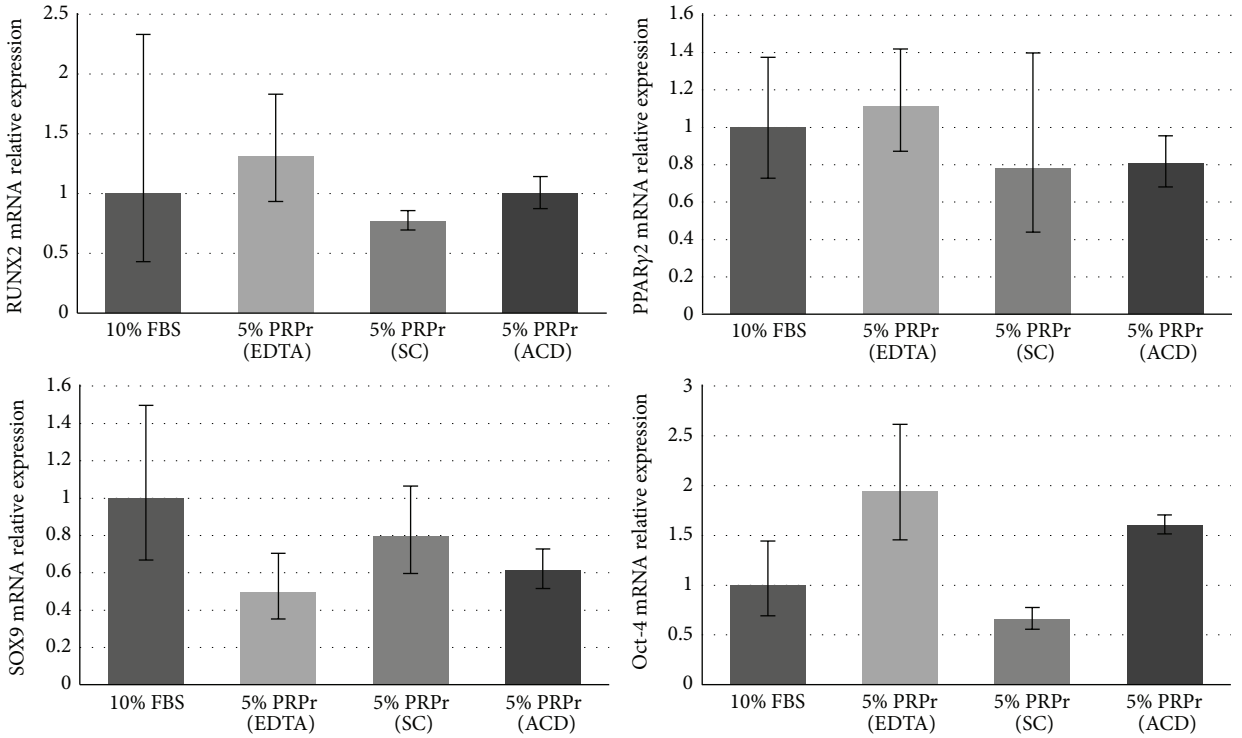

Figure 6: Relative gene expression. RUNX2, PPARγ2, SOX9, and Oct-4 gene expression in cells cultured in medium supplemented with 10% FBS (control group) or 5% PRPr obtained with EDTA, SC, or ACD. Data are expressed as relative quantification of gene expression (RQ). Upper and lower error bars correspond to RQ maximum and RQ minimum, respectively.

discussion in the literature regarding the PRP effects on chondrogenesis, with some groups claiming its inducing effects [70, 71] and others its inhibitory effects [72, 73]. Recently, our group has showed that it can be dependent on the concentration of PRPr used in cell medium, with lower concentrations inducing chondrogenesis and higher concentrations inhibiting it [74]. Oct-4 expression presented an intense variability, with upregulation in EDTA and ACD groups and downregulation in SC group. Whether this can be an indicator of the maintenance of cells stemness or not, it must be further investigated. In general, gene expression was similar between PRP groups, although SC was the group that presented the smaller variation compared to 10% FBS culture, evidencing that cells presented the lightest changes in their phenotype with this treatment.

5. Conclusion

The literature provides a variety of methodologies to obtain PRP. The first variation is the methodology to collect blood and the anticoagulant used. In this paper, we analyzed the effects of three different anticoagulants, obtained in commercially available tubes, on PRP obtaining. Although no significant change in the amount of growth factors released was observed, some features could be highlighted. The blood collection in tubes containing EDTA resulted in higher platelet yield in the whole blood. However, this was accompanied by an increase of MPV following the centrifugation steps, which is an indicator of change in platelet morphology. On the other hand, the use of tubes containing citrate solutions resulted in a greater induction of MSC proliferation. Particularly, the obtaining in SC resulted in the higher platelet recovery after the first centrifugation step. If ACD is used, the reduction of the tube size may increase platelet recovery. In addition, the PRP obtained in SC presented the smallest variation in MSC gene expression compared to cells cultured in the presence of 10% FBS. Therefore, in order to obtain a bigger amount of platelets and induce MSC proliferation without dramatically interfering with their phenotype, we suggest the use of SC as anticoagulant for PRP acquisition.

Competing Interests

The authors declare no competing interests.

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Research Article

Fabrication of Core-Shell PEI/pBMP2-PLGA Electrospun Scaffold for Gene Delivery to Periodontal Ligament Stem Cells

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Bone tissue engineering is the most promising technology for enhancing bone regeneration. Scaffolds loaded with osteogenic factors improve the therapeutic effect. In this study, the bioactive PEI (polyethylenimine)/pBMP2- (bone morphogenetic protein-2 plasmid-) PLGA (poly(D, L-lactic-co-glycolic acid)) core-shell scaffolds were prepared using coaxial electrospinning for a controlled gene delivery to hPDLSCs (human periodontal ligament stem cells). The pBMP2 was encapsulated in the PEI phase as a core and PLGA was employed to control pBMP2 release as a shell. First, the scaffold characterization and mechanical properties were evaluated. Then the gene release behavior was analyzed. Our results showed that pBMP2 was released at high levels in the first few days, with a continuous release behavior in the next 28 days. At the same time, PEI/pBMP2 showed high transfection efficiency. Moreover, the core-shell electrospun scaffold showed BMP2 expression for a much longer time (more than 28 days) compared with the single axial electrospun scaffold, as evaluated by qRT-PCR and western blot after culturing with hPDLSCs. These results suggested that the core-shell PEI/pBMP2-PLGA scaffold fabricated by coaxial electrospinning had a good gene release behavior and showed a prolonged expression time with a high transfection efficiency.

1. Introduction

Periodontal disease involves the deterioration of periodontal bone and is the primary cause of tooth loss in adults. At present, periodontal disease treatments focus on promoting periodontal tissue regeneration by controlling inflammation and formatting new attachment [1–3]. While periodontal treatments such as scaling, surgical cleaning, bone grafts, and guided tissue regeneration are generally successful, the ability to regenerate the damaged tissues predictably still remains a major unmet objective for conventional treatment strategies [4, 5]. Therefore, Langer in 1993 proposed tissue engineering as a possible technique for periodontal tissue regeneration [6, 7]. Tissue engineering mimics the natural healing process to reconstruct or regenerate damaged tissue using three fundamental tools: progenitor cells, signaling molecules, and scaffolds.

Mesenchymal stem cells (MSCs), as multipotent progenitor cells, can be isolated from adult bone marrow or prenatal tissues. As one of the MSCs from dental origin, periodontal ligament stem cells (PDLSCs) have been isolated and tested for their ability to develop into various types of tissues in *in vitro* and *in vivo* studies [8]. Due to this multipotent differentiation ability, PDLSCs can be used in regenerative medicine because they provide a source of cells not only for dental tissue regeneration but also for repair of nondental structures such as bone and nerves [9]. Human PDLSCs (hPDLSCs) have proven stem cell characteristics, including osteogenic-differentiating and immune-modulating properties [10]. More importantly, hPDLSCs can be harvested from medical waste materials such as discarded extracted teeth without additional surgery that may cause patients to experience physical deformity, pain, and considerable expense. Therefore, hPDLSCs are considered a promising cell type for

cell-based gene delivery and periodontal tissue engineering applications [11].

Bone regeneration is regulated by various bioactive agents such as platelet derived growth factor (PDGF), transforming growth factor-beta (TGF-β), bone morphogenetic proteins (BMPs), and insulin-like growth factor (IGF), which are important factors involved in the osteogenic process. Among the many BMPs, BMP2, a member of the highly conserved transforming growth factor-β superfamily, is a potent inducer of osteogenesis and plays an important role in bone formation and remodeling [12–15]. Although successful bone regeneration was induced by BMP2 in preclinical and clinical application, supraphysiological dosage of BMP2 was required, which may cause low efficiency and potentially toxic effect. Thus, a new modality for BMP2 treatment is required to overcome these shortcomings. Gene delivery using various vectors has been suggested as a novel approach, in which target gene materials are injected into and around the wound to infect the host cells, enabling them to produce the therapeutic proteins. This strategy can avoid the disadvantages caused by the direct application of exogenous growth factors such as BMP2 [16]. Genetically modified carriers are mainly divided into viral and nonviral vectors. The drawback of the viral vectors is that they cause a strong body immune response; they are infectious and potentially carcinogenic. In addition, virus carrier preparation is difficult and expensive, and the carrier capacity is small. Despite lower price and virus-free carrier, the traditional nonviral vector, such as liposomes, chitosan, and calcium phosphate, produces less antigenicity, lower transduction efficiency, and shorter expression duration. Thus, it is difficult to obtain a significant gene expression with traditional nonviral vector systems. Compared to other nonviral systems and especially viral vectors, cationic nonviral delivery systems have several advantages such as low toxicity and antigenicity due to only biological lipids composition and long-term expression with less risk of insertional oncogenesis. Polyethylenimine (PEI) is a cationic polymer that can compact the long DNA chains into submicron particles [17–19]. PEI is a potent nonviral transfection agent, which has high transfection activity *in vitro* and moderate activity *in vivo*.

Gene activated matrix (GAM), which combines gene delivery with scaffold has been commonly discussed as a powerful strategy that can provide opportunities to more effectively apply gene delivery for tissue engineering [20, 21]. However, GAM fabrication techniques such as freeze-drying emulsion, solution casting method, leaching, gas foaming method, and thermally induced phase separation method have the limitation of reducing plasmid activity by organic solvent; thus, the gene release behavior is uncontrollable and transfection efficiency is not ideal [8–10]. Electrospinning is a simple method of preparing nanofibers. By altering polymer solution composition and parameters, the mechanical, biological, and kinetic properties of the electrospun nanofibrous scaffold can be easily controlled. Electrospun nanofibrous membrane has reticular structure with high specific surface area, high porosity, and interconnect pores, which is similar to the natural extracellular matrix (ECM) and can enhance the adhesion, proliferation, and growth of cells [22]. Preparation of GAM by common single electrospinning method consists of mixing gradable polymer material with plasmid vector. In this way, the plasmid is inevitably exposed to organic solvents during the electrospinning process, which will reduce its activity. In addition, the sudden release of plasmid from this kind of nanofibers may lead to a short valid time in the initial period. Coaxial electrospinning is an innovative extension of electrospinning, which uses two concentrically aligned capillaries to enforce the formation of the fibers with a core-shell structure, where multiple biomolecules at each layer can be designed to diffuse out sequentially. Differences in the diffusion pathways of gene vectors through two layers composed of different materials can alter the release rates of the incorporated gene vectors in each layer, which have already been observed in many drug delivery studies using core-sheath structures [23–25].

This study aimed to fabricate a core-shell PEI/BMP2 plasmid (pBMP2)-poly(D, L-lactic-co-glycolic acid) (PLGA) electrospun scaffold for gene delivery as GAM by coaxial electrospinning in order to protect pBMP2 from direct exposure to organic solvents and control its release. The characterization and mechanical properties of the GAM were evaluated, and the effects of this nanofibers scaffold loaded with pBMP2 on cell viability and BMP2 expression in hPDLSCs were investigated.

2. Materials and Methods

2.1. Materials. Poly(D, L-lactic-co-glycolic acid) (PLGA, MW 100,000) with a 75 : 25 monomer ratio (lactic acids : glycolic acids) was provided by Daigang Company (Shandong, China). PEI and 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) were obtained from Aladdin Industrial Corporation (Shanghai, China). Phosphate-buffered saline (PBS) pH 7.4 containing 0.1 M sodium phosphate and 0.15 M sodium chloride, used for *in vitro* release study, was purchased from Sigma-Aldrich (St. Louis, MO, USA). The plasmid containing human BMP2 and enhanced green fluorescent protein gene (GFP) was generously donated by Cell Engineering Research Center, Fourth Military Medical University (Xi'an, China). Plasmid extraction kit (QIAGEN Plasmid Maxi Kit) was purchased from QIAGEN Company (Germany).

2.2. Electrospinning. PLGA was dissolved in HFIP at 12% wt and stirred overnight at room temperature to prepare the sheath solution of nanofibers. The pBMP2 was amplified in the *Escherichia coli* strain DH5α and then purified by QIAGEN Plasmid Maxi Kit. Next, the PEI/pBMP2 nanoparticles were created as a result of static attraction between PEI and pBMP2 to form the core solution of core-shell fibers. The size of pBMP2 encapsulated particle was mainly evaluated by N/P ratio [26]. PEI/pBMP2 nanoparticles prepared with an N/P ratio of 10 tended to have higher thermal dynamics ability. pBMP2 solution was prepared at the concentration of 500 μg/mL in 0.1x TE buffer (pH 7.3). Then 2 mL of the pBMP2 aqueous solution and 750 μg of PEI solution (99% purity) were quickly mixed together and stirred for 5 min.

The electrospinning equipment (SH2535, UCALERY Co., Beijing, China) was used to prepare the electrospun core-shell scaffolds. The setting to prepare the core-shell scaffolds consisted of two coaxial syringe pumps with different flow rates. In this study, the PLGA solution was used to form the outer shell and the PEI/pBMP2 solution was used to form the inner core. These two solutions were connected to the coaxial needles, a 16 G (ID = 1.6 mm) outer needle and a 25 G (ID = 0.5 mm) inner needle, respectively, and then concentrically placed. The electrospinning followed the same conditions (applied voltage: 25 kV; 2 mL/h for the sheath flow rate and 0.6 mL/h for the core flow rate; air gap, 15 cm). The electrospinning process lasted for 5 h. Under the same condition, PEI/pBMP2-loaded PLGA blended nanofibers and pure PLGA nanofibers were also fabricated by single axial electrospinning as single axial scaffolds group and PLGA scaffolds group, respectively. The prepared membranes were vacuum-dried for 24 h and stored in a refrigerator at 4°C.

2.3. Scaffold Characterization

2.3.1. Morphology Characterization. The surface morphology of the electrospun membrane was observed using HITACHI S-4800 SEM. The samples were mounted on metal stubs and sputter coated with palladium-platinum-gold for 60 s. Images were taken at an accelerating voltage of 5 kV. The fibers diameters and pore sizes were measured using Image-Pro Plus software ($n = 100$).

The samples were placed on the copper meshes directly obtained during coaxial electrospinning process and observed using a JEM-3010 TEM (JEOL Co., Japan), with an accelerating voltage of 300 kV. Bright field images were collected with an 11-megapixel SC1000 Orius CCD camera (Gatan, Inc.). Image analysis was performed using Digital Micrograph (Gatan, Inc.).

2.3.2. Mechanical Properties. The tensile properties of the electrospun membrane were carried out using a universal tensile tester (EZ-SX, Shimadzu, Japan). Different scaffolds with varying compositions and thicknesses were sectioned into 1 cm × 6 cm sections (dog-bone shaped) and tested at a speed of 1 mm/min.

2.3.3. In Vitro Release Behavior. The two groups of electrospun scaffolds containing PEI/pBMP2 (the core-shell scaffolds and the single axial scaffolds) were cut into round sections at a diameter of 10 mm, sterilized with 70% ethanol, and washed with PBS, and each section was incubated with 2 mL PBS in Eppendorf tubes placed on a constant-temperature shaker (CHA-S, Guohua, China). No PEI/pBMP2 nanoparticles were present in the pure PLGA scaffolds; thus, we did not test the PLGA release behavior. At scheduled time interval, eluents of each group were obtained and stored at 4°C until the end of the release assay. The amount of released plasmid BMP2 was quantified using the PicoGreen Assay. Solutions were excited at 485 nm wavelength, and emission was measured at 530 nm in a microplate reader (Tecan Infinite 200 PRO, Switzerland). Optical density (OD) value of each sample was recorded. DNA concentration was calculated using the standard curve.

2.4. In Vitro Study

2.4.1. Cell Culture. Primary hPDLSCs used in this study were generously donated by Cell Engineering Research Center of Fourth Military Medical University (Xi'an, China) and cultured in MEM alpha medium (Hyclone, USA) supplemented with 10% fetal bovine serum (Hyclone, USA) and 1% penicillin/streptomycin. The hPDLSCs were maintained in a humidified incubator at 37°C and 5% CO_2 and used in the following experiments at passage three or four.

2.4.2. Cell Viability. To evaluate cell viability, three groups of scaffolds were analyzed by MTT assay (Sigma-Aldrich). Cells were seeded into 24-well plates at an initial density of 5×10^4/well with 1 mL growth medium and cultured with the three groups of scaffolds. One cell group was cultured without any scaffold as a blank control. After 1, 3, 5, and 7 days of culture, 100 μL of MTT solution was added to each well and incubated for 4 h. After removing the medium containing the MTT, 1 mL dimethyl sulfoxide (DMSO) (Sigma-Aldrich) was added to each well to extract the formazan crystals under gentle shaking. The absorbance intensities were measured at 595 nm using a microplate reader.

2.4.3. Transfection Efficiency. No PEI/pBMP2 nanoparticles were present in the PLGA scaffolds; thus, we did not test the PLGA transfection efficiency. The control group was designated as an equivalent amount of pBMP2. Gene transfection efficiency was evaluated by GFP expression. The two groups of scaffolds containing PEI/pBMP2 were cut into round sections at a diameter of 10 mm, sterilized with 70% ethanol, washed with PBS, and placed into 6-well plates containing 2 mL PBS. The PBS was replaced by an equal amount of α-MEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 0 d, 2 d, 4 d, 6 d, 13 d, 20 d, and 27 d, and hPDLSCs were seeded in these 6-well plates at the concentration of 3×10^5 cells per well when the PBS was replaced. After 24 h, GFP expression within cells was observed using confocal microscopy (FV1000, OLYMPUS, Japan) and the final transfection efficiency was determined by flow cytometry (FCM) (BD FACS Aria Flow Cytometer, Beckman, USA). A number of 10,000 cells were counted for each sample.

2.4.4. Osteogenic Activity. In order to study the osteogenic activity, BMP2 expression ability was evaluated only in the two PEI/pBMP2-loaded scaffolds groups. The two groups of scaffolds were cut into round sections at a diameter of 10 mm, sterilized with 70% ethanol, washed with PBS, and placed into the wells of 6-well plates. hPDLSCs were seeded in these 6-well plates at a concentration of 3×10^5 cells per well and cultured using the same method as described above. The plated cells were collected at 3 d, 7 d, 14 d, 21 d, and 28 d. The controls were designated as 0 days. The expression of mRNAs was quantitatively detected using DyNamo SYBR Green qPCR

Table 1: Sense and antisense primers' sequences used to amplify gene transcripts by RT-PCR.

Gene	Sense sequence	Antisense sequence
Cbfa1/Runx2	ACA ACC ACA GAA CCA CAA G	TCT CGG TGG CTG GTA GTG A
BMP2	TAT GCT CGA CCT GTA CCG C	CAC TTC CAC CAC AAA CCC

Table 2: Mechanical properties of the three groups of electrospun scaffolds (mean ± standard deviation).

	Core-shell scaffold	Single axial scaffold	PLGA scaffolds
Ultimate strength (MPa)	3.51 ± 0.5	2.57 ± 0.31	4.60 ± 0.46
Elastic modulus (MPa)	11.34 ± 3.22	10.23 ± 4.08	15.57 ± 0.96
Ultimate strain (%)	29.87 ± 3.98	26.45 ± 2.53	35.46 ± 3.92

kit (Finnzymes, Espoo, Finland). Experiments were repeated three times for each sample. The primer's sequences of the targeted genes are listed in Table 1. Runx2 and BMP2 protein expression were assayed by western blotting. Calcium (Ca) concentration at four weeks was evaluated in the cell lysates from two groups as previously described. Ca concentration was quantitatively detected using calcium ion content assay kit (Sigma-Aldrich).

2.5. Statistical Analysis. Quantitative values were averaged and expressed as mean ± standard deviation. Student's t-test was used to evaluate the difference between experimental groups and $P < 0.05$ was considered statistically significant. The SPSS statistical software (IBM; version 20.0) was used for data analysis.

3. Results

3.1. Characterization of Nanofibrous Scaffolds

3.1.1. Morphological Characterization. As shown in Figure 1, both the single axial and the core-shell scaffolds were highly porous and had a smooth surface. The average diameters of the PLGA nanofibers, single axial nanofibers, and core-shell nanofibers were 287 ± 63 nm, 297 ± 103 nm, and 481 ± 103 nm, respectively.

The interior structure of the composite fibers was investigated by TEM (Figure 2). As we expected, the core-shell nanofibers had a clear core/sheath structure, while the pBMP2 microspheres were randomly dispersed throughout the single axial nanofiber.

3.1.2. Mechanical Properties. Table 2 shows the mechanical properties of the core-shell scaffold. The values were approximately 11 MPa for tensile modulus, 29% ultimate strain, and 3.5 MPa for ultimate stress, which were slightly lower than those of the PLGA scaffolds ($P < 0.05$). However, there was no significant difference between core-shell scaffold and single axial scaffold group ($P > 0.05$).

3.1.3. In Vitro Release Behavior. Figure 3 shows the pBMP2 release behavior of two groups of scaffolds. After a slight burst release occurred within the first 24 h, the pBMP2 release became stable and was sustainable. A sudden release was observed in the first day, with 31.98% and 42% from the core-shell scaffolds and the single axial scaffolds, respectively. Compared with the single axial scaffolds, release of pBMP2 in the core-shell scaffolds increased significantly for 2, 3, 4, 5, 6, 7, 10, 14, 21, and 28 days ($P < 0.05$).

3.2. In Vitro Studies

3.2.1. Cell Viability. To confirm the biocompatibility, hPDLSCs were cultured with the three groups of scaffolds (the core-shell scaffolds, the single axial scaffolds, and the PLGA scaffolds) compared with the blank control. After culture, the scaffolds with pBMP2 did not show a negative effect on cell proliferation. The optical density value was increased in a time-dependent manner from day 1 to day 7 in all groups. Indeed, the optical density in PEI/pBMP2-loaded scaffolds was increased in the same extent as PLGA scaffolds and blank control ($P > 0.05$).

3.2.2. Transfection Efficiency. To evaluate gene expression efficiency in hPDLSCs, transfection experiments were carried out using GFP as a reporter gene. Figure 5 shows pBMP2 transfection observed by confocal microscopy during the 7 days. The core-shell scaffolds and the single axial PEI/pBMP2-loaded PLGA scaffolds showed a significant increase in transfection efficiency compared to the control group which only contained the pBMP2 plasmid without PEI. In addition, the core-shell scaffolds showed a large number of GFP-positive cells homogeneously distributed across the electrospun meshes on day 7, while the other groups showed few GFP-positive cells.

The ability of the electrospun fibers to enhance the cell transfection was evaluated by FCM. Transient transfection efficiency in Figure 6 showed a steady time-dependent decrease in both groups, although the core-shell scaffolds showed stable transfection efficiency for a longer time, more than 28 days. Compared to the single axial scaffolds, the core-shell scaffolds had significantly higher transfection efficiency for 7, 14, 21, and 28 days ($P < 0.05$).

3.2.3. Osteogenic Activity. Cbfa1/Runx2 and BMP2 mRNA expression was quantitatively evaluated by real-time PCR. In the core-shell scaffolds group, BMP2 mRNA levels were increased approximately 13-fold more than the control after

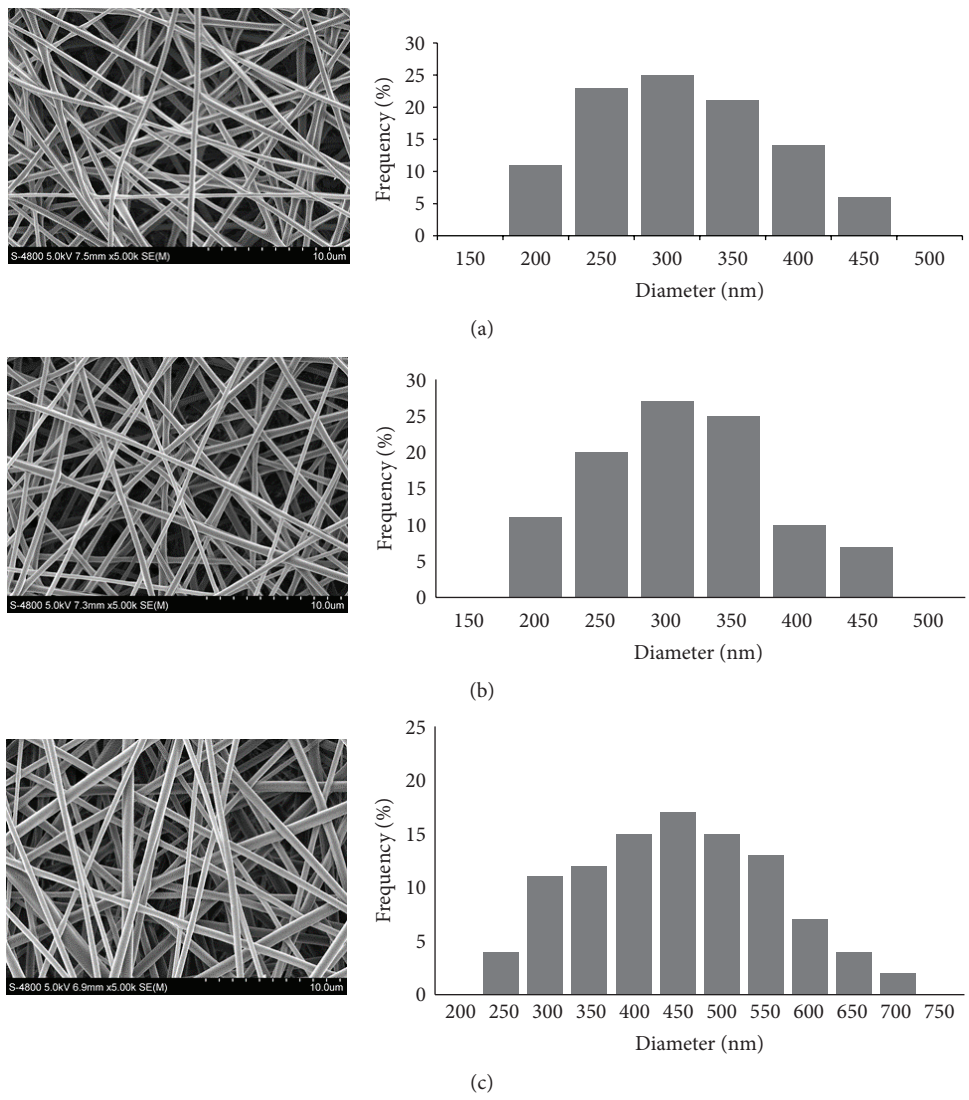

Figure 1: Morphology of electrospun scaffolds. (a) PLGA nanofibers. (b) Single axial nanofibers. (c) Core-shell nanofibers.

3 days. After 7 days, BMP2 mRNA expression was 8.5-fold higher than the control, and it was still 3.5-fold higher than the control after 28 days. The Runx2 levels increased 11-, 8.5-, 6.4-, 4.2-, and 4-fold more than the control on days 3, 7, 14, 21, and 28, respectively. In the hPDLSCs cultured with the single axial scaffolds group, BMP2 gene expression showed a 10- and 5-fold increase, while Runx2 levels showed a 12- and 5.7-fold increase at days 3 and 7, respectively, compared to the control. After approximately 10 days, the BMP2 and Runx2 levels were similar to the control group (Figures 7(a) and 7(b)). The expression of both BMP2 and Runx2 proteins involved in osteogenesis was evaluated by western blot (Figure 7(c)). BMP2 and Runx2 protein expression was increased during the first week, but the single axial scaffolds group showed a clear decrease also during the second week. After 14 days, both proteins expressions were also significant in the core-shell scaffolds. The level of protein expression was consistent with the mRNA expression. The core-shell scaffolds clearly showed a prolonged expression time. Furthermore, the higher Ca concentration was observed in the core-shell scaffolds group than that in the single axial scaffolds group ($P < 0.05$) (Figure 7(d)).

4. Discussion

Periodontal tissue consists of four component structures: alveolar bone, periodontal ligament, cementum, and gingiva. Periodontal regeneration is a complex process. Appropriated and environmental signals are needed to activate cells growth and differentiation, usually working in a controlled temporospatial manner. Successful periodontal tissue engineering needs several factors including cells, biodegradable scaffold, and biological factors to mimic the critical aspects of these biological processes. GAM combines gene therapy and tissue engineering to create a novel solution with a great potential for the restoration of structure and function of damaged or dysfunctional tissues. In this study, a core-shell

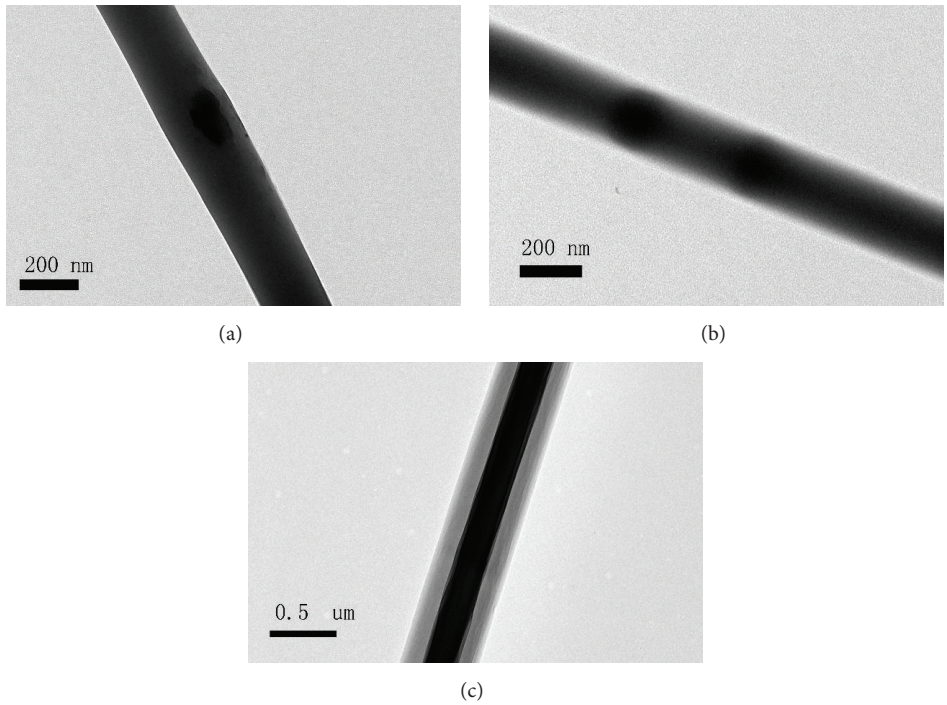

Figure 2: TEM images. (a) PLGA scaffolds. (b) Single axial scaffolds. (c) Core-shell scaffolds.

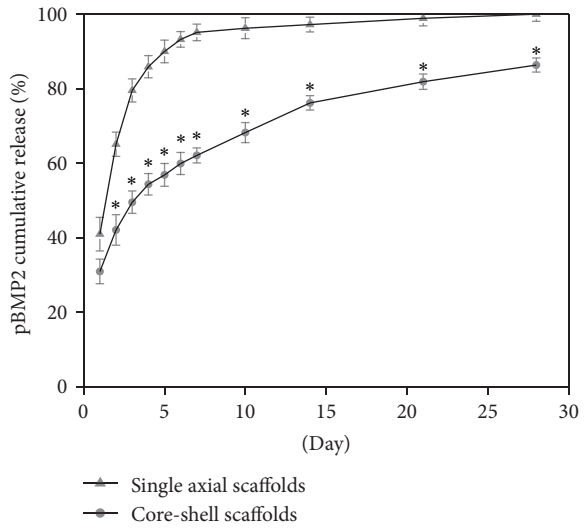

Figure 3: *In vitro* release curves of the single axial scaffolds and core-shell scaffolds at days 1, 2, 3, 4, 5, 6, 7, 10, 14, 21, and 28 ($n = 3$, $^{*}P < 0.05$).

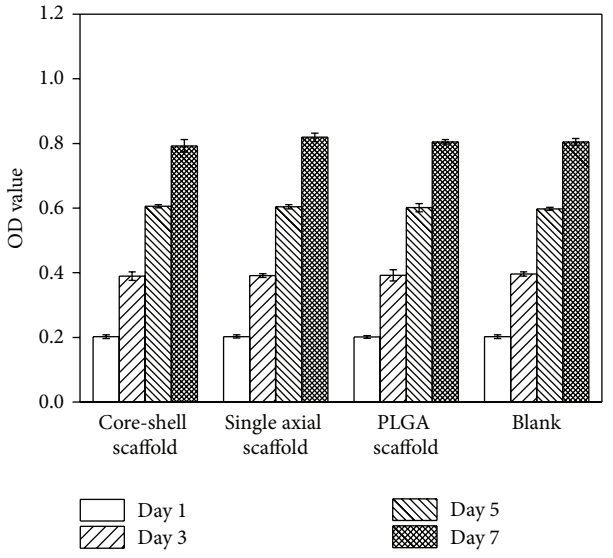

Figure 4: hPDLSCs cultured with core-shell scaffolds, single axial scaffolds, PLGA scaffolds, and tissue culture plates (blank). The OD values of all the groups were increased until day 7, and there were no significant differences between groups ($n = 3$, $P > 0.05$).

PEI/pBMP2-PLGA electrospun scaffold for gene delivery was fabricated by coaxial electrospinning.

Coaxial electrospinning was firstly demonstrated by Sun et al. [27]. In this study, two solutions, PLGA and PEI/pBMP2, underwent coaxial and simultaneous electrospinning through different feeding capillary channels and flow rates in one needle to generate core-shell composite nanofibers. The structure and morphology of the produced fibers are determined by a synergetic effect of formulation and process parameters. Despite the formation of a stable Taylor cone during fabrication of the fiber meshes for all the formulations, the core-shell fibers showed a greater distribution of diameters as showed in Figure 1. Both these observations suggest that the inclusion of highly charged moieties, such as a cationic gene delivery vector and an anionic plasmid DNA, significantly affect the electrospinning

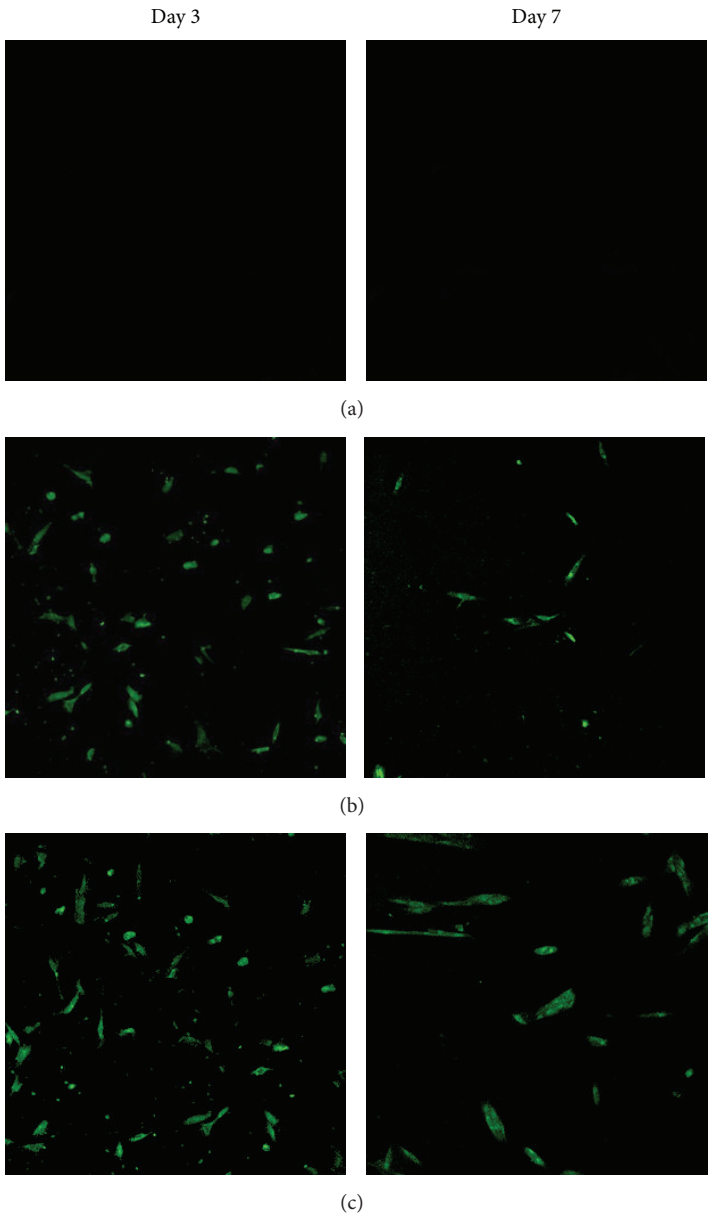

Figure 5: GFP expression in the three groups at days 3 and 7. (a) pBMP2 without PEI. (b) Single axial scaffolds. (c) Core-shell scaffolds.

properties of PLGA. pBMP2 can be incorporated in the electrospun nanofibrous structures, as illustrated in Figure 2(b); PEI/pBMP buffers with PLGA polymer solutions in organic solvents, followed by electrospinning the nanofibers. But the core-sheath structures, as showed in Figure 2(c), can protect PEI/pBMP2 from direct exposure to organic solvents and control the release of pBMP2.

The mechanical properties of GAM are important in tissue engineering. As we know, the potential periodontal regeneration scaffold should provide sufficient mechanical stability during the process of tissue regeneration and structural degradation. It also should be easily handled and applied around the affected tooth root and bone. In Table 2, average elongation at break and average tensile strength demonstrated that scaffold groups which incorporated pBMP2 have lower strength than PLGA scaffolds group. Compared to PLGA nanofibers with uniform circular cross-sections, core-shell nanofibers containing PEI/pBMP2 nanoparticles with nonuniform cross-sections showed different ductility. This probably also stems from the diameter increase, the slight decrease in porosity, and the interfusion of PEI/pBMP2 nanoparticles different from PLGA after assembly, which could result in an increase in the modulus. However, in view of the future clinical application, the mechanical strength data of these two incorporated pBMP2 groups are still within the satisfactory limits and comparable to the data

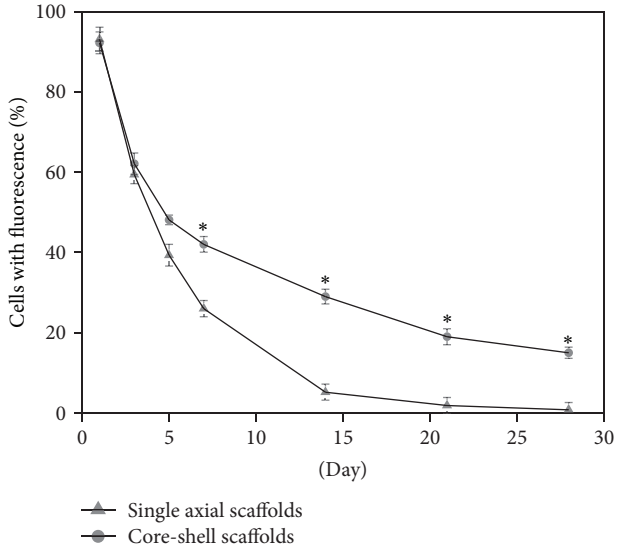

Figure 6: Cells fluorescence percentage after culture with core-shell scaffolds and single axial scaffolds at 1, 3, 5, 7, 14, 21, and 28 days ($n = 3$, $^{*}P < 0.05$).

of commercially available GTR membranes in the previous papers [28, 29].

To achieve a successful gene delivery, another important aspect that should be considered is to ensure the release of gene within the time frame of tissue regeneration. The sustained release of the plasmid DNA, whose release kinetics was determined by both the contents of the copolymers used to produce the nanofibers and the fiber structure itself [30, 31]. In our study, pBMP2 was incorporated into PEI and then combined with the PLGA by electrospinning for delivery to a target site. To confirm whether pBMP2 can be continuously released from the scaffolds, pBMP2 release profiles were evaluated in the electrospun scaffolds. As showed in Figure 3, 42% pBMP2 was released from the single axial scaffolds and only 31.98% was released from the core-shell scaffolds in the first day. Compared with the single axial scaffolds, release of pBMP2 in the core-shell scaffolds also decreased significantly for 2, 3, 4, 5, 6, 7, 10, 14, 21, and 28 days ($P < 0.05$). It indicated that the core-shell scaffolds can provide an efficient means to control the sequential release of multiple vectors and can simultaneously protect gene vectors in the core-layer against the relatively harsh processes.

PEI is one of the most positively charged dense polymers, which have high transfection activity *in vitro* and moderate activity *in vivo*. One disadvantage of PEI is its nonbiodegradable nature and its toxicity *in vivo*, which limits its application in *in vivo* delivery [32–34]. There are conflicting associations between the gene delivery efficiency and PEI toxicity. The presence of pBMP2 triggered biochemical reactions which changed cell function and behavior and finally increased cell proliferation [35, 36]. MTT-test is a method of investigating the proliferation of cells. As showed in Figure 4, there was no difference for hPDLSCs proliferation among the core-shell scaffolds group, single axial scaffolds group, and control groups. We did not observe signs of PEI cytotoxicity during transfection; this could be due to the PEI amount used in our experiment, which was insufficient for an appreciable toxicity. The other possible reason is that the core-shell structure of the nanofibers limits direct contact with hPDLSCs.

Similar to growth factor delivery, another important issue for gene delivery is to modulate transfection efficiency. To achieve gene transfection successfully, the effective concentration of target gene-vector complex should be released into the cell-surrounding microenvironment within an optimal time frame. Equivalent amounts of pBMP2 without PEI added directly to the cell medium revealed no green cells as showed in Figure 5, suggesting that there was no cellular transfection. The results were fairly accorded with experimental results reported in literature, in which the transfection efficiency of the PLGA scaffold with DNA without PEI is very low, less than 1% [22]. At an early time, the two scaffolds groups (the core-shell scaffolds and the single axial scaffolds) showed high transfection efficiency. Subsequently, the transfection efficiency of the single axial scaffold was reduced because of the pBMP2 concentration decrease in the nutrient due to its release. Compared to the single axial scaffolds, the core-shell scaffolds had significantly higher transfection efficiency for 7, 14, 21, and 28 days. The use of PEI/pBMP2 in combination with coaxial electrospinning enhanced the transfection efficiency.

The last important issue for gene delivery is the expression of the gene. The PEI/pBMP2 in hydrophilic core-layers followed by encapsulation with hydrophobic shell-layers in PLGA can prevent the direct contact of gene vectors with organic solvents. PEI/pBMP2 can be continuously released from the core-shell scaffolds, to avoid the PEI/pBMP2 complex burst release and then disassembled in the nutrient in a short time. The core-shell scaffolds could exhibit a long expression duration. Runx2/Cbfa1 is a global regulator of osteogenesis and is crucial for regulating the expression

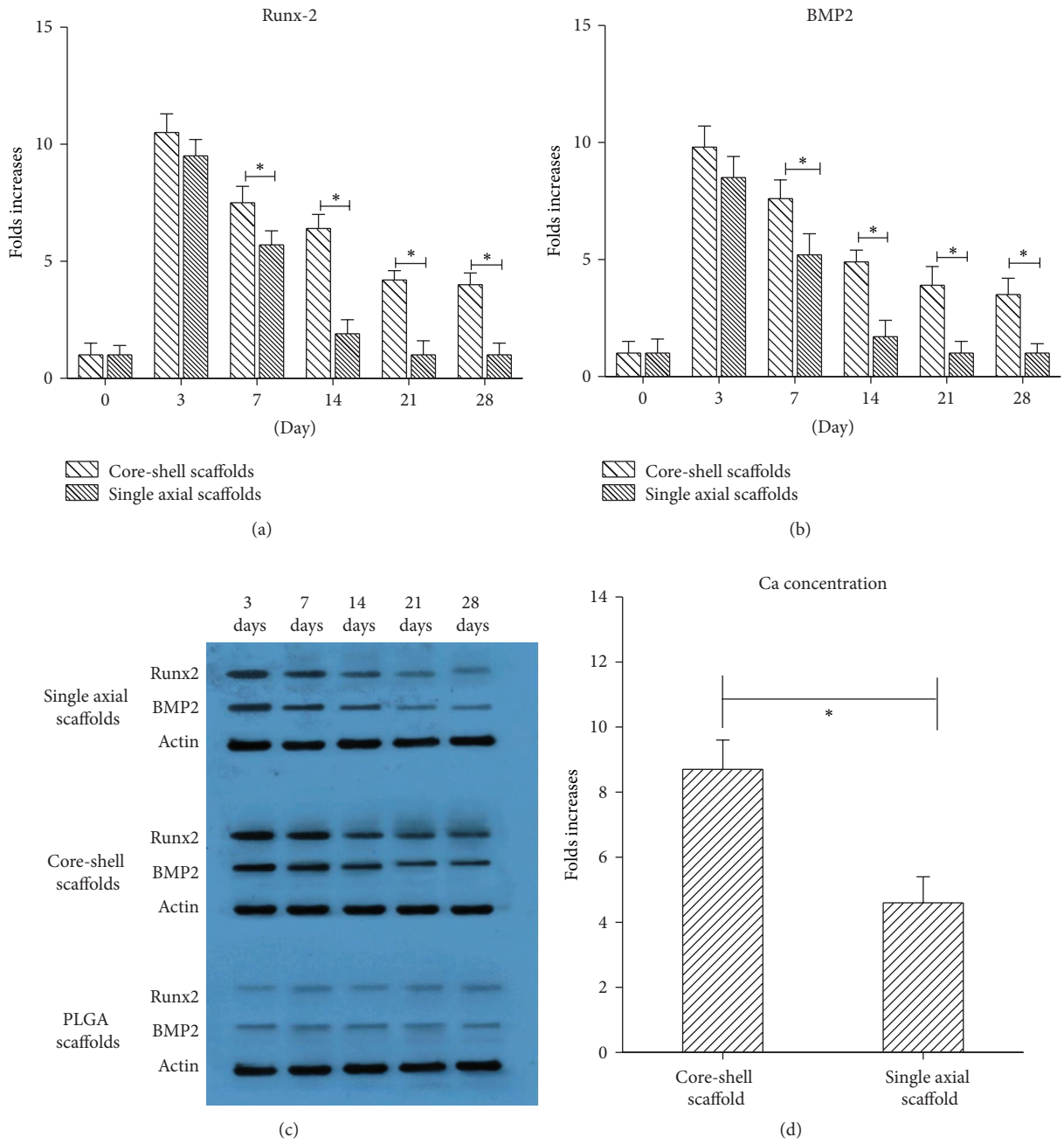

Figure 7: Runx2 (a) and BMP2 (b) mRNA expression was evaluated by qRT-PCR at several time points after transfection. (c) Runx2 and BMP2 protein expression evaluated by western blot. (d) Ca levels measured after 4-week culture ($^{*}P < 0.05$, $n = 3$).

of bone-specific genes. Runx2 is a major target of the BMP pathway. BMP2 signaling stimulates Runx2 acetylation, increasing transactivation activity of Runx2 [37, 38]. Cbfa1/Runx2 and BMP2 mRNA expression was quantitatively evaluated by real-time PCR. Compared with the single axial scaffolds, Runx2 and BMP2 mRNA expression in the core-shell scaffolds increased significantly after 7 days ($P < 0.05$), although the pBMP quantity in the same area of the two scaffolds groups was not always the same. Assayed by western blotting, Runx2 and BMP2 protein expression also indicated that the hPDLSCs cultured with core-shell scaffolds clearly showed more protein expression and longer expression time. Calcification is another important indicator of osteoblast differentiation, which plays a major role in the formation of mineral deposits in the matrix during bone tissue engineering. As the cell messenger, its regularity of change can adjust a variety of physiological processes. Ca concentration was measured as a marker of degree of calcification and can indirectly reflect the degree of osteogenic differentiation, evaluating the osteogenic activity of cells [39, 40]. The present

study can also show that the Ca concentration increase is more pronounced for the hPDLSCs cultured with the core-shell scaffolds compared with that cultured with the single axial scaffolds after 28 days ($P < 0.05$).

5. Conclusion

This study demonstrated that PEI/pBMP2 nanoparticles could be incorporated into a core-shell electrospun membrane, maintaining sustained release capability and biological activity. The prepared electrospun membrane showed satisfactory mechanical properties. Finally, the core-shell scaffold showed a good gene release behavior and prolonged expression time with high transfection efficiency. Therefore, our results could represent new insights to promote periodontal tissue regeneration.

Competing Interests

The authors declare that they have no conflict of interests.

Acknowledgments

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Review Article

Cell-Based Strategies for Meniscus Tissue Engineering

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Meniscus injuries remain a significant challenge due to the poor healing potential of the inner avascular zone. Following a series of studies and clinical trials, tissue engineering is considered a promising prospect for meniscus repair and regeneration. As one of the key factors in tissue engineering, cells are believed to be highly beneficial in generating bionic meniscus structures to replace injured ones in patients. Therefore, cell-based strategies for meniscus tissue engineering play a fundamental role in meniscal regeneration. According to current studies, the main cell-based strategies for meniscus tissue engineering are single cell type strategies; cell coculture strategies also were applied to meniscus tissue engineering. Likewise, on the one side, the zonal recapitulation strategies based on mimicking meniscal differing cells and internal architectures have received wide attentions. On the other side, cell self-assembling strategies without any scaffolds may be a better way to build a bionic meniscus. In this review, we primarily discuss cell seeds for meniscus tissue engineering and their application strategies. We also discuss recent advances and achievements in meniscus repair experiments that further improve our understanding of meniscus tissue engineering.

1. Introduction

As a worldwide medical problem, treatment of meniscus injuries has long been a research focus [1–6]. In adult, the distribution of meniscus neurovascular is complex with heterogeneity [7]. The outside region (red-red zone) is full of neurovascular tissues, while the inside region (white-white zone) lacks neurovascular tissue, and the region (red-white zone) between the former two areas displays a transitional characteristic from both the red-red and white-white regions. The neurovascular distribution is often associated with the prognosis of patients with meniscus tear. It is usually difficult to repair the injuries in the white-white zone [8]. The meniscus performs important functions in load bearing, shock absorption, joint lubrication, and joint stability [9–13]. Injury to this structure can greatly influence joint motion and daily living [14, 15]. According to one report, the incidence of meniscus injury resulting in meniscectomy is 61/100,000, of which the medial meniscus represents 81% and the lateral meniscus, 19% [1]. And it will be higher in athletes [16–18].

Meniscectomy is an effective way to relieve pain and joint swelling. However, follow-up surveys show that this type of surgery may damage knee stability and accelerate the development of osteoarthritis, so that many patients undergoing meniscus resection eventually have to accept a total knee arthroplasty [19–22]. Therefore, this choice may be beneficial for short-term purposes but may cause more long-term damage. An arthroscopic partial meniscectomy may be a better method to reduce joint damage [23, 24]. To maintain the stability of the knee, the surgeon may perform a minimally invasive arthroscopic meniscorrhaphy to repair a lacerated meniscus, which results in improved function compared to a meniscectomy and allows earlier joint motion [25–27]. In young patients, to better protect articular cartilage and restore knee function, an "ideal" solution would be meniscus replacement or regeneration [28]. Meniscal allograft transplantation (MAT) can improve knee function and result in good clinical outcomes; however, further evidence is necessary to determine whether it is

chondroprotective [29, 30]. Furthermore, it is difficult to resolve resource, shape matching, and ethical issues. Tissue engineering using natural or synthetic matrices as a scaffold to guide tissue repair or regeneration in three dimensions shows promising prospects for meniscus regeneration [31].

In meniscus tissue engineering, a scaffold is the basis for regenerating a new structure. Scaffold materials are typically selected from polymeric synthetic materials, such as polyglycolic acid (PGA) and poly-L-lactic acid (PLA), and natural biological products, such as silk, collagen type I, and proteoglycans [32–35]. The production process has ranged from "traditional" molding to electrospinning and more recently to three-dimensional (3D) printing technology [32, 33, 36, 37]. Research regarding scaffold preparation has made great progress. Scientists at Columbia University successfully built a meniscus with polycaprolactone (PCL) via 3D printing technology, which was loaded with connective tissue growth factor (CTGF) and transforming growth factor- (TGF-) $\beta 3$. It was shown to induce internal stem cell migration and differentiation to regenerate a new meniscus [37]. This treatment has been successful in sheep. Stone et al. [38, 39] developed a collagen type I scaffold isolated from bovine tendon that has been used clinically. Growth factors such as TGF and fibroblast growth factors (FGF) play important roles in regulating cell growth and cell differentiation and are regarded as key elements in tissue engineering [40–42]. The growth factors were also used to induce cells to differentiate from stem cells to obtain more cell resources for meniscus tissue engineering [34, 43–45]. Moreover, the studies also revealed that a hypoxic environment is able to slow down cell dedifferentiation process, and mechanical stimulation can improve collagen and glycosaminoglycan (GAG) secretion [44, 46–48]. A bioreactor, which simulates the knee microenvironment, can provide a better platform for meniscus tissue engineering research [49, 50].

The study has shown that a cell-based tissue-engineered meniscus achieves better repair results than cell-free ones [51]. Therefore, current research on meniscus tissue engineering has focused on the use of cells. As one of the three elements of tissue engineering, the choice of cell seed is particularly important. In early research, cells used in meniscus tissue engineering were mainly of a single cell type, and it was difficult to copy the native tissue features. Inspired by the coexistence of cells in the knee and their interaction, researchers carried out cell cocultures to build a tissue-engineered meniscus with better mechanical and cartilaginous properties. This method also solved cell supply issues to some extent. After further understanding the complex structure of the meniscus, combined with the theory of cell coculture, zonal recapitulation was proposed to mimic the organizational heterogeneity of the meniscus. To increase cell interactions and reduce the interference of nonbiological components, research was also conducted to build a tissue-engineered meniscus with cell self-assembly alone without any scaffolds. In this review, we review the reports on cell-based meniscus repair and discuss cell application strategies from single cell type, cell coculture, zonal recapitulation, and scaffold-free cell self-assembly, respectively (Table 1).

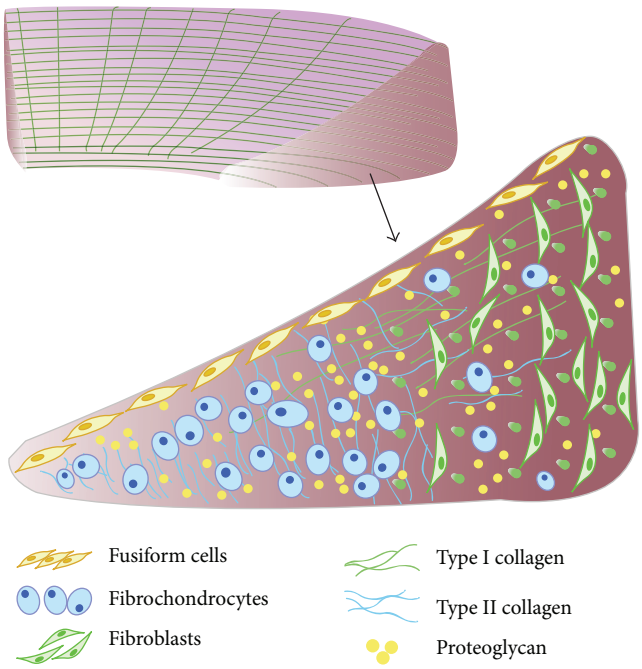

Figure 1: Schematic diagram of meniscus internal ultrastructure. Most of collagen fibers bundles oriented circumferentially; only few of them presented a radial alignment. Cells of the superficial zone are fusiform in shape and located below the tissue surface; the cells in the outer one-third region or vascular zone are mainly elongated fibroblasts, and collagen I accounts for >90%; for inner two-thirds region or avascular zone, round or oval shaped fibrochondrocytes are interspersed, the ratio of collagen I and collagen II is about 2 : 3.

2. Single Cell Type with a Scaffold

As a "classical" strategy for a tissue-engineered meniscus, this method has been used in many studies and as the basis for further cell-based developments. The cells, which include fibrochondrocytes, chondrocytes, synoviocytes, or stem cells, are all involved in building a construct with scaffolds. Herein, we discuss the application of these cells one by one to find an appropriate seed for meniscus tissue engineering.

3. Meniscus Fibrochondrocytes

The structure of the meniscus is complex with heterogeneity (Figure 1). Meniscal extracellular matrix (ECM) mainly comprised collagen fibers and proteoglycans. Most of collagen fibers bundles oriented circumferentially; only few of them presented a radial alignment. The oriented structure of the fibrillar collagens is closely associated with meniscus biomechanical properties. Therefore, it is important to restore the microarchitecture of the collagen fibers for successfully repairing or regenerating the meniscus [52]. The populations of meniscus cells can be divided into three distinct groups: fibrochondrocytes are cells with a round or oval shape mostly located in the inner two-thirds of the meniscus; they express both collagen I and collagen II. Fibroblast-like cells are elongated and found mainly in the outer one-third, the ECM of which is mainly collagen I. Cells of the superficial zone are

Table 1: Cell application in meniscus tissue engineering.

Strategy	Cell application	Cell resource	Author
Single cell type	MC	Human (18–84 years)	Baker et al., 2009 [58]
		New Zealand white rabbits	Gunja and Athanasiou, 2010 [41]
		Allogeneic rabbit cell	Kang et al., 2006 [32]
		New Zealand white rabbits	Esposito et al., 2013 [62]
	AC	Swine	Yoo et al., 2011 [63]
		Autologous sheep chondrocytes	Kon et al., 2008 [64]
		Autologous and allogenic swine chondrocytes	Weinand et al., 2006 [66]
	SMC	Adult equine	Fox et al., 2010 [70]
		Canine	Warnock et al., 2014 [71]
	SC	Rat bone marrow	Yamasaki et al., 2005 [77]
		Femoral and tibial bone marrow of calves	Nerurkar et al., 2011 [78]
		Femoral and tibial bone marrow of calves	Baker et al., 2011 [44]
		Human iliac crest (27–55 years)	Petri et al., 2012 [48]
		Adult human synovial membranes	Sakimura et al., 2006 [81]
Cell coculture	MC and AC	Knee joint of rabbit	Gunja and Athanasiou, 2009 [87]
	MC and MSCs	Human meniscus and human MSCs	Cui et al., 2012 [92]
	MC and BMSC	Human meniscus and iliac crest of patients	Diao et al., 2013 [91]
	MC and BMSC	Human meniscus and human MSCs	Matthies et al., 2013 [93]
	MC and SMSC	The knees of pigs	Tan et al., 2010 [95]
Zonal recapitulation	AC and FBC	Human donors (<35 years)	Mandal et al., 2011 [33]
	AC and MC	Stifle joint of calves	Higashioka et al., 2014 [97]
Scaffold-free	SMC	Synovial villi from canine with stifle osteoarthritis	Warnock et al., 2014 [72]
	SMSC	Porcine synovial membranes	Moriguchi et al., 2013 [102]
	MC and AC	Knee joints of calves	Huey and Athanasiou, 2011 [104]
	MC and AC	Knee joints of calves	Hadidi et al., 2015 [106]

MC: meniscus cell, AC: articular chondrocyte, MSC: mesenchymal stem cells, BMSC: bone marrow mesenchymal stem cells, SMC: synovial membrane cell, SC: stem cell, SMSC: synovial membrane mesenchymal stem cells, FBC: fibroblast cells, LPA: phospholipid lysophosphatidic acid, FBS: fetal bovine serum, and ADSC: adipose-derived stem cells.

fusiform in shape and located below the tissue surface [53–55].

Currently, most studies focusing on cells isolated from the meniscus involve fibrochondrocytes [56, 57]. One advantage of a substitute built with in situ derived cells is better histocompatibility. Baker et al. [58] conducted an experiment in which meniscus cells isolated from 10 human donors who underwent knee surgery were expanded to passage two in monolayer culture and then seeded onto PCL fiber-aligned biodegradable nanofibrous scaffolds and cultured for 10 weeks. The results showed that all constructs seeded with meniscus cells increased in dry weight, DNA, collagen, and GAG contents in a time-dependent manner, and the mechanical properties of the meniscus-derived cell-laden constructs strongly correlated with collagen content, but not donor age. Correlation analysis between collagen content in constructs and the mechanical properties suggests that increasing the collagen deposition of constructs may further enhance their mechanical properties. Similarly, this indicated that autologous cells derived from the patients themselves could represent a potential cell resource for meniscus tissue engineering. However, the number of primary cells isolated from the meniscus was not sufficient to satisfy the needs for tissue engineering. To overcome this, an approach often used is to expand primary cells to passage two or three in monolayer culture until the cell number is sufficient. However, cellular phenotypes and gene expression levels can be changed during passaging, indicating that cells are in a dedifferentiation process [59]. Other studies have shown that the addition of fibroblast growth factor (FGF) into the culture solution or as a coating on the scaffold can inhibit cell dedifferentiation and promote secretion of the ECM [41, 60, 61]. Culturing cells in hypoxic conditions also have a similar effect [46, 47]. Kang et al. [32] used fibrochondrocytes seeded on PGA/PLGA scaffolds to repair rabbit models with a total meniscectomy in vivo. After 6 and 10 weeks, a neomeniscus formed with the appearance of fibrocartilaginous tissue. Moreover, the histological and immunohistochemical structures in the neomeniscus were similar to those of the native meniscus. After 36-week transplantation, histochemical and immunohistochemical structures of neomeniscus were more aligned than those of 10 weeks. Similarly, in the anterior areas, biomechanical properties in neomeniscus were higher than those of native meniscus. However, biomechanical properties of neomeniscus at middle and posterior sites were lower than those of native meniscus. Esposito et al. [62] preseeded fibrochondrocytes on PLDLA/PCL-T scaffolds for three weeks, at which point the constructs were implanted

to replace the medial meniscus in rabbits. The constructs showed good compatibility with surrounding tissues, and fibrocartilaginous tissue with mature collagen fibers appeared in histology results after 24 weeks. These experiments indicate the feasibility of regenerating meniscus with meniscus cells using tissue engineering.

4. Articular Chondrocytes

The limited supply of allogeneic or autologous meniscus cells limits their application for meniscus tissue engineering, and monolayer expansion can lead to significant changes in cellular phenotype [59]. Cartilaginous cells, such as articular chondrocytes, are regarded as a promising cell source. Fibrochondrocytes and chondrocytes are both from cartilage tissue, and a study showed their similar cell membrane markers and high expression of collagen II, the major component of the ECM [55]. These similarities in cellular phenotype and molecular biology make it possible for chondrocytes to be used as cell seeds for meniscus tissue engineering. Yoo et al. [63] embedded PLGA scaffolds implanted with chondrocytes subcutaneously in nude mice for seven days to evaluate the fibrocartilage formation ability of the construct. Histology results showed fibrocartilaginous neotissue generation between meniscus discs, demonstrating the feasibility of chondrocytes as cell seeds for meniscus tissue engineering. Moreover, significantly more cartilaginous tissue and complete healing of the meniscus defects was observed in a platelet-rich plasma- (PRP-) treated hyaluronic acid/PCL scaffold seeded with autologous chondrocytes in a total meniscectomy sheep model; the author believed that seeding of the scaffolds with autologous chondrocytes provided additional benefits for fibrocartilaginous tissue repair [64, 65]. However, harvesting autologous cells could cause additional trauma to patients. Weinand et al. [66] compared the repair capacity of autologous and allogeneic cells for meniscus lesions and found that the remediation results between the two cell types showed no significant difference. This experiment demonstrated the essentially equal potential of autologous and allogeneic chondrocytes for meniscus tissue engineering and will likely broaden the possible cell sources for cartilaginous tissue engineering if ethical issues can be resolved. Extensive research on chondrocytes and their successful application in cartilage repair will be beneficial for their application in meniscus tissue engineering.

5. Synoviocytes

As the most abundant tissue in the knee joint, the synovial membrane can affect the development of osteoarthritis by producing microRNAs and other factors [67]. Synoviocytes are considered a resource for meniscus tissue engineering due to their chondrogenic behavior. Studies have shown that synoviocytes can form a meniscus-like matrix in vitro, and FGF promotes collagen II formation and a chondrocytic cell phenotype [68, 69]. Fox et al. [70] investigated the feasibility of fibroblast cells derived from synovial tissue to generate a meniscus construct in vitro. Equine synoviocytes were seeded onto a PGA/PLLA scaffold and cultured with growth factors in a rotating bioreactor. Reverse transcription polymerase chain reaction (RT-PCR) results indicated that fibroblast cells exhibit fibrochondral characteristics, and growth factors improve the expression of collagen II and GAGs. As previously mentioned, bioreactors, which provide mechanical stimulation, have a positive effect on cell differentiation, cell viability, ECM production, and compressive biomechanical properties. As research showed culturing a synoviocyte-seeded PGA scaffold in a rotating bioreactor resulted in much better cell and matrix characteristics than when cultured in static conditions [71]. Furthermore, the research has shown that synoviocytes derived from osteoarthritic joints can produce meniscal fibrocartilage ECM components at levels similar to those of normal synoviocytes [72]. These achievements demonstrate the feasibility of synoviocytes as a potential cell source for meniscus tissue engineering.

6. Stem Cells

Stem cells exhibit features of multidirectional differentiation. Stem cells can be divided into embryonic stem cells and adult stem cells. The latter, such as bone marrow mesenchymal stem cells (BMSC) and adipose-derived stem cells (ADSC), have been a focus of research due to their wide distribution and species diversity [73, 74]. Studies have shown that stem cells can differentiate into cartilaginous cells with abundant matrix; therefore, stem cells can be used as cell seeds for meniscus tissue engineering [75, 76]. Yamasaki et al. [77] built a construct similar to a native meniscus using rat BMSC and a decellularized meniscus scaffold. Nerurkar et al. [78] and Baker et al. [44] built a meniscus substitute with bovine BMSC and aligned PCL electrospun scaffolds and found that dynamic culture conditions and mechanical stimulation were beneficial for stem cells infiltration and proliferation, collagen deposition, gene expression, and reinforcing mechanical properties. The ECM generated in the structure was sufficient and resembled the collagen and GAG content in the native meniscus. It is noteworthy that the duration of preculture may play a role in BMSC responding to the mechanical stimulation, because the condition of preculture can provide a sufficient time for cell colonization and ECM deposition. In addition, human adult BMSC were also investigated to generate a meniscus substitute. The results showed that BMSC improved the tensile strength of collagen scaffold, and mechanical stimulation enhanced the mechanical properties of the structure [34, 48].

Many MSCs exist in synovial membranes and can be induced to differentiate into chondrocytes under appropriate culture conditions [79, 80]. Sakimura et al. [81] found synergistic effects of TGF-β1 and Insulin-like Growth Factor-1 (IGF-1) on synovial MSC in cartilaginous differentiation and GAG production for repairing meniscus defects. Some researchers believe that MSCs in synovial tissue can migrate to damaged areas to promote restoration, just like the scaffold loaded with CTGF and TGF-β3 can regenerate a meniscus via stem cell recruiting [37]. Injections of MSC derived from the synovial membrane have been used to repair meniscus defects in rat, rabbit, and pig models, resulting in effective new fibrocartilage generation [82–84]. Due to their quantity,

ease of acquisition and multidirectional differentiation, synovial MSC may represent good prospects in future meniscus tissue engineering.

Direct injection of stem cells into the knee joint for the treatment of meniscus defects and osteoarthritis has been assessed in clinical trials [85]. However, tissue engineering is a better method to infuse a large number of stem cells. Not only does a scaffold provide a specific space for cell attachment and proliferation to maintain high levels of stem cells in a particular area, but also loading growth factors induce stem cell homing to the defect to improve repair and regeneration results.

7. Cell Coculture

According to previous research, with a tissue-engineered meniscus based on a single cell type, it is difficult to copy microstructural features of the native meniscus. Cell coculture, culturing two cell types, is a strategy used to improve the biochemical and biomechanical properties of a tissue-engineered meniscus through cellular interactions and mitigates the need for cell expansion [86]. There are two types of cell coculture systems: direct and indirect. The former is a mixed cell culture, which works through direct cell interaction, while the latter involves separating the cell culture in one system and works through the transfer of growth factors; this is also known as paracrine secretion.

Research has shown that collagen dominates the tensile response while GAG is important for compressive properties [43]. One study found that meniscus fibrochondrocytes alone were not able to produce enough collagen type II and GAG, while pure chondrocytes were unable to deposit sufficient collagen type I to sustain tensile strength. Could coculturing two types of somatic cells have a synergistic effect? Gunja and Athanasiou [87] built a construct by coculturing meniscus cells and chondrocytes on PLLA scaffolds and found that coculturing not only increased ECM secretion but also generated a meniscus structure with better mechanical properties. The compressive properties in cell-seeded constructs approached those of the native meniscus. However, much work needs to be done which enhances the tensile properties in the cell-seeded constructs towards those of the native tissue.

MSCs have the potential to differentiate along the chondrogenic pathway in appropriate conditions, as well as deliver trophic effects to differentiated cells [88–90]. In a coculture system, somatic cells provide inductive stimulation for stem cell differentiation, while stem cells supply various growth factors to maintain cellular phenotype and regulate cell proliferation. Previous studies reported that coculture of MSC with chondrocytes enhances ECM production and inhibits hypertrophy [91], so why not with meniscus cells? Cui et al. [92] cocultured meniscus cells with MSCs at different ratios and found that coculturing not only promoted levels of collagen type I and GAG production but also prevented cell hypertrophy, with meniscus cells and MSC at a 75 : 25 ratio showing the best results. The studies also found that coculturing meniscus cells isolated from osteoarthritic patients with BMSC in a pallet contribute to similar results as normal meniscus cells [93, 94]. These studies strengthen the combined application of meniscus cells and MSCs as a cell source for meniscus tissue engineering. Tan et al. [95] cocultured meniscus cells with synovium-derived stem cells on small intestine submucosa to engineer a meniscus substitute construct and found that this coculture-based tissue construct exhibited higher GAG and collagen II levels, resulting in a concomitant increase in mechanical properties. MSCs derived from synovial tissue secrete IGF-1 with anti-inflammatory effects, which is beneficial in preventing osteoarthritis [96]. Thus, cell coculture not only enriches the choice of cell seeds but also serves as an effective method to maintain cellular phenotype and improve repair effects and may represent a promising strategy for meniscus tissue engineering.

8. Zonal Recapitulation

Compositional differences in the meniscus inner and outer regions make it difficult to fabricate a bionic meniscus. To generate a tissue-engineered meniscus successfully, mimicking its complex internal architecture is important. The heterogeneity of cell types and matrix components in the inner and outer regions makes it nearly impossible for meniscus reconstruction and complete regeneration via tissue engineering methods, especially when relying on a method to build a construct with a single cell type and scaffold. Hence, the idea of seeding different cell types on different regions of a scaffold to create a biomimetic substitute in both structure and content has been developed. This strategy simultaneously creates an indirect coculture system when effectively applying the cells' different physiological characteristics. Mandal et al. [33] have designed a multilayer meniscus scaffold fabricated with silk to mimic native meniscus structure; human articular chondrocytes and dermal fibroblast cells were seeded at the periphery and center of the scaffold, respectively. Scanning electron microscopic (SEM) results reveled that the top two layers showed circular pores, while the bottom third layer had laminar channels. After culturing for four weeks in vitro, the results showed that the cell-seeded construct improved mechanical properties, increased cell populations, enhanced ECM deposition, and maintained a chondrocytic phenotype. Thus, the author suggested that a construct with multiple layers and porosity can act as an effective medium to direct cell orientation and neotissue formation, resembling the structural and mechanical anisotropy of the native meniscus. Higashioka et al. [97] used agar without cell adhesion to build an anisotropic graft with zonal variations; the inner one-third was seeded with 100% chondrocytes and the outer region was generated by coculturing chondrocytes with meniscus cells. The two zones of the structure integrated well and exhibited significantly different mechanical and biochemical properties after culturing. This is a case that combined zonal recapitulation with cell coculture. With the application of 3D printing technology, it may be more conducive to regenerate a construct that is more biomimetic of the native meniscus. Zonal recapitulation is a more suitable method to mimic the complex internal architecture of the meniscus and may be a better cell application strategy for meniscus tissue engineering.

9. Scaffold-Free Tissue Engineering

In "traditional" meniscus tissue engineering, the scaffold is an important factor. Biodegradability, biocompatibility, and certain mechanical strength are necessary for a scaffold [39]. However, it is difficult to copy the complex internal structure of the meniscus with the technologies available today. Some researchers have proposed generating a new meniscus relying on self-assembling cells in a specific artificial environment, simulating the tissue generation process; this is also referred to as scaffold-free culture [98]. High-density cells form a tissue through close interactions in a specific environment, which not only reduces the obstruction of the scaffold but also is more conducive to crosslinking collagen fibers in the ECM to promote mechanical strength [99]. This method can also be divided into single cell type self-assembly and coculture self-assembly.

10. Single Cell Type Self-Assembly

Single cell type self-assembly is mostly used to regenerate the inner part of the meniscus, in which fibrochondrocytes represent the main cell type. A series of experiments were conducted to explore the possibility of generating a neotissue via cell self-assembly. Ballard et al. [100] compared the properties of bioscaffolds generated by meniscus fibrochondrocytes and synoviocytes and determined they had similar collagen and GAG content. He also found that treating synovial neotissue with chondrogenic growth factors (bFGF, TGF-β1, and IGF-1) or mechanical stimulation showed greater fibrocartilage-like matrix content and better biomechanical properties [101]. In addition, he demonstrated the fibrochondrogenic potential of synoviocytes from osteoarthritic joints, making them a potential cell source for meniscal tissue engineering [72]. These studies confirm the feasibility of a synovial bioscaffold as a replacement therapy for meniscus defects. Moreover, Moriguchi et al. [102] used an allogeneic synovial MSC bioscaffold to repair swine meniscus defects. The defects implanted with the bioscaffold were consistently repaired by fibrocartilaginous tissue with good tissue integration and cartilage protection after six months. These results suggest that a scaffold-free tissue-engineered construct could represent a promising cell-based implant to repair meniscus lesions.

11. Cells Coculture Self-Assembly

Cell coculture not only offers more options to generate a meniscus construct but also promotes biochemical and biomechanical properties of the tissue-engineered meniscus. Hoben et al. [103] compared constructs with self-assembling chondrocytes, meniscus fibrochondrocytes, and cocultures of fibrochondrocytes and chondrocytes and found that the coculture resulted in a mixed collagen I and collagen II matrix similar to the native meniscus. To achieve a scaffold-free construct with better mechanical properties mimicking the native meniscus, Huey and Athanasiou [104] combined the self-assembly process with the catabolic enzyme chondroitinase-ABC and TGF-β1 and resulted in a mature neotissue with a higher radial and compressive modulus. This study revealed that self-assembly approach can produce a tissue-engineered construct that is similar to the biochemical and biomechanical characteristics of native meniscus. Hadidi and Athanasiou [105] applied the phospholipid lysophosphatidic acid (LPA) to enhance the mechanical properties of a construct generated by chondrocytes and meniscus cells via a self-assembling process. Moreover, this research further revealed that LPA mainly depended on inducing cytoskeletal reorganization and cell-matrix traction to enhance the mechanical properties. He also investigated the appropriate number of cells to create a scaffold-free structure for meniscus tissue engineering and found that coculturing chondrocytes and meniscus cells at a ratio of 1 : 1 with lower seeding density resulted in beneficial effects on self-assembling fibrocartilage [106]. These studies have shown the feasibility of generating an effective substitute without a scaffold, and the results demonstrated favorable biomechanical properties of the neotissue. From these achievements, we conclude that cells are the most important factor for scaffold-free culturing, and obtaining a sufficient number of cells is the key for applying this technology.

In the future, it will be vital to solve the meniscus regeneration problem, regardless of how the meniscus is created, especially the injuries in the white-white areas of the meniscus, because the role vessels play in maintaining the meniscus in this areas is inadequate. This dilemma might be addressed by the following approaches. In the one side, creating some vascular channels to the scaffolds or direct injection of some stem cells may enhance the meniscus regeneration to some extent; on the other side, loading some growth factors or PRP to the scaffolds may also be beneficial to form a tissue-engineered meniscus. However, some drawbacks exist in the application of constructs. For scaffolds with a single cell type or cell coculture, the mechanical properties are sufficient if the construct is only needed for avascular region regeneration, but it will be difficult for fixation and ideal results, whereas if the construct is needed for whole regeneration, it will be difficult to satisfy the required radial tensile strength. For zonal recapitulation, the mechanical difference between the inner and outer regions is large, and the transition zone will tear easily. For scaffold-free culturing, improving the mechanical properties remains important.

Considering all the current advances, we can envisage an ideal tissue-engineered meniscus in the future: regarding the meniscus internal structure as a blueprint and using the meniscus ECM/cells mixture as material for 3D printing technology, it will be possible to build a tissue-engineered meniscus with zonal heterogeneity, while growth factors within the construct will not only promote cell proliferation and maintain cellular phenotype but also induce recruiting of stem cells in vivo to promote meniscus regeneration. However, for a cell-scaffold construct in vivo, cell supply issues and how to mobilize the migration of stem cells are problems that still need to be resolved.

12. Conclusions

Meniscus tissue engineering is a main focus but remains difficult. It will be essential to create a structure mimicking

the architectural and mechanical properties of the native meniscus. Because the natural complex internal architecture is still not absolutely clear, a method based on zonal recapitulation may be more promising with the further development of 3D printing technology. However, scaffold-free cell self-assembly methods to generate a functional meniscus graft with good mechanical properties may be a possibility if an appropriate environment can be created. In conclusion, three main parts, scaffolds, cells and growth factors, need further clarification in order to create an ideal tissue-engineered meniscus. We still have a long way to go.

Disclosure

Wei Niu, Weimin Guo, and Shufeng Han are the coauthors, while Shuyun Liu and Quanyi Guo were the corresponding authors, because Dr. Weimin Guo has done a lot of work in revising the paper.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Wei Niu, Shufeng Han, Shuyun Liu, and Weimin Guo contributed equally to this work.

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Research Article

A Comparative Evaluation of the Mechanical Properties of Two Calcium Phosphate/Collagen Composite Materials and Their Osteogenic Effects on Adipose-Derived Stem Cells

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Adipose-derived stem cells (ADSCs) are ideal seed cells for use in bone tissue engineering and they have many advantages over other stem cells. In this study, two kinds of calcium phosphate/collagen composite scaffolds were prepared and their effects on the proliferation and osteogenic differentiation of ADSCs were investigated. The hydroxyapatite/β-tricalcium phosphate (HA/β-TCP) composite scaffolds (HTPSs), which have an additional β-tricalcium phosphate, resulted in better proliferation of ADSCs and showed osteogenesis-promoting effects. Therefore, such composite scaffolds, in combination with ADSCs or on their own, would be promising for use in bone regeneration and potential clinical therapy for bone defects.

1. Introduction

Bone loss due to trauma, inflammation, and surgical processes has posed great difficulty in the aesthetic reconstruction of a functional alveolar bone [1, 2]. Tissue engineering and biomaterials, which can promote alveolar bone regeneration, have become a popular focus of current studies [3–6].

As an important element in alveolar bone tissue engineering, osteogenic cells, in addition to growth factors and scaffolds, have been studied extensively to regenerate new bones and to repair large bone defects. Embryonic, osteoblastic, and adult stem cells have been adopted for such applications [7]. Mesenchymal stem cells (MSCs) from the bone marrow stem cells (BMSCs) and from the adipose-derived stem cells (ADSCs) are two of the most extensively explored cell sources for bone regeneration [8]. However, the collection of the bone marrow is a painful operation and the yield of MSCs from this source is relatively low [9]. Adipose tissue is an abundant source of ADSCs and can be easily obtained by using liposuction, which is less traumatic than bone marrow collection [10]. ADSCs can undergo osteogenic differentiation in vitro under certain conditions [11, 12] and yield 100- to 500-fold more MSCs than BMSCs after initial harvest [13]. ADSCs preserve their proliferative and differentiation potentials even in older patients, which would be helpful for repairing bone defects in these patients [7]. In addition to these properties, the nonimmunogenic properties [14] and capacity to migrate to the injury site in ADSCs make them a potential candidate for use in alveolar bone regeneration [15, 16].

The materials used for the preparation of bone grafts must be biocompatible, osteoinductive, and osteoconductive. Additionally, the structure and mechanical properties of these materials should be similar to those of natural bones [17].

Among the different kinds of biomaterials, calcium phosphate-based materials including hydroxyapatite (HA), α-tricalcium phosphate (α-TCP), and β-tricalcium phosphate (β-TCP) have been widely explored for use as scaffolds in bone regeneration [18–20]. As a major mineral constituent of natural bones, HA can maintain space for the proliferation of osteogenic cells and for the exchange of substances due to long-term resorption, while TCP can form a porous structure and release calcium and phosphorus ions by dissolving, which contributes to osteogenic activity and thus to new bone formation [21, 22]. A composite material comprising HA and β-TCP was developed, which incorporated the advantages of both these materials, showing better mechanical properties and allowing better degradation [23, 24]. Recently, many researchers have attempted to bring about improvements in such composite scaffolds by modifying many parameters such as the HA/TCP ratio as well as phase composition, formulation, sizes, and shapes [25–27].

While extensive research has been conducted on the effects of HA and TCP on BMSCs [28–31], relatively fewer studies have revealed the influence of these biomaterials on the osteoblastic differentiation capacity of ADSCs. Hicok et al. showed that, in combination with ADSCs, HA and β-TCP could enable the formation of osteoids in mice with severe combined immunodeficiency (SCID) [32]. Moreover, Lee et al. revealed that HA could induce osteogenic differentiation [33]. Further, ADSCs seeded on HA-TCP scaffolds could form a higher percentage of osteoids in mice with SCID compared to that observed with the use of HA alone [32, 34]. Additionally, a β-TCP matrix could achieve similar effects when used with osteogenic culture media [32]. Based on these data, we hypothesized that a HA/β-TCP composite scaffold could better promote osteogenic differentiation than a scaffold comprising only either one of these biomaterials.

However, because of the brittleness of such inorganic materials, better effects could be achieved by additionally using organic constituents of natural bone, such as collagen [35]. Collagen, especially type-I collagen, which accounts for a major part of the extracellular matrix (ECM), has become one of the most widely explored tissue engineering scaffolds due to its excellent biocompatibility, degradation properties, and absorbability [36]. Collagen-based materials result in excellent osteogenic differentiation when used with different stem cells [37, 38]. Besides, in collagen-calcium phosphate composite materials, the poor mechanical properties of both the organic and the inorganic components, such as stability [39] and the mechanical "wet" properties [40], can be strengthened partly owing to the energy dissipation that occurs through a multiplicity of "sacrificial bonds," which are covalent bonds formed within the collagen molecular chain itself or due to the crosslinking between different chains in the composite materials [41, 42]. In addition, ions or hydrogen bonds [43], the sliding of layered water films [44], and other hypothetical mechanisms of mineral-collagen interactions have been proposed to explain the improved nature of the composite materials. A combination of the adhesion property of collagen and the osteoconductive ability of HA and TCP can be used in such composite biomaterials to increase the proliferation and osteogenic differentiation of ADSCs [32, 43, 44].

In this study, we prepared pure HA particles (HAP) using the traditional solution precipitation method and HA/β-TCP particles (HTP) by burning the bovine bone and composited both these particles with type-I collagen by freeze-drying. In addition to the composition, microstructure, and mechanical and wet properties of these composite biomaterials, their effects on the proliferation and osteogenic differentiation potential of ADSCs were examined to pave the way for further application of ADSCs as seed cells to improve bone regeneration.

2. Materials and Methods

2.1. Materials. CaO (analytical reagent, AR), H_3PO_4 (AR), H_2O_2 (AR), NaOH (AR), and absolute alcohol (AR) were all purchased from Beijing Chemical Works (Beijing, China); type-I purity collagen was purchased from Collagen Biotechnology Co. Ltd. (Hebei, China); KBr (spectroscopically pure) was purchased from Botianshengda Co. Ltd. (Tianjin, China); phosphate buffer solution (PBS), Dulbecco's Modified Eagle Medium (DMEM), and fetal bovine serum (FBS) were all purchased form Thermo Fisher Scientific (USA); MTT and DMSO were purchased from Sigma (USA); Trizol reagent was purchased from Invitrogen (USA); SYBR Green I and the PrimeScript™ RT reagent Kit was purchased from TaKaRa (Japan); and FITC phalloidin was purchased from Alexis (USA).

2.2. Preparation of the Composite Scaffolds

2.2.1. Preparation of HAP. HAP was synthesized using a reaction of $Ca(OH)_2$ and H_3PO_4 in water, based on the method described by Antebi et al. [45]. Briefly, in a CO_2-free atmosphere filled with Ar, 1 M of CaO was added to 500 mL distilled water, and 300 mL of a H_3PO_4 aquatic solution (2 mol/L) was slowly added (at about 0.5 mL/min) to the $Ca(OH)_2$ slurry to obtain a final Ca/P molar ratio of 1.67. After boiling for 2 days, the precipitated solid was collected by centrifugation and washed with distilled water. The precipitate was then redispersed in boiled distilled water. These washing and boiling procedures were repeated until the pH of the supernatant was approximately 7. Then, the HA precipitate was collected by centrifugation, washed with acetone, and dried at 110°C. The HA crystals were then ground and filtrated through an 80-mesh screen.

2.2.2. Preparation of HTP. HTP was prepared by calcining bovine cancellous bone [46], which was cut into cubes of 4 mm × 4 mm × 10 mm. After being incubated in 30% H_2O_2 solution for 30 min, the bone cubes were then stewed in a NaOH solution for 1 h to remove collagen and proteins. The bone cubes were then washed with water and immersed in 1% H_3PO_4 solution and heated at 125°C for 2 h. This was followed by washing with 100% alcohol to remove moisture, which had resulted in small cracks during the heat treatment. The bone cubes were then air-dried for 12 h and heated in a furnace (siliconit muffle furnace, Kwangsung Science Co., Korea) at a

rate of 10°C/min up to 1000°C. This process was continued for 2 h. Finally, the bone cubes were ground and filtered through an 80-screen cloth to obtain HTP particles with a diameter of 0.25–1 mm.

2.2.3. Preparation of the HAP- and HTP-Collagen Composite Scaffolds (HAPSs and HTPSs, Resp.). The composite scaffolds were prepared using the freeze-drying approach [47]. Because an acidic solution, such as acetic acid, would cause tissue inflammation, pure water was used to dissolve the collagen. Pure type-I collagen (Collagen Biotechnology Co. Ltd., China) was dispersed in distilled water at a concentration of 4 g/100 mL. A homogenizer (IKA, Germany) was used to achieve full dispersion. After the collagen was mixed with water thoroughly, HAP and HTP were added into slurries with constant stirring at 45 rpm for 24 h. The concentration of the bone particles was 36 g/100 mL. After degassing, the slurry was injected into stainless steel molds with dimensions of 5 mm × 5 mm × 10 mm. In order to densify the material, it was subjected to a constant pressure of 105 kPa for 2 h. The molds were then exposed to a temperature of −10°C for 2 h to freeze, and the mixture was then finally freeze-dried in vacuum to obtain HAPS and HTPS. The scaffolds were then exposed to 25 kGy gamma radiation for sterilization at room temperature.

2.3. Characterization of HAPS and HTPS

2.3.1. Morphology Analysis. The surface morphologies of HAPS and HTPS were examined by SEM using a Hitachi S-4800 SEM (Hitachi, Japan) with 15 kV accelerating voltage. Before the examination, both samples were heated at 40°C for 2 h to remove residual moisture and then underwent spray-gold.

2.3.2. Microstructure Analysis. In order to study the microstructures of the two samples, their N_2 adsorption isotherms were measured using an AUTOSORB-1 adsorption analyzer (Quantachrome, USA) at −196°C. The specimens were then dried at 200°C for 24 h in a N_2 atmosphere to remove the water on the solid surface. The specimens were then exposed to a temperature of 25°C in vacuum to attain a residual pressure of 10^{-4} Pa. The multipoint BET method was used to measure the specific surface area (S_A), pore volume (V_p), and average pore radius (P_R) of the two specimens [46]. Each group had five samples and the results were displayed in the form of mean ± standard deviation.

2.3.3. EDX Analysis. An Energy Dispersive X-ray (EDX) spectrometer (Hitachi S-4800) connected to the SEM was used to determine the elemental combination of the composite material. The specimens did not undergo spray-gold before examination.

2.3.4. FTIR Analysis. FTIR analysis using Shimadzu FTIR-8400S was conducted for the chemical analysis of HAPS and HTPS. The spectral range was set as 4000 to 400 cm^{-1} with a resolution of 4 cm^{-1} and scan time of 100. The samples were diluted to a concentration of 1% by mixing with KBr (infrared grade).

2.3.5. XRD Analysis. X-ray diffraction spectroscopy (XRD) was conducted to examine the crystal phase composition of the samples. A 0.5 mm × 0.5 mm area of each sample was randomly selected and the XRD spectra were acquired at room temperature using an X-ray diffractor (D/max-II; RIGAKU, Japan) with Cu Kα radiation. The range was 10–90° with a 0.2° step and 1 s/step scan speed (40 kV, 40 mA).

2.3.6. Analysis of the Mechanical Properties of HAPS and HTPS. To evaluate the strength of HAPS and HTPS, Young's moduli of all the specimens were determined using a Universal Testing Machine (Instron 300DX, US). Additionally, the Bio-Oss Collagen Young's moduli were also calculated as a positive control. The dimensions of each specimen were measured using a vernier caliper. These scaffolds were then placed on plates and compressed uniformly. They were then subjected to a force at the rate of 1 mm/min exerted on the top of the scaffolds. The load was augmented until the scaffolds crushed. Force and displacement data were recorded in order to generate the stress-strain curves. By measuring the slope of the stress-strain curve in the elastic region, Young's modulus could be calculated. Each group had five samples and the results were displayed in the form of mean ± standard deviation.

2.3.7. Water Absorption Rate Assay. The water absorption rates of HAPS and HTPS were also determined. Before the water was soaked, the specimens were weighed using an analytical balance (M_0) round to 0.001 g. The specimens were then immersed in 0.01 mol/L PBS (pH 7.4) for 15 min to achieve complete water absorption. The surface was then dried using filter paper and the specimens were weighed using an analytical balance (M_1) with the same accuracy. The water absorption rate was calculated using the formula $(M_1 - M_0)/M_0$. Each measurement for both the groups was repeated three times.

2.4. Evaluation of the Biological Effect of HAPS and HTPS on ADSCs

2.4.1. Cell Culture. ADSCs (Poietics Human Adipose-Derived Stem Cells) were purchased from ScienCell (ScienCell, USA) and cultured in 100 mm dishes with Dulbecco's Modified Eagle Medium (DMEM, GIBCO, USA) supplemented with 10% (v/v) fetal bovine serum (FBS, GIBCO, USA). The medium was replaced every other day and cells were expanded until passage 3. The cells were then digested using a trypsin-EDTA solution (Sigma, USA) and cell number was calculated using a blood counting chamber. The cell suspension was then centrifuged for 5 min at 1200 rpm at room temperature and resuspended in DMEM (10% FBS) to adjust the cell number to 1×10^7/mL. After placing the scaffolds in a 24-well tissue culture plate (CORNING, USA), each well was seeded with 200 μL of the cell suspension. After 1 h of cell adhesion at 37°C with 5% CO_2, 700 μL of DMEM (10% FBS)

Table 1: Primer sequences for RT-qPCR.

Primer number	Primer sequence (5′-3′)	Target gene	Product size (bp)
osgD145	CGACAGCAAGCCCAAGAG	ALP	110
osgD146	GTGGAGACGCCCATACCA		
osgD155	ACATCCGCTCCACAAACG	BMP-2	132
osgD156	GGTGCCACGATCCAGTCA		
osgD149	CTTCTCAGAGCCTCAGTCC	OCN	129
osgD150	ACCGTAGATGCGTTTGTAG		
ACTINF	ACTCGCTGCGCTCGGTCGTT	ACTIN	125
ACTINR	CCTTTTGCTGGCCTTTTGCTCAC		
osgD286	CACTCCAATCGTCCCTAC	OPN	127
osgD287	GTCCTCATCTGTGGCATC		
osgD316	CCCTGGACAGCCTGGACTT	COL1A1	95
osgD317	CATAGGACATCTGGGAAGCAA		
osgD290	ACCTTGGACGCCATGAAA	COL2A	102
osgD291	CTTGCTGCTCCACCAGTTT		

was added to each well and the medium was replaced every day.

2.4.2. Cell Proliferating Assay. After culturing for 1, 3, 5, and 7 days, cell proliferation was determined using the MTT method. In brief, each well was aspirated and washed with PBS three times. Next, 700 μL of DMEM (with 10% FBS) and 70 μL of MTT (0.5 mg/mL of water) solution was added into each well and incubated at 37°C at 5% CO_2 for 3 h in the dark. The medium was then aspirated and 700 μL of DMSO was added into each well to dissolve the formazan. After shaking gently on a shaker in the dark for 15 min, the absorbance at 450 nm was measured using a microplate reader (biokinetics reader, Bio-Tek Instruments, USA). Cells grown on the culture plates only were used as the control group. The measurements were performed in triplicate for each group.

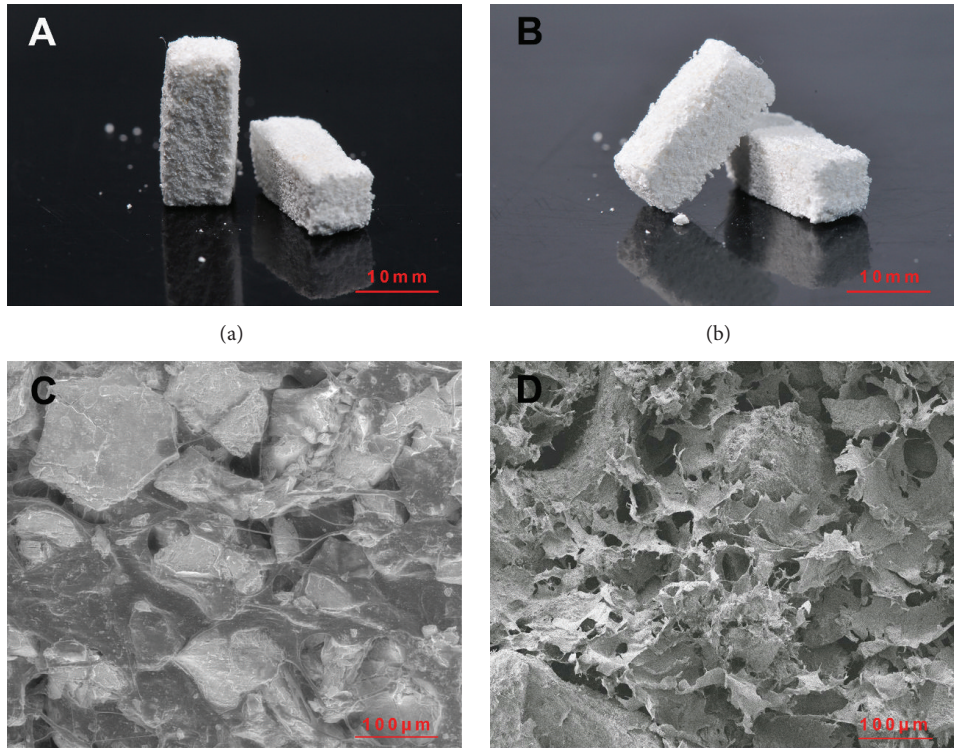
2.4.3. Quantitative Real-Time Polymerase Chain Reaction (RT-qPCR) Assay. Total RNA was extracted from the ADSCs after 3 and 5 days of culture on HAPS and HTPS using Trizol reagent (Invitrogen, USA) by following the manufacturer's protocol. SYBR Green I (TaKaRa, Japan) was used for quantitative real-time polymerase chain reaction and the PrimeScript RT reagent Kit was used for reverse transcription. The target PCR primers for ALP, BMP-2, OCN, OPN, COL1A1, COL2A, and ACTIN were designed using the Basic Local Alignment Search Tool® (Table 1). For each data point, there were three replicates. From each sample, cDNA was synthesized from 1 μg of total RNA using reverse transcriptase (TaKaRa). The qPCR mixture in each tube contained 10 μL of 2x SYBR Green I qPCR Mix (TaKaRa), 250 nM of each primer, PCR grade water, and 20 μg of cDNA. The PCR cycling program was set as follows: 95°C for 5 min initially, followed by 40 cycles at 95°C for 15 s, 58°C for 30 s, and 95°C for 15 s. The comparative Ct method (also known as the $2^{-\Delta\Delta Ct}$ method) was applied to analyze the dissociation curves.

2.4.4. Immunofluorescence Assay. In order to observe the morphology of the cells after seeding them on the scaffolds, the immunofluorescence staining of the cytoskeleton of the ADSCs was conducted. After 24 h of incubation on both scaffolds, the cells were gently washed with 0.01 M PBS three times and then fixed in 4% glutaraldehyde PBS solution for 20 min at room temperature. The cells were then again washed with PBS three times, and 0.5% Triton X-100 PBS solution was applied on the cells for 5 min to increase cell membrane permeability. After washing again with PBS, the cells were incubated with FITC phalloidin (Alexis, USA) for 20 min in the dark at room temperature. The cells were then washed with PBS three more times and then incubated in DAPI (5 μg/mL) in the dark at room temperature for 5 min to allow visualization of the nucleus. After washing with PBS again three times, the samples were mounted with 95% glycerin PBS solution. A confocal microscope (Olympus IX71, Fluoview, Japan) was used to obtain the images. An excitation filter at 490 nm and an emission filter at 530 nm were used to visualize FITC staining and an excitation filter at 340 nm and an emission filter at 488 nm were used for DAPI staining.

2.5. Statistical Analysis. The data are represented as the mean ± SD. In terms of statistical comparison, this study adopted the t-test or one-way ANOVA as well as the LSD test. $^{*}P < 0.05$ was considered to indicate statistical significance.

3. Results

3.1. SEM Observation. The pictures and SEM images of HAPS and HTPS are shown in Figure 1. The macroscopic shapes (Figures 1(a) and 1(b)) indicate that HAP and HTP were distributed in the collagen uniformly. The SEM images (Figures 1(c) and 1(d)) show that both the scaffolds share a similar structure with mineral crystals dispersing homogeneously in the collagen fiber grid. The diameters of the mineral crystals were in the range of 150–200 μm without significant



(a) (b) (c) (d)

Figure 1: Pictures and SEM images of HTPS and HAPS. (a) HTPS group; (b) HAPS group; (c) SEM images of the HTPS group, 500x; and (d) SEM images of the HAPS group, 500x.

differences between HTPS and HAPS, and HTPS was more porous than HAPS.

3.2. EDX Analysis. The EDX spectra were obtained for HAPS and HATS to determine the element composition. The EDX results (Table 2) show that both samples had similar elemental compositions, including carbon, oxygen, and nitrogen, which were derived from collagen and calcium and phosphorus were derived from the mineral components. HTPS had a lower calcium/phosphorus ratio than HAPS (HTPS: 1.58 : 1 and HAPS: 1.66 : 1), possibly because, during the preparation of HTP, HA reacted with H_3PO_4 and generated $Ca_3(PO_4)_2$ in an acidic environment:

$$Ca_{10}(PO_4)_6(OH)_2 + H^+ \longrightarrow Ca^{2+} + PO_4^{3-} \longrightarrow Ca_3(PO_4)_2 \quad (1)$$

In the HAPS, the molar ratio of Ca/P accords with HA (1.67). In the HTPS, the molar ratio was less than 1.67. After calculation, the HA/β-TCP ratio in HTPS was found to be 1 : 1.

3.3. FTIR Analysis. The FTIR spectra (Figure 2) of both groups show typical peaks at 1030 cm^{-1} and 602/566 cm^{-1}, which are indicative of the v_3 and v_4 vibrations of PO_4^{3-}, and at 1547 and 1600 cm^{-1}, which represent collagen (COO^-). Such patterns are consistent with the collagen and mineral (HA or HA/TCP) components in each of the samples. However, the peaks at 1547 and 1600 cm^{-1} demonstrated different patterns in HTPS and HAPS. In HTPS, the peaks were sharper than those in HAPS because of the relatively low hydroxyl content, which could form hydrogen bonds with carbonyl and result in weakened vibrations of carbonyl. Therefore, the peaks of COO^- would become less sharp with higher hydroxyl content, and the addition of β-TCP, which lacks the hydroxyl group, would sharpen the characteristic peaks of COO^-.

Table 2: Results of EDX analysis for HTPS and HAPS.

Element	At% HTPS	At% HAPS
CK	39.64	48.49
NK	05.40	06.12
OK	34.17	28.74
NaK	00.69	00.56
MgK	00.44	00.30
PK	07.47	05.62
ClK	00.15	00.93
CaK	12.04	09.28

3.4. XRD Analysis. The XRD spectra (Figure 3) indicate significant peaks the RDin the 23.4°–23.7° and 26.9°–27.4° ranges for HTPS (International Center for Diffraction Data, Joint Committee on Powder Diffraction Standards, CPDS number 41-0487) and a much lower peak at 40.4°–40.9°, which

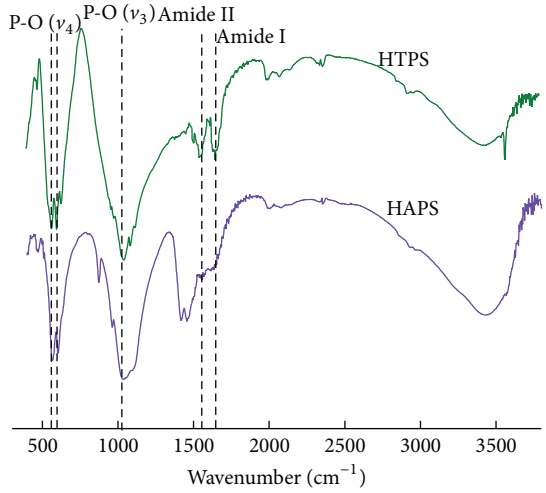


Figure 2: FTIR patterns of HTPS and HAPS. Both the groups share the same organic and inorganic composition. Note: green line: HTPS; purple line: HAPS. Dashed lines indicate similar symbolic peaks of the two groups: 1030 cm^{-1}: v_3 vibration of PO_4^{3-}; 602, 566 cm^{-1}: v_4 vibration of PO_4^{3-}; 1547 cm^{-1}: Amide II; and 1600 cm^{-1}: Amide I.

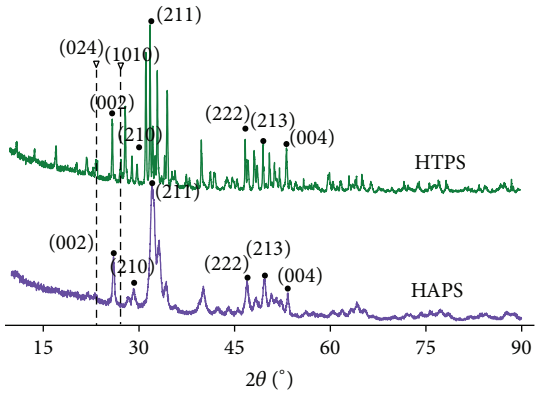


Figure 3: XRD spectra of HTPS and HAPS. Note: green line: HTPS; purple line: HAPS. Note: round symbols indicate the symbolic peaks of hydroxyapatite and triangles at 23.4°–23.7° and 26.9°–27.4° demonstrate the symbolic peaks of β-TCP in CHTS.

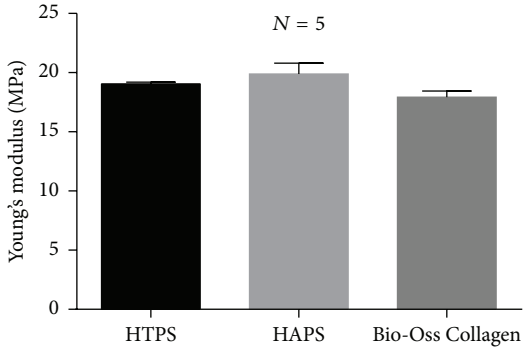


Figure 4: Young's moduli of HTPS and HAPS.

indicates the TCP phase; HAPS does not have such features. The HA peaks of HTPS overlapped with those of HAPS due to a similar $Ca_5(PO_4)_3(OH)$ phase. The peaks between 25° and 35° in both the groups indicated the amorphous structure of collagen [36].

3.5. Mechanical Properties (Young's Modulus). Figure 4 indicates that Young's modulus was 19.05 ± 0.088 (Mpa) in HTPS, 19.88 ± 0.530 (Mpa) in HAPS, and 17.93 ± 0.292 (Mpa) in Bio-Oss Collagen, respectively. No significant difference was observed between the three groups.

3.6. Porosity Analysis. The S_A, V_p, and P_R of HTPS and HAPS are shown in Figure 5. The S_A and V_p of both groups were similar. Although the values of S_A, V_p, and P_R of HAPS were less than those of HTPS, no significant differences were observed (HTPS: S_A: 77.17 ± 0.68 mm^2, V_p: 0.3520 ± 0.004 cm^2, and P_R: 18.64 ± 0.23 nm; HAPS: S_A: 76.03 ± 0.97 mm^2, V_p: 0.3433 ± 0.01 cm^2, and P_R: 18.04 ± 0.43 nm).

3.7. Water Absorption Behavior of HTPS and HAPS. The water absorption rate of HTPA and HAPS is shown in Figure 6. The absorption rate of HTPS was slightly lower than that of HAPS (HTPS: 1.793 ± 0.08410 and HAPS: 1.709 ± 0.05476), but the difference was not significant. This indicates that the water absorption ability and stability in

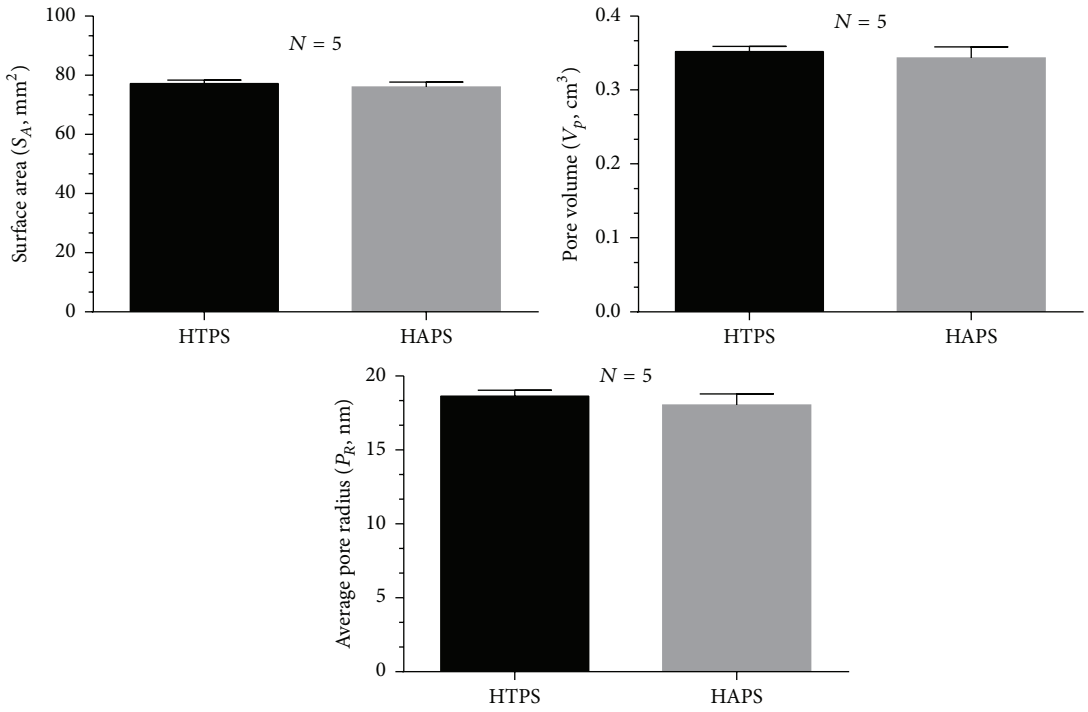


Figure 5: Porosity analysis of HTPS and HAPS.

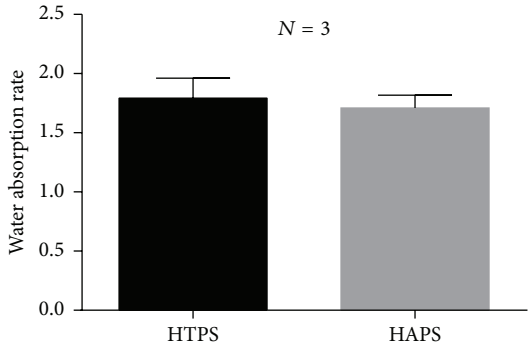


Figure 6: Water absorption rate of HTPS and HAPS.

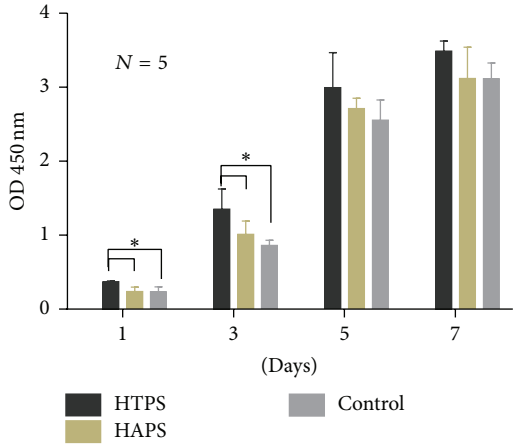


Figure 7: MTT line graph of HTPS and HAPS.

aqua was similar for HAPS and HTPS. Such differences could be attributed to the higher porosity of HTPS, which would also enhance the exchange of substances in a cell growth environment.

3.8. MTT Assays. Cell proliferation on the scaffolds can reflect the toxicity and biocompatibility of the scaffold materials. MTT assays were, therefore, conducted to determine the proliferation of ADSCs on both HAPS and HATS. The growth curves (Figure 7) indicated that the overall growth tendencies in both groups were similar during the culture process. However, the cell proliferation was higher on HTPS from day 1 to day 7; particularly, significant differences could be detected on the first and third day. This is partly because the fast degradation of β-TCP led to an increase in the concentration of calcium and phosphate during this period. The growth tendency of the cells on HAPS and in the control group did not show significant differences at all the four indicated time points.

3.9. Gene Expression and Immunofluorescence Assay. Osteogenesis-related gene expression could reflect the differentiation of ADSCs into osteoblasts, the key seed cells in bone tissue engineering. After the culture of ADSCs for 3 and 5 days, the mRNA levels of ALP, BMP-2, OCN, OPN, COL1A1, COL2A, and ACTIN were examined using RT-qPCR, and the results are shown in Figure 8. The expression of ALP in the cells cultured with HTPS on day 3 was significantly higher than that in the cells cultured with HAPS. On day 5, both groups showed similar ALP expression. OCN expression

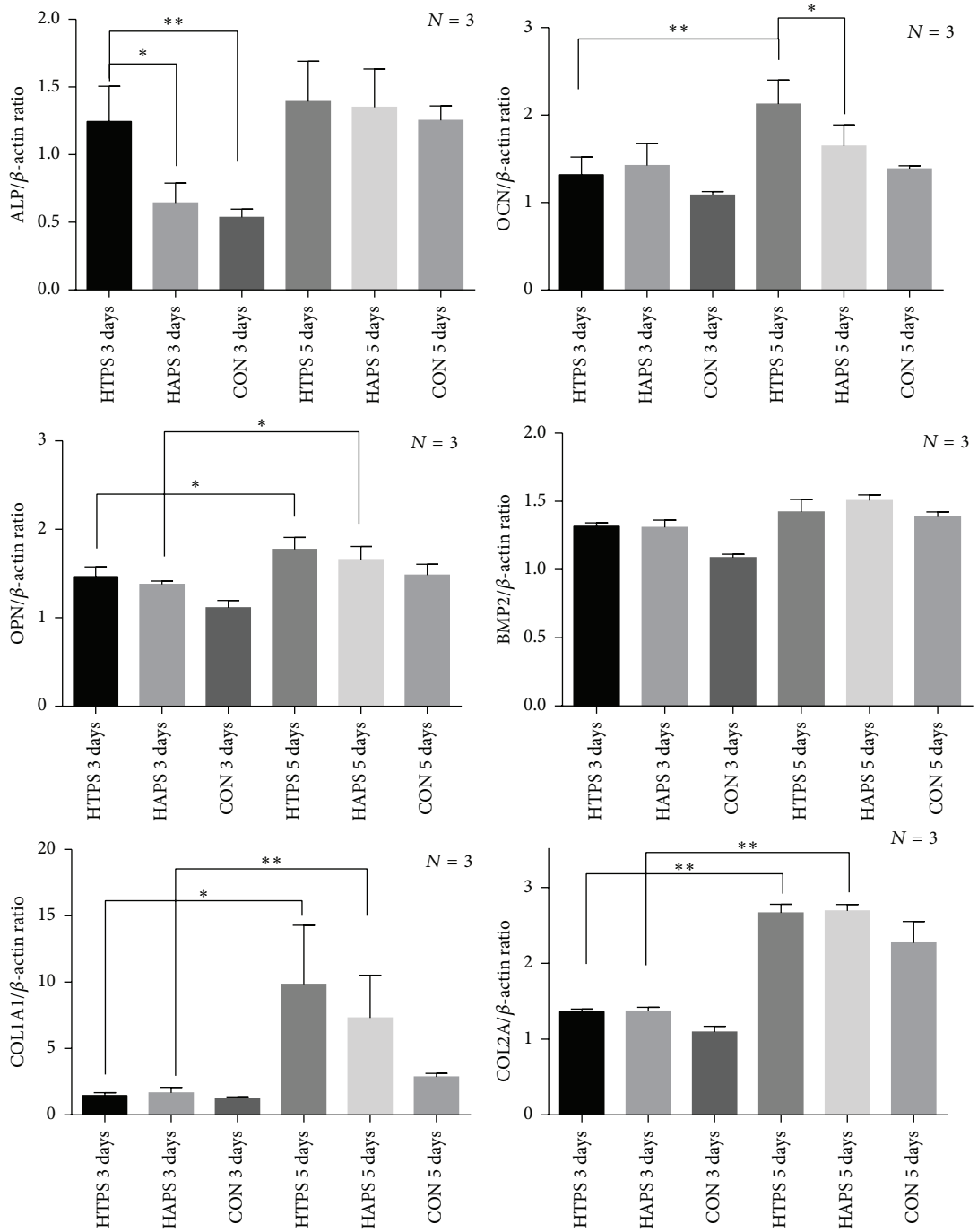


Figure 8: Gene expression of ALP, BMP-2, OCN, OPN, COL1A1, and COL2A in ADSCs cultured on HAPS and HTPS for 3 and 5 days. Note: $^{*}P < 0.05$, $^{**}P < 0.01$.

increased significantly from the third to the fifth day in both groups, and the cells cultured with HTPS showed a significant higher level than those cultured with HAPS. The OPN mRNA level in both groups increased significantly from the third to the fifth day, but the difference between the groups was not significant. Similarly, the expression of BMP2 increased slightly in both groups from the third to the fifth day, but no significant differences were observed between the groups. The expression of both COL1A1 and COL2A increased significantly from the third to the fifth day in both groups, but the level of COL1A1 was much higher than that of COL2A.

The cytoskeleton is a dynamic system of many cellular functions and could be an excellent indicator of the behavior of the ADSCs (Figure 9). By staining the cytoskeleton and nucleus, the morphology of the ADSCs could be easily detected with fluorescence microscopy. At 24 h, the cells on HAPS (Figures 9(a) and 9(b)) exhibited an elongated spindle

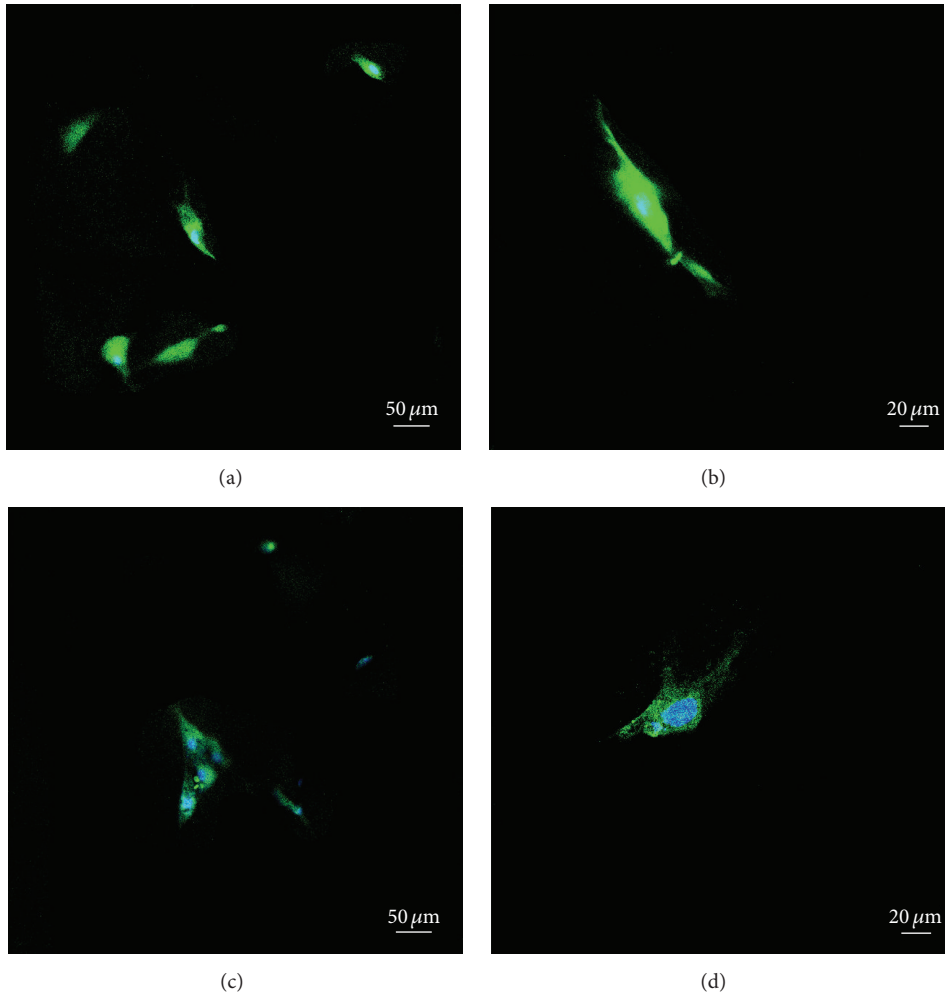


Figure 9: Fluorescence images of the ADSC seeded on HAPS and HTPS. Note: (a and b): ADSCs on HAPS at 24 h, 10x and 20x objective lenses, respectively, and (c and d): ADSCs on HTPS at 24 h, 10x and 20x objective lenses, respectively.

shape, which is typical of MSCs. However, the cells on HTPS started to show typical osteoblast-like morphologies with a big nucleus and polygonal appearance (Figures 9(c) and 9(d)).

4. Discussion

Bioscaffolds have played an important role as one of three main elements of tissue engineering [47] and have been explored widely. In this study, type-I collagen composite scaffolds with two different mineral particles were prepared. Pure water was used to dissolve the collagen with mechanical stirring instead of an acid solution (e.g., acetic acid) to avoid the irritation caused by acidic solutions. To improve the mechanical property of collagen, crosslinking using glutaraldehyde [48] or EDC/NHS [49] was conducted. However, the inevitable residual would cause cytotoxicity and influence the healing process. Therefore, mechanical pressing was used to enhance the strength of the composite scaffolds without any negative effects on the biocompatibility.

Compared with HA prepared by chemical synthesis, inorganic bovine bone has better bone formation ability because its components and microstructure are similar to that of the natural bone [50–52]. In this study, we prepared two mineral particles, HAP and HTP. In the preparation of HTP, the addition of H_3PO_4 enabled a portion of HA to be transformed into TCP. As a result, the proportion of calcium and phosphorus in HTP was lower than that in HAP, as determined from the EDX spectrum (1.58 : 1 versus 1.66 : 1). XRD and FTIR analyses also confirmed the existence of β-TCP. HTP degrades and resolves faster because of the high biodegradation rate and solubility of β-TCP, which is suitable for tissue engineering applications [53]. With the addition of the β-TCP solution, the concentration of calcium and phosphate in the surroundings promotes osteogenesis by favoring the synthesis of osteoinductive growth factors and by upregulating adenosine signaling in phosphate metabolism [54–56]. The differentiation of osteoblasts and the subsequent bone formation, including the secretion and mineralization of ECM, can also be facilitated with the use of β-TCP [57].

Therefore, the use of β-TCP in HTPS could attain better biological effects than using HA alone.

Excellent mechanical property not only provides a mechanical support for bone formation, but also makes the materials conducive for use in clinical applications. Both HAPS and HTPS have Young's moduli similar to that of Bio-Oss Collagen, which has been widely used in clinical practice [58, 59]. Although we were unable to attain a Young modulus equivalent to that of the natural bone, the mechanical strength achieved by using these biomaterials is stronger than that obtained by using collagen alone [60] and is enough for dental applications in which the tensile stress is relatively lower [46]. Properties such as water absorption and porosity can allow the blood to infiltrate the graft quickly when implanted in the recipient and allow material exchange, which could promote cell proliferation. Besides, good water absorption can enable doctors to manipulate grafts conveniently by mixing the materials with normal saline or blood. No significant difference was detected in the water absorption rate between the two groups.

Stem cell-based therapy has become a promising tool in some fields such as craniofacial bone regeneration and spine surgery, and a randomized controlled clinical trial has shown positive effects of such therapy [61]. In our study, we tested the effects of the two types of biomaterial scaffolds on the proliferation and osteogenic differentiation capacity of ADSCs. Additionally, fluorescence images provided direct evidence indicating that the ADSCs cultured on HTPS could achieve osteogenesis earlier than those on HAPS. The MTT assays also suggest that the cells in both the groups showed higher (HTPS) or similar (HAPS) proliferation activity compared with the control group. However, from the first day, cell proliferation on HTPS was higher than that on HAPS, and this trend was observed until day 7. RT-PCR results indicate that the expression of all the osteoblastic genes except BMP2 was upregulated in both the groups. ALP is an extensively studied enzyme that is produced by osteoblasts, making it an important osteoblastic indicator. Osteocalcin (OCN) appears in the late stage of osteoblastic differentiation, while osteopontin (OPN) appears in the early stage [60]. In the HTPS group, ALP was upregulated significantly both on the third and on the fifth day. Both groups showed similar ALP expression on the fifth day. Similarly, both OCN and OPN were upregulated on the fifth day. The expression of these genes was in accordance with the results reported in previous literature on the osteogenic differentiation of mesenchymal cells on a TCP matrix [62–64]. COL1 and COL2 are two important components of the ECM. The expression of COL1 and COL2 increased in both the groups, indicating an increase in the synthesis of nonmineralized ECM, which is an important step in bone formation [60]. This phenomenon could be attributed to the function of collagen, whose ability to enhance collagen synthesis has been studied extensively [65]. Therefore, although both the scaffolds could enhance the osteogenic differentiation capacity of the ADSCs, HTPS could achieve a better result, which was also verified by the fluorescence images. The osteogenic differentiation also occurred earlier in the HTPS group than in the HAPS group.

5. Conclusion

In this study, two composite collagen scaffolds, HAPS and HTPS, were prepared using mineral particles distributed homogeneously in type-I collagen grids. HTPS contained 50% β-TCP, which enabled better performance with regard to the promotion of the osteogenic differentiation of the ADSCs. Therefore, HTPS can be a novel candidate for use in stem cell-based therapy.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

Qing Li and Tong Wang equally contributed to this paper.

Acknowledgments

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Review Article

Applications of Mesenchymal Stem Cells and Neural Crest Cells in Craniofacial Skeletal Research

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Craniofacial skeletal tissues are composed of tooth and bone, together with nerves and blood vessels. This composite material is mainly derived from neural crest cells (NCCs). The neural crest is transient embryonic tissue present during neural tube formation whose cells have high potential for migration and differentiation. Thus, NCCs are promising candidates for craniofacial tissue regeneration; however, the clinical application of NCCs is hindered by their limited accessibility. In contrast, mesenchymal stem cells (MSCs) are easily accessible in adults, have similar potential for self-renewal, and can differentiate into skeletal tissues, including bones and cartilage. Therefore, MSCs may represent good sources of stem cells for clinical use. MSCs are classically identified under adherent culture conditions, leading to contamination with other cell lineages. Previous studies have identified mouse- and human-specific MSC subsets using cell surface markers. Additionally, some studies have shown that a subset of MSCs is closely related to neural crest derivatives and endothelial cells. These MSCs may be promising candidates for regeneration of craniofacial tissues from the perspective of developmental fate. Here, we review the fundamental biology of MSCs in craniofacial research.

1. Introduction

Developmental origins are beginning to be elucidated through rigorous studies in stem cell biology. Recent studies have demonstrated that the basis of regenerative medicine can be found in developmental biology. Indeed, many applications in regenerative medicine mimic the development and healing of specific tissues.

Mesenchymal stem cells (MSCs) are commonly used in both basic and clinical studies because they can be easily identified in adult tissues. MSCs were first identified as fibroblast-like cells in the bone marrow [1], resemble colony forming unit-fibroblasts (CFU-Fs) at clonal density, and have the capacity for differentiation into mesenchymal lineages, such as bone, cartilage, and fat [2]. Notably, MSCs and neural crest cells (NCCs) are both used in various approaches in craniofacial biology because of their developmental similarities. Indeed, the craniofacial mesenchyme developmentally originates from NCCs [3–5], and NCCs are developmentally identified at the embryonic stages [6]. It is difficult to isolate NCCs because of their limited accessibility and ethical concerns; therefore, it is difficult to directly use NCCs in patients. In contrast, MSCs are present in easily accessible adult tissues, such as bone marrow, fat tissues, and synovium, enabling facile isolation. Importantly, MSCs and NCCs have similar self-renewal and differentiation potential, and MSCs are capable of differentiating into neuronal cells [7, 8]. Furthermore, MSCs can also differentiate into endothelial cells [9–11] and are indispensable for tissue formation [12, 13]. Similar findings have also been reported in skeletal tissues [14, 15], suggesting that adult MSCs may be useful in clinical applications associated with the regeneration of skeletal tissues, particularly because of the necessity for synchronized neural tissue formation and vascularization.

Skeletal stem cells (SSCs), which were recently identified [16, 17], have been shown to contribute to the construction

of skeletal tissues during development and wound healing. However, the formation and regeneration of skeletal tissues involve not only construction of bone, but also vascularization and neural synchronization [18–20]. Despite this fact, few studies have evaluated these processes with regard to SSCs.

NCCs, MSCs, and SSCs are all isolated using culture and exhibit overlapping self-renewal and multipotent differentiation potential. Thus, clarifying the specific characteristics of each cell type will improve the clinical application of these types of stem cells. In this review, we discuss the fundamental biology of stem cells in craniofacial research.

2. Stem Cells in Craniofacial Research

Skeletal tissues are composed of a network of hard tissues, including bone and cartilage. The jawbone and teeth comprise the craniofacial region, and many individuals suffer from skeletal diseases, such as metastasis of oral malignant tumors into the bone, congenital craniofacial malformation, severe periodontitis, and medication-related osteonecrosis of the jaw [21]. These diseases cause eating difficulty, aesthetic disorders, respiratory distress, and speech disorders, leading to decreased quality of life. Current fundamental approaches to these diseases include surgery and subsequent reconstruction using artificial materials or xenobiomaterials. However, natural bone formation and healing using autologous cells are preferable. Therefore, development-based medicine and approaches are desired.

During the most recent decade, stem cell research has made great advances in clarifying the mechanisms of tissue development. Indeed, many stem cell researchers have focused on developmental biology and regenerative medicine, and applications of stem cells in craniofacial research have been proposed. In particular, MSCs and NCCs have been studied extensively in craniofacial research. MSCs partially originate from NCCs [7, 22–25]; therefore, some MSCs may also differentiate into neuronal cells [26–28], suggesting that specific subsets of MSCs may have the same potential as NCCs. Moreover, because MSCs are present in several adult tissues [2], they are easy to isolate and expand *in vitro*.

Notably, dental-specific MSCs have been identified in craniofacial tissues. Several research groups have reported the presence of dental MSCs in dental pulp stem cells (DPSCs) [29], stem cells from exfoliated deciduous teeth (SHED) [30], periodontal ligament stem cells (PDLSCs) [31], and stem cells from apical papilla (SCAP) [32]. These dental MSCs may have applications in degenerative and intractable diseases [33]. Furthermore, conditioned medium (CM) from dental MSCs supplies paracrine factors and may be effective for injured areas [34, 35]. Osugi et al. reported that SHED-CM promoted the growth of bone mass in a calvarial defect model. Additionally, the use of conditioned medium from dental tissue-derived MSCs is a unique approach for craniofacial regenerative medicine without cell transplantation. This approach may reduce costs and time/labor requirements and may alleviate safety concerns [36].

Bone marrow MSCs are utilized as a typical model for clinical studies and basic biology research, and they are classically defined by conventional culture, as MSCs show vigorous expansion and multipotent differentiation. However, the mechanisms of regeneration in conventional MSCs cannot be traced back to developmental fate, and it is difficult to predict which subsets of a crowded cell population contribute to the development of specific target tissues. Thus, mixed cultures of conventional MSCs may not provide consistent, predictable therapeutic outcomes from a developmental biological viewpoint. Further studies are needed to clarify the development and degeneration of craniofacial skeletal tissues using stem cells, particularly for analysis of the clonal phenotype of NCCs and MSCs.

3. Purified MSCs Are Partially Derived from NCCs

Cranial NCCs constitute a major part of facial tissues [3, 37]. Moreover, NCCs are localized in the neural folds of fetal tissues, migrate into various tissues, and regulate skeletal tissues [38]. Cranial NCCs form the first pharyngeal arch innerved by the trigeminal nerve and the second pharyngeal arch innerved by the facial nerve [39–41]. Then, the first and second pharyngeal arches interact with each other and form craniofacial tissues [42, 43]. Craniofacial tissues are mainly composed of bone, muscle, and tendon together with the neurovascular bundle [44]. Therefore, improving the understanding of neurogenesis and vascularization in skeletal tissues is essential for discussions of craniofacial tissue formation. To identify and track the process of cranial tissue specification, visualization of the neural crest and its derivatives is needed.

Within the last few decades, transgenic technologies have been developed that enable visualization of target cells for prospective and retrospective analyses [45]. Transgenic animal studies can be used to elucidate what and how specific cells contribute to form target tissues. This approach has also been applied to the craniofacial field, where NCCs and their derivative cells are labeled and their fates are analyzed [4, 46, 47]. This transgenic technology provides clear information on the development of NCCs in the craniofacial field and enables the visualization of NCCs and their derivatives within dental and craniofacial tissues as shown in Figure 1(a).

Some researchers have focused on cells that are easier to obtain than embryonic NCCs, such as bone marrow stem cells or pluripotent stem cells. The bone marrow is a typical source of MSCs and contains two distinct types of stem cells, hematopoietic stem cells (HSCs), and MSCs [48]. Transgenic animal studies have demonstrated that MSCs partially originate from NCCs. NCCs delaminate from neuroepithelial cells, which can differentiate into cells expressing the MSC marker platelet-derived growth factor receptor alpha (PDGFRα) (CD140a) [22]. Thus, neuroepithelial cells are a source of MSC differentiation. Moreover, Nagoshi et al. showed that NCCs migrate though the aorta-gonad-mesonephros region and circulate into the bone marrow, demonstrating how neural crest-derived stem cells can travel to the bone marrow [23]. Additionally, Morikawa

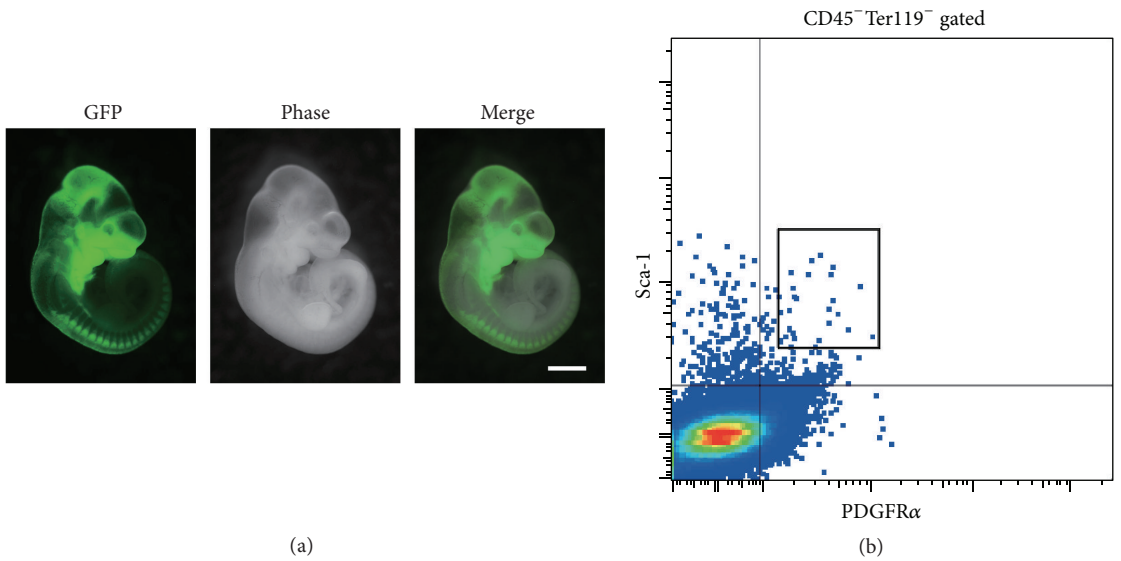

Figure 1: (a) Neural crest lineage labeling mouse (Wnt1-Cre/GFP) clearly demonstrates green fluorescence-positive NCCs in craniofacial tissues at embryonic day 11. Scale bar, 1 mm. (b) Murine bone marrow cells were analyzed by flow cytometry. The chart shows that cells expressing hematopoietic lineage markers (CD45 and Ter119) were negatively gated, and the highly potent murine MSC marker-expressing (PαS) subset is indicated by the black-colored box.

et al. reported a subset of highly potent murine MSCs, characterized by the cell surface marker combination of PDGFRα, stem cell antigen-1 (Sca-1/lymphocyte activation protein; Ly-6), CD45, and Ter119 (Ly-76). This specific subset, PDGFRα^+Sca-1$^+$CD45$^-$Ter119$^-$ (PαS), could be found in the bone marrow as shown in Figure 1(b) [49]. The PαS subset partially originates from NCCs as shown by developmental fate analysis using transgenic mice [7]. Thus, application of cell tracking systems in mice has clarified the relationship between MSCs and NCCs, supporting the application of MSCs in craniofacial skeletal tissue research.

The PαS subset of MSCs has been identified as the perivascular niche and has differentiation potential to both mesenchymal and neural crest lineages [7]. Indeed, the PαS subset promotes neural crest-derived periodontal tissue regeneration [50]. PαS cells are closely related to SSCs, which are identified by rigorous assays. Bone marrow MSCs also contain stem cells that can only differentiate into skeletal tissues. Thus, SSCs represent a reservoir for bone-forming cells and have the potential to shape and regulate the local microvascular network in the bone marrow [51].

Worthley et al. reported that Gremlin-1 functions as a specific marker of skeletal stem cells in the long bone marrow. Gremlin-1 is a bone morphogenic peptide (BMP) antagonist, and the transforming growth factor (TGF)-β/BMP signaling pathway regulates osteoblastogenesis and bone formation [52]. In Gremlin-1-overexpressing transgenic mice, Gremlin-1$^+$ cells differentiate into bone, cartilage, and reticular stromal cells. Postnatally, Gremlin-1$^+$ cells also contribute to development. Gremlin-1$^+$ cells are not further enriched for PαS cells. However, the PαS subset also contributes to the formation of skeletal tissues [53]. Therefore, some MSCs in the PαS subset may have the differentiation potential of SSCs. Consistent with this notion, Gremlin regulates developing limbs, and Gremlin-1$^+$ and PαS cell subsets have been identified in the long bone [17, 49, 54, 55]. Additionally, recent reports have shown that cranial MSCs are different from long bone marrow MSCs [56].

Zhao et al. reported that glioma-associated oncogene homolog-1 (Gli-1) is a marker of craniofacial-specific MSCs in cranial bones [57]. Gli-1$^+$ cells are not coexpressed with classical MSC markers *in vivo*. Although the specific characteristics of these cells have not been defined *in vivo*, Gli-1$^+$ cells show typical phenotypes of MSCs, such as vigorous expansion, expression of classical MSC markers, and differentiation to mesenchymal lineages *in vitro* [44, 57]. However, Gli-1 is not always expressed in MSCs during development, in contrast to PDGFRα. Notably, no direct gene regulation mechanism has been identified between Gli-1 and PDGFRα [58]. Gli-1 is induced by sonic hedgehog (Shh) signaling and is associated with transient Sox2 expression during tooth formation [59]. Shh signaling has also been detected in Hertwig's epithelial root sheath (HERS) and found to lead to tooth root formation [60], suggesting that Shh signaling may promote Gli-1 expression in craniofacial-specific mesenchyme. Although Gli-1$^+$ cells are not present in the perivascular niche [57], MSCs in long bones are regulated by the perivascular niche [61–65]. The leptin receptor (LepR) (CD295) has been reported as an excellent marker for MSCs. LepR$^+$ cells are identified around sinusoids [61, 66]. In contrast, MSCs in the cranial bone suture are found around the midline of the suture structure. This specificity of craniofacial MSCs does not correspond

to MSCs in long bone marrow. These differences in murine craniofacial and long bone MSCs should be examined in greater detail in future studies.

4. Purified MSCs Can Be Derived from Induced Pluripotent Stem Cells (iPSCs)

To achieve the transition of stem cell research from the bench to the bedside, more studies investigating human cells are required. Human MSCs can be found in several types of tissues. The classical definition of MSCs is the same in mice and in humans, and conventional MSCs can be contaminated by other cell lineages. To avoid such problems, more studies of cell markers and selection of target cells are needed. Previously, Mabuchi et al. identified the highly specific human MSC markers, low-affinity nerve growth factor receptor (LNGFR), and thymocyte antigen 1 (THY-1) [67]. Cells expressing LNGFR and THY-1 have been identified in the bone marrow, and the combination of LNGFR and THY-1 cell surface markers characterizes a distinct subset of MSCs with a hematopoietic lineage. $LNGFR^{+}THY\text{-}1^{+}$ cells have high colony forming potential and differentiate into mesenchymal lineages. These specific cells have also been identified in decidua, fat tissues, synovium, and dental pulp [67–69]. The $LNGFR^{+}THY\text{-}1^{+}$ subset shows highly potent self-renewal and differentiation capacity both *in vitro* and *in vivo* and is also associated with the expression of other classical MSC markers, such as CD29 (integrin beta 1), CD44 (homing cell adhesion molecule; HCAM), CD73 (ecto-5′-nucleotidase), CD105 (endoglin), CD146 (melanoma cell adhesion molecule; MCAM), and CD166 (activated leukocyte cell adhesion molecule; ALCAM) [67, 69]. Efficient procedures for purification will improve the ability to analyze MSCs.

In craniofacial diseases, there is a great need for reconstruction of large areas affected by disease or injury. Thus, it is necessary to obtain large numbers of MSCs for such clinical applications. This must be achieved without damaging original tissues; therefore, specific cells derived from pluripotent stem cells based on developmental research may help to overcome this problem.

Human iPSCs were first generated in 2007 and have been used extensively in biomedical studies [70]. Human iPSCs have the capacity for self-renewal and can be expanded relatively easily. The strong potential of iPSCs can also be applied in craniofacial research. MSCs can be derived both directly from iPSCs and indirectly from neural crest like cells using specific markers and culture conditions [24, 71]. MSCs induced from iPSCs have been used in the regeneration of periodontal tissues [72]. Thus, these findings suggest that iPSC-derived MSCs have the capacity for use in craniofacial tissue regeneration.

5. Application of Human MSCs in Craniofacial Research

Craniofacial connective tissues originate from neural crest-derived ectomesenchyme, which is a source of many craniofacial bone and cartilage structures. Umeda et al. reported the generation of ectomesenchymal cells through neural crest-like progeny from human iPSCs [73]. Sensory innervation is necessary for maintaining sound bone. In dentistry, sensory innervation of craniofacial tissues involves the trigeminal nerve. Previously, several methods were reported for induction of peripheral nerve formation using NCCs [74, 75]. Application of sensory neuronal cells to craniofacial skeletal tissues requires neural crest-derived craniofacial-specific sensory neuronal induction. Dincer et al. reported that the craniofacial placode can be used to identify the craniofacial trigeminal nerve [76]. However, induction procedures for craniofacial target tissues are a relatively new approach in regenerative medicine and disease-specific iPSC technology. Further studies are needed to provide clear information on neural crest biology and craniofacial specificity. Methods for induction of target cells must take advantage of the generation of a sufficient number of cells, and induction based on the basic knowledge of developmental biology may provide an evidence-based approach for application of stem cells in the clinical field.

For application of human iPSC-derived cells in the regeneration of craniofacial tissues, prevention of teratoma formation represents a major challenge. iPSC lines show variations in the patterns of teratoma formation in iPSC-derived neural progenitor cells [77, 78]. Surprisingly, despite the exclusion of pluripotent markers, iPSCs may form teratomas in some cases. Lee et al. reported that iPSC-derived neural crest-derived stem cells, which exhibit downregulation of polysialic acid-neural cell adhesion molecule (PSA-NCAM), tend to form teratomas [79]. These findings suggest the importance of basic and preclinical studies of iPSC-derived NCCs. Recent studies have shown the safety of iPSC-derived cells in preclinical models [80]. Reconstruction of the target craniofacial tissues in nonhuman primate models is essential before human clinical studies using iPSCs can be initiated. Prescott et al. showed that *cis*-regulatory divergence is associated with differences in quantitative expression in human and chimpanzee cranial NCCs derived from iPSCs [81]. It is quite important that this study demonstrated the novel application of iPSC-derived NCCs for analyzing evolutionary cellular anthropology in the context of craniofacial development.

Thus, iPSC technology may facilitate future applications in regenerative medicine if the risk of iPSC-derived tumor formation can be minimized.

6. Conclusion

Skeletal tissues are composed of bone, cartilage, and tendon. These mesenchymal tissues are generated from NCC-derived MSCs and exhibit neurogenesis and neovascularization. MSCs are part of the perivascular niche and overlap with neural crest-derived stem cells [25]. MSCs have potential for differentiating into skeletal cells, neuronal cells, and endothelial cells, suggesting that MSCs may be useful for craniofacial tissue regeneration. Indeed, MSCs have been applied for the treatment of craniofacial diseases, such as periodontitis and osteonecrosis of the jaw in small animal models [82, 83]. The potential of stem cells is typically demonstrated using mice and rats because they are easy to breed and handle, and there

is a variety of well-established disease models recognized by the scientific community. However, the craniofacial anatomy of these animals is different from that of humans. Several research groups reported stem cell approaches using larger animals such as pigs, dogs, and chimpanzees [32, 81, 84]. In animal studies, establishment of live cell imaging systems can be used to clearly visualize the potential for migration and differentiation. Such imaging systems have been utilized in the craniofacial field [69, 85]. Demonstration of cell tracking in large animal models such as nonhuman primates provides essential evidence for human preclinical studies that expected to take place in the near future.

Human clinical application of stem cells has already started [86, 87]; however, the current protocols for clinical application mainly utilize conventionally cultured cells. Conventional MSCs contain various types of cells within adherent culture, resulting in contamination of the MSCs with other cell lineages. Moreover, MSCs show great differences in characteristics between long bones and craniofacial tissue, and these differences should be evaluated in detail in future studies.

For further analyses of the applications of MSCs in humans, the developmental fate of human MSCs must be elucidated. Sufficient quantities of purified MSCs from adult tissues for reconstruction of large spaces in the craniofacial region are difficult to collect. Human iPSC technology may be used to overcome this problem. Furthermore, more analyses of MSCs, NCCs, SSCs, and iPSCs are required based on a developmental biological approach, which will be expected to provide evidence-based methods for the treatment of various craniofacial diseases.

Conflict of Interests

Hideyuki Okano is a paid scientific advisor of San Bio Co. Ltd. The other authors have no conflict of interests to declare.

Authors' Contribution

Satoru Morikawa and Takehito Ouchi contributed equally to this work.

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Research Article

Lavandula angustifolia Extract Improves the Result of Human Umbilical Mesenchymal Wharton's Jelly Stem Cell Transplantation after Contusive Spinal Cord Injury in Wistar Rats

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Introduction. The primary trauma of spinal cord injury (SCI) results in severe damage to nervous functions. At the cellular level, SCI causes astrogliosis. Human umbilical mesenchymal stem cells (HUMSCs), isolated from Wharton's jelly of the umbilical cord, can be easily obtained. Previously, we showed that the neuroprotective effects of *Lavandula angustifolia* can lead to improvement in a contusive SCI model in rats. *Objective.* The aim of this study was to investigate the effect of *L. angustifolia* (Lav) on HUMSC transplantation after acute SCI. *Materials and Methods.* Sixty adult female rats were randomly divided into eight groups. Every week after SCI onset, all animals were evaluated for behavior outcomes. H&E staining was performed to examine the lesions after injury. GFAP expression was assessed for astrogliosis. Somatosensory evoked potential (SEP) testing was performed to detect the recovery of neural conduction. *Results.* Behavioral tests showed that the HUMSC group improved in comparison with the SCI group, but HUMSC + Lav 400 was very effective, resulting in a significant increase in locomotion activity. Sensory tests and histomorphological and immunohistochemistry analyses verified the potentiation effects of Lav extract on HUMSC treatment. *Conclusion.* Transplantation of HUMSCs is beneficial for SCI in rats, and Lav extract can potentiate the functional and cellular recovery with HUMSC treatment in rats after SCI.

1. Introduction

There are approximately 200,000 spinal cord injuries (SCIs) annually in the United States, the vast majority of which are caused by motor vehicle accidents [1]. SCI can result in severe damage to the motor, sensory, and autonomic nervous systems and their function and may lead to paraplegia and severe disabilities [2].

The pathogenesis of SCI after the primary trauma plays an important role in initial tissue disruption, and the subsequent series of secondary cellular processes can lead to long-term spinal deficits [3, 4]. Increased oxidative stress [5] and activation of redox transcription factors, as well as elevated expression of inflammatory mediators, may play some of the most important roles [6] in promoting secondary injuries after SCI. SCI may be followed by the degeneration of axons, the loss of neurons as well as glia, and demyelination around the lesion site. Axonal regeneration in the central nervous system (CNS) is impeded partly by myelin-associated inhibitors [7, 8] and the formation of a postlesion scar barrier [9]. The extent of intrinsic cell renewal alone [10], even after the application of mitogenic agents, such as epidermal growth factor and fibroblast growth factor-2 [11, 12], is not sufficient to allow substantial recovery following SCI [13]. Therefore,

therapeutic strategies such as exogenous cell replacement should be considered. Human umbilical mesenchymal cells (HUMSCs) from Wharton's jelly possess stem cell properties [14] and express type I MHC molecules, MSC markers (SH2 and SH3), and adhesion molecules (CD44 and CD105), but not type II MHC and hematopoietic markers (CD34 and CD45) [15]. HUMSCs from Wharton's jelly are primitive, uncontaminated, and immunotolerable [15] and are a low-cost source of stem cells that can be easily obtained and propagated in culture without invasive medical procedures or ethical issues [16]. These cells can be induced to form other cell lineages, such as neurons and glial cells [17, 18].

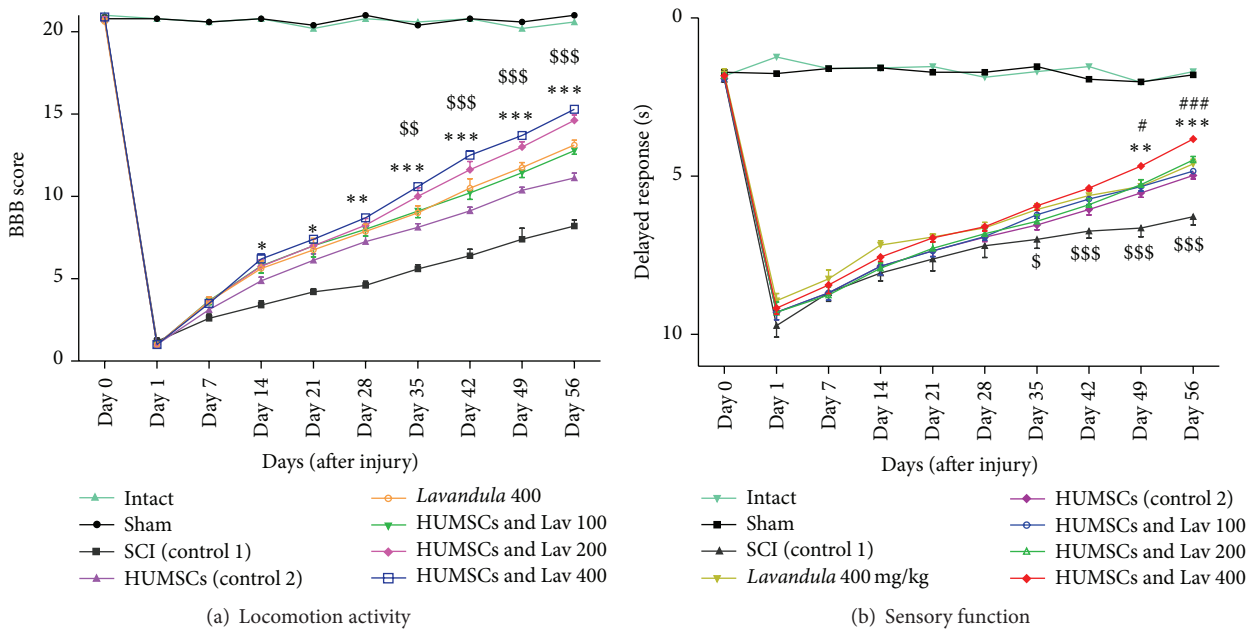
HUMSCs are also capable of differentiating into osteogenic, chondrogenic, adipogenic, and myogenic cells *in vitro* [19]. HUMSCs might be a good stem cell source for transplantation [20]. There is ample evidence that stem cell therapy could be effective in SCI [21], but we need a strategy to potentiate these stem cell transplantation results. As there has been some interest in finding natural agents that may help to prevent the inflammation and degeneration of neural cells in SCI, one of the well-known herbal drugs that has demonstrated antioxidant effects is Lavender. Lavender, or *Lavandula angustifolia* Mill. (Lamiaceae), commonly known in Iran as "Ostokhoddous," is a widely distributed aromatic herb [22]. It has been used widely for nervous system problems in Iranian traditional medicine [23], and it has recently been demonstrated to have important effects on the central and peripheral nervous systems, including anti-inflammatory, antiapoptosis, antioxidant, antimutant, and neuroprotective effects [24]. Gas chromatography-mass spectrometry analysis extraction of *L. officinalis* L. from Urmia, Iran, showed totals of 60 and 100 compounds, respectively, in 96% and 70% ethanol solvent extractions [25]. The most abundant constituents observed in ethanol 96% extraction included ethane (29.80%), methanecarboxylic acid (9.01%), p-vinylguaiacol (4.45%), pentadecanoic acid (3.67%), and dimethylamine, N,N-dimethyl methanesulfonamide (2.06%) [25]. Yuanyuan et al. identified 17 compounds in lavender from Xinjiang, China, with linalool (44.54%), geraniol (11.02%), lavandulyl acetate (10.78%), 3,7-dimethyl-2,6-octadien-1-ol (10.35%), and isoterpineol (6.75%) as the main components [26]. It is known that linalool is responsible for important therapeutic effects [27, 28]. Each of these constituents can vary significantly in oils derived from different cultivars, and variations can affect the medical properties; therefore, this study aimed to assess the effect of *L. angustifolia* extract from Iran on SCI treated with HUMSCs. Previously, we showed that the effective dose of *L. angustifolia* was 400 mg/kg in an SCI contusive model, and this *Lavandula* extract was effective at improving behavioral, sensory, and cellular function after SCI. We hypothesized that *L. angustifolia* may play a role in preventing the harmful effects and neural damage triggered by SCI, promoting axonal regeneration, and potentiating stem cell transplantation effects on behavioral, sensory, and cellular function after SCI. The aim of this study was to assess the effect of *L. angustifolia* extract on the outcome of transplantation of HUMSCs from Wharton's jelly after contusive SCI in Wistar rats.

2. Materials and Methods

2.1. Drug Treatments and Experimental Outline. Sixty rats were divided into eight groups as follows: group I: intact ($n = 6$); group II: sham-operated/saline ($n = 6$); group III: control 1, subjected to SCI ($n = 7$); group IV: Lav 400 mg/kg ($n = 8$); group V: control 2, subjected to HUMSC ($n = 7$); group VI: SCI treated with HUMSC + Lav 100 mg/kg ($n = 8$); group VII: SCI treated with HUMSC + Lav 200 mg/kg ($n = 10$); and group VIII: SCI treated with HUMSC + Lav 400 mg/kg ($n = 8$). Lav and saline, respectively, were injected intraperitoneally in the Lav and sham groups starting one day after injury and then daily for 14 days. We used the SCI group as control 1 for comparison with the HUMSC group, and we used the HUMSC group as control 2 for comparison with the HUMSC + Lav-treated groups. As we determined the effective dose (400 mg/kg) of *L. angustifolia* in SCI in our previous study, we used that dose for this study.

2.2. Intraspinal Cord HUMSC Transplantation: Preparation of HUMSCs. After considering all ethical aspects and receiving permission from the parents, fresh umbilical cords were aseptically collected from full-term infants after cesarean section. The study adhered to the policies of Baqiyatallah Hospital and received approval from the Ethical Committee of Baqiyatallah University of Medical Sciences, Tehran, Iran. The experiment followed the Iranian Ministry of Health and Medical Education's guidelines for laboratory animals. The fresh human umbilical cords were collected in Hanks' Balanced Salt Solution (HBSS) (Gibco, USA) at 4°C. Following disinfection in 75% ethanol for 30 sec, the umbilical cord vessels were cleaned off while still in HBSS. The mesenchymal tissue in Wharton's jelly was then diced into cubes of about 0.5 cm each and centrifuged at 250 g for 5 min. Following removal of the supernatant fraction, the precipitate (mesenchymal tissue) was washed with serum-free DMEM (Gibco) and centrifuged at 250 g for 5 min. Following aspiration of the supernatant fraction, the precipitate (mesenchymal tissue) was treated with collagenase at 37°C for 18 hours, washed, and further digested with 2.5% trypsin (Gibco) at 37°C for 30 min. Fetal bovine serum (FBS; Hyclone, USA) was then added to the mesenchymal tissue to stop trypsinization. The cells were incubated for 2-3 days until the cells reached confluency, and the culture was repeated for four passages (P4), with one week for each passage. At P4, the cells were checked for the properties of bone marrow stem cells (BMSCs) using fibronectin (+), CD44 (+), CD90 (+), and CD45 (−) immunostaining. The dissociated mesenchymal cells were further dispersed in 10% FBS-DMEM and counted under a microscope with the aid of a hemocytometer (Figure 5). The mesenchymal cells were then used directly for cultures or stored in liquid nitrogen for later use. These cells were then separately transplanted into three positions on the injured rat spines 24 hours after injury.

2.3. Intraspinal Transplantation of HUMSCs. The animals were reanesthetized as described before, and the laminectomy site was reexposed. The sham group animals were injected 24 h after laminectomy with 9 μL of normal saline



(a) Locomotion activity

(b) Sensory function

FIGURE 1: Administration of *L. angustifolia* extract improves motor and sensory function impairment in the rat spinal cord contusion model treated with HUMSCs. Administration of *L. angustifolia* extract (i.p.) daily for 14 consecutive days after injury significantly improved BBB scores (a) and sensory function (with decreased delayed response in the hot-water test) in HUMSCs treated animals (b). Data are represented as mean ± SEM. (a) *, **, and *** show significant differences of BBB scores between HUMSCs, HUMSCs + Lav 100, 200, and 400, and SCI group (control 1) ($P < 0.05$, $P < 0.001$, and $P < 0.0001$, resp.). $^{\$}$, $^{\$\$}$, and $^{\$\$\$}$ show significant differences of BBB scores between HUMSCs + Lav 100, 200, and 400, and HUMSCs treated group (control 2) ($P < 0.05$, $P < 0.001$, and $P < 0.0001$, resp.). (b) ** and *** show significant differences of sensory function between HUMSCs and SCI group (control 1) ($P < 0.001$ and $P < 0.0001$, resp.). $^{\#}$ and $^{\#\#\#}$ show significant differences of sensory function between HUMSCs + Lav 400 and HUMSCs treated group (control 2) ($P < 0.05$ and $P < 0.0001$, resp.). $^{\$}$, $^{\$\$}$, and $^{\$\$\$}$ show significant differences of sensory function between HUMSCs + Lav 400 and SCI group (control 1) ($P < 0.05$, $P < 0.001$, and $P < 0.0001$, resp.).

using a 10 μL Hamilton syringe. The HUMSC-treated group was injected 24 h after injury. The marked HUMSCs (3×10^5 cells/μL) with 5-bromo-2′-deoxyuridine (BrdU) in 9 μL of normal saline were sucked into a Hamilton syringe and then injected slowly at a rate of 0.25 μL/min with a microinjector, into three separate locations of the lesion area (epicenter, distal, and proximal) at a depth of 1.2 mm.

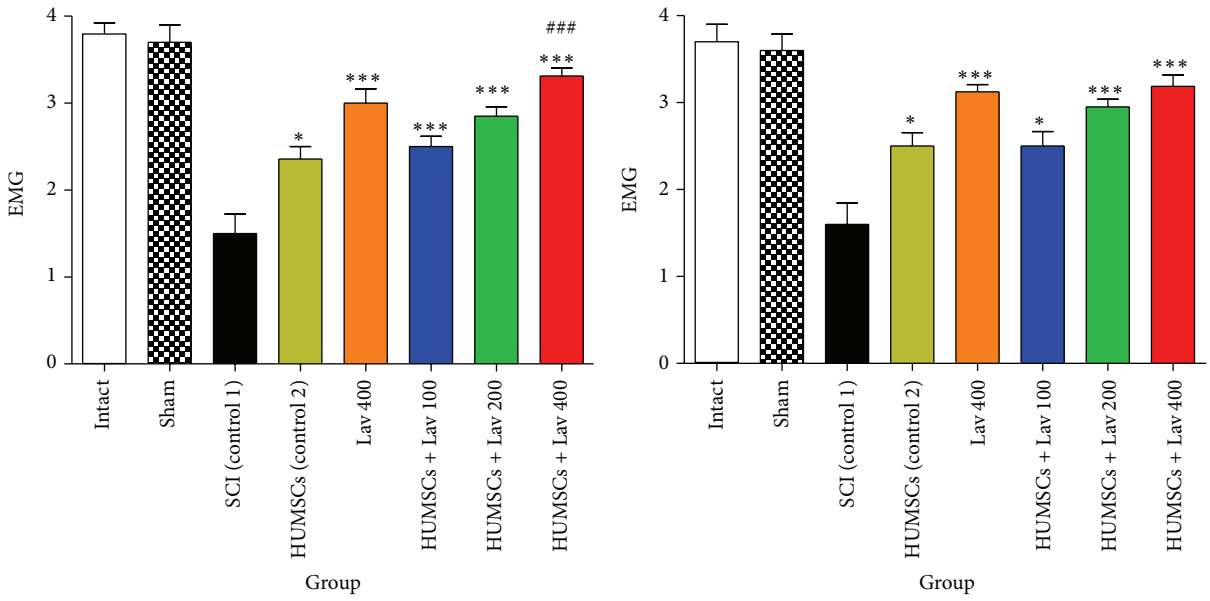
The HUMSCs were previously labeled with BrdU in order to facilitate identification of the cells within the subsequent histological specimens. After a 5 min delay, the fascia were sutured. The animals were kept on warming pads (37°C) until recovery, and after recovery they were returned to their previous cages. All procedures were done under aseptic conditions with complete anesthesia.

3. Results

3.1. Effects of L. angustifolia Extract on Locomotor Recovery after SCI Treatment with HUMSCs. While the SCI resulted in immediate paraplegia (loss of hindlimb movement), there were no significant differences in locomotion scores (BBB) of the sham-operated rats in comparison with the intact animals. HUMSC treatment significantly improved locomotor function compared to the control group. However, when we added intraperitoneal Lav treatment (100, 200, or 400 mg/kg) one day after injury, locomotor function was significantly improved compared to the control, HUMSC, and Lav groups. Application of two-way ANOVA showed significant interaction between variables, such as HUMSC therapy, Lav treatment (100, 200, and 400 mg/kg), and time ($F(81, 585) = 57.27$, $P < 0.0001$).

Application of Bonferroni's post hoc multiple-comparison test revealed significant improvement in motor function following HUMSC therapy from day 14 after injury ($P < 0.01$) until day 56 ($P < 0.001$). Adding Lav extract improved the BBB scores in comparison with the SCI group from days 14 through 56 after injury ($P < 0.001$) and also improved BBB scores in comparison with the HUMSC group on day 56 after injury ($P < 0.01$) at the dose of 100 mg/kg. Lav extract at doses of 200 and 400 mg/kg were more effective and resulted in more improved BBB scores in comparison with the HUMSC group after day 35 at both doses ($P < 0.001$). However, the Lav dose of 400 mg/kg with HUMSCs also resulted in better BBB scores on days 14 ($P < 0.05$), 21 ($P < 0.05$), and 28 ($P < 0.01$) after injury. HUMSCs with Lav extract potentiated the BBB scores in comparison with the SCI, Lav, and HUMSC groups (Figure 1(a)).

3.2. Effects of L. angustifolia Extract on Sensory Recovery after SCI Treated with HUMSCs from Wharton's Jelly. Statistical evaluations revealed that the mean latency time of response



(a) Left hindlimb EMG

(b) Right hindlimb EMG

Figure 2: Stem cell therapy and administration of *L. angustifolia* improved locomotor and EMG impairment in the rat spinal cord contusion model. Intraperitoneal administration of *L. angustifolia* extract daily for 14 consecutive days after injury significantly improved the EMG results in left (a) and right hindlimbs (b) in HUMSCs treated groups. Data are represented as mean ± SEM. (a) * and *** show significant differences between HUMSCs + Lav 100, 200, and 400 compared to the SCI group (control 1) ($P < 0.05$ and $P < 0.0001$, resp.). $^{\#\#\#}$ shows significant differences between HUMSCs + Lav 400 (control 2) and HUMSCs ($P < 0.0001$). (b) * and *** show significant differences between HUMSCs + Lav 100, 200, and 400 compared to the SCI group (control 1) ($P < 0.01$ and $P < 0.0001$, resp.).

to painful stimuli was significantly decreased in the HUMSC group versus the control group. However, when we added intraperitoneal Lav treatment (100, 200, and 400 mg/kg) one day after injury, sensory recovery was significantly improved compared to the control, HUMSC, and Lav groups. Two-way ANOVA showed significant interactions between variables, including SC, Lav dose (100, 200, or 400 mg/kg), and time ($F(81, 621) = 20.22$, $P < 0.0001$).

Application of Bonferroni's post hoc multiple-comparisons test revealed significant improvement in sensory function following HUMSC therapy in comparison with the SCI group on days 49 ($P < 0.01$) and 56 ($P < 0.001$) after injury. However, when we added intraperitoneal Lav treatment (100, 200, or 400 mg/kg) one day after injury, sensory function was significantly improved compared to the control, HUMSC, and Lav groups, separately.

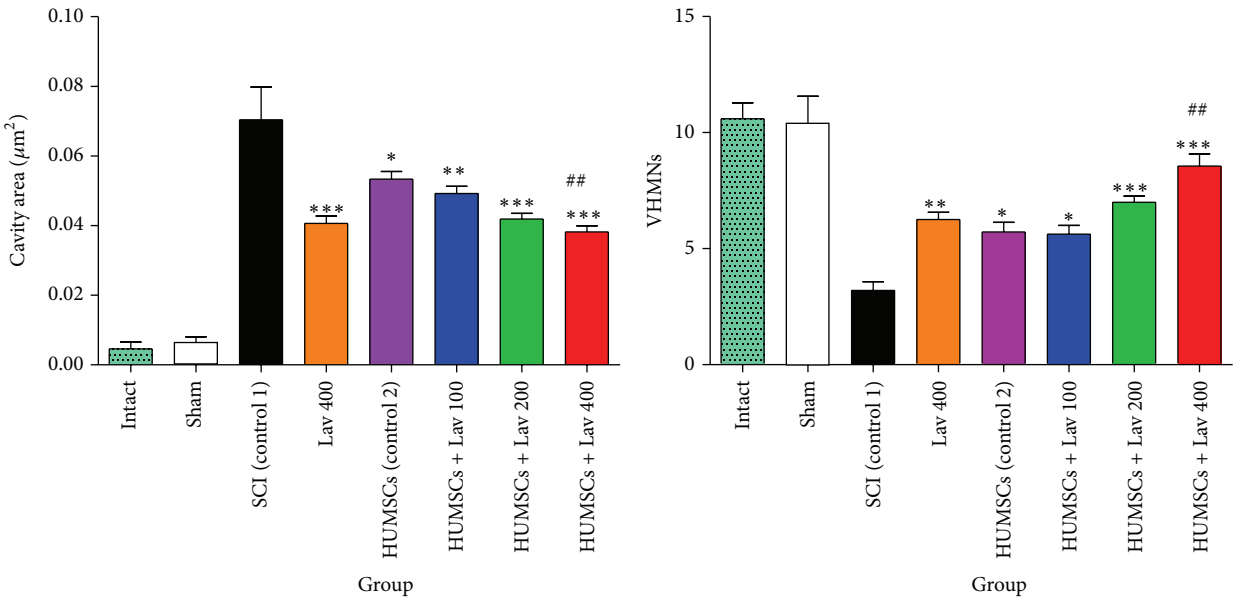
There were no significant differences between the HUMSC group and the HUMSC + Lav 100 and HUMSC + Lav 200 groups, but there was significant improvement in sensory function in the SC + Lav 400 group on days 49 ($P < 0.01$) and 56 ($P < 0.001$) after injury (Figure 1(b)).

3.3. Effects of L. angustifolia Extract on Electrophysiological Recovery after SCI Treatment with HUMSCs from Wharton's Jelly. Statistical analysis showed that the mean recruitment indexes were increased significantly for the left ($F(9, 64) = 13.63$, $P < 0.0001$) and right ($F(9, 64) = 20.44$, $P < 0.0001$) hindlimbs in the Lav + HUMSC groups versus the control HUMSC and SCI groups.

Application of Bonferroni's post hoc multiple-comparisons test, as well as Bartlett's test for equal variances, revealed significant improvement in electrophysiological activity of the right hindlimb following stem cell therapy and HUMSC + Lav 100, HUMSC + Lav 200, and HUMSC + Lav 400 ($P < 0.0001$) and the left hindlimb following stem cell therapy in the HUMSC + Lav 100, HUMSC + Lav 200, and HUMSC + Lav 400 ($P < 0.0001$) treatment groups compared with the SCI group (Figure 2). Although there were some improvements in the HUMSC + Lav treatment groups in comparison with the control HUMSC group, these improvements were not significant in the right hindlimb but were significant in the HUMSC + Lav 400 group left hindlimbs in comparison with HUMSC therapy alone ($P < 0.0001$). The most-recovered hindlimb results were in the HUMSC + Lav 400 group, as there were no significant differences between the sham-operated left and right hindlimb EMG results versus the HUMSC + Lav 400 group's results.

3.4. Effects of L. angustifolia Extract on Histomorphological Findings after SCI Treatment with HUMSCs from Wharton's Jelly. Statistical evaluations revealed that the mean cavity size was significantly reduced in the HUMSC group ($F(3, 20) = 53.45$, $P < 0.0001$) and the HUMSC + Lav 100, 200, and 400 treatment groups ($F(6, 44) = 46.75$, $P < 0.0001$) compared to the SCI group (Figure 3(a)).

There were significant differences between the HUMSC group and the HUMSC + Lav-treated groups compared to the SCI group. Cavity sizes were significantly reduced in



(a) Cavity area

(b) Number of ventral horn motor neurons

FIGURE 3: HUMSCs transplantation and administration of *L. angustifolia* improved histomorphological results, decreased cavity area (a), and increased the number of VHMNs (b) in the rat spinal cord contusion model. Intraperitoneal administration of *L. angustifolia* extract daily for 14 consecutive days after injury significantly improved histomorphological results including cavity area and VHMNs results. Data are represented as the mean ± S.E.M. *, **, and *** show significant differences of HUMSCs, HUMSCs + Lav 100, 200, and 400 compared to SCI (control 1) group ($P < 0.05$, $P < 0.0001$, and $P < 0.0001$, resp.). $^{\#\#}$ shows significant differences between HUMSCs + Lav 400 and HUMSCs (control 2) ($P < 0.001$). Cavity area significantly decreased in HUMSCs and HUMSCs + Lav-treated groups (a) (Per 35625 μm^2). Number of ventral horn motor neurons significantly increased in HUMSCs and HUMSCs + Lav-treated groups (b) (Per 5700 μm^2).

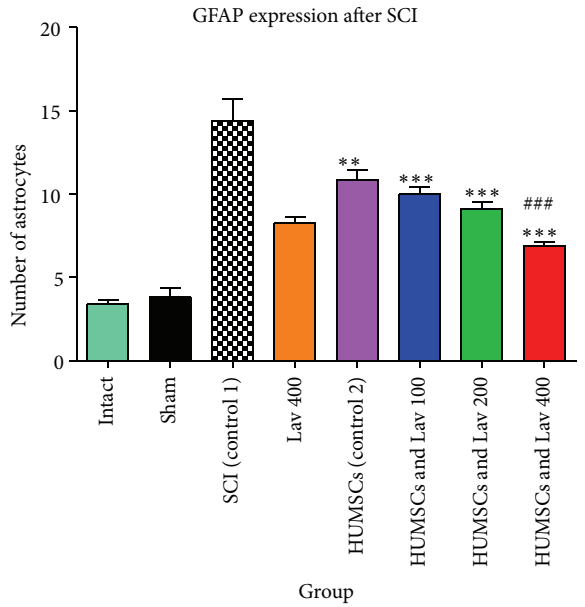
the HUMSC ($P < 0.05$), HUMSC + Lav 100 ($P < 0.001$), and HUMSC + Lav 200 and HUMSC + Lav 400 ($P < 0.0001$) groups. No statistically significant differences were found in cavity volume between the HUMSC group and the HUMSC + Lav 100 or HUMSC + Lav 200 groups.

In addition, application of one-way ANOVA showed significant differences between the sham, HUMSC, HUMSC + Lav, and control groups in the number of ventral horn motor neurons ($F(6, 45) = 17.45$, $P < 0.0001$). Application of Bonferroni's post hoc multiple-comparisons test, as well as Bartlett's test for equal variances, revealed significant increases in the number of ventral horn motor neurons in the HUMSC ($P < 0.05$), HUMSC + Lav 100 ($P < 0.05$), and HUMSC + Lav 200 and HUMSC + Lav 400 ($P < 0.0001$) groups compared to the SCI group (Figure 3(b)). No significant differences were observed between the HUMSC group and the HUMSC + Lav 100 and HUMSC + Lav 200 groups, but there were significant differences between the HUMSC and HUMSC + Lav 100 groups and the HUMSC + Lav 400 group ($P < 0.001$).

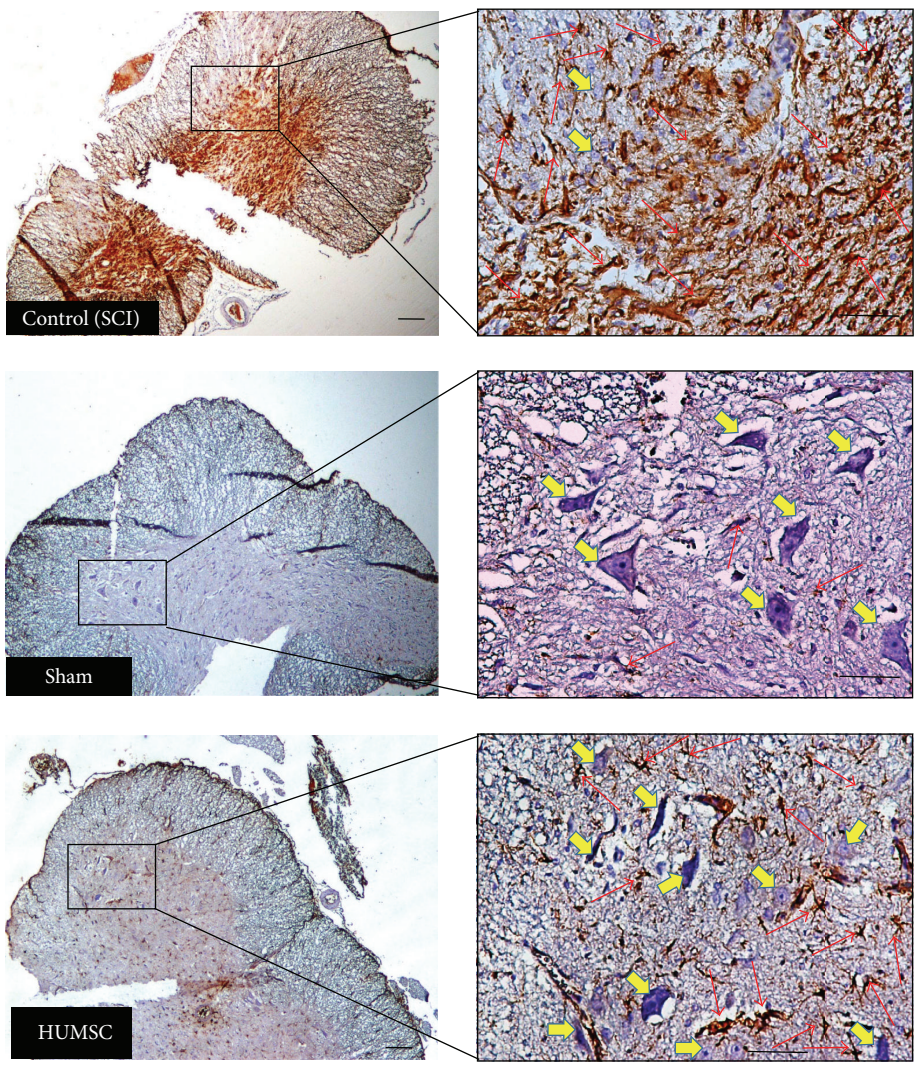
3.5. Effects of L. angustifolia Extract on GFAP Expression after SCI Treatment with HUMSCs from Wharton's Jelly. Strong immunostaining for GFAP was demonstrated in the control group (Figure 3(a)); however, this activation was significantly attenuated in the HUMSC and HUMSC + Lav groups ($F(9, 66) = 25.57$, $P < 0.0001$). Application of Bonferroni's post hoc multiple-comparisons test, as well as Bartlett's test for equal variances, revealed significantly decreased GFAP expression in the HUMSC ($P < 0.001$) group and in the HUMSC + Lav 100, HUMSC + Lav 200, and HUMSC + Lav 400 groups (all $P < 0.0001$) compared to the SCI group (Figure 3(b)). No statistically significant difference was found in GFAP expression between the HUMSC + Lav 200 and HUMSC + Lav 400 groups, but there was significantly decreased GFAP expression in the SC + Lav 400 group compared to the HUMSC-treated groups ($P < 0.0001$) (Figure 4).

4. Discussion

Some studies have shown unsuccessful effects of stem cell transplantation for SCI treatment [29], but ample evidence demonstrates that stem cells are effective in such treatments. Therefore, controversy exists, as some reviewers believe that stem cells have little effect on treating SCI [30]. However, stem cell therapy is administered to SCI patients by surgeons in operating rooms throughout the world [21, 30]. We decided to potentiate the effects of transplanted stem cells by adding a neuroprotective and effective drug to induce curative effects in SCI-induced rats. *L. angustifolia* has neuroprotective and neurotrophic effects [31], including enhancement of functional recovery [31], suggesting that it has a therapeutic effect on neurodegenerative disease [28, 31, 32] (Figure 6). Our previous research demonstrated that administration of *L. angustifolia* extract itself improved behavioral, sensory, and



(a)



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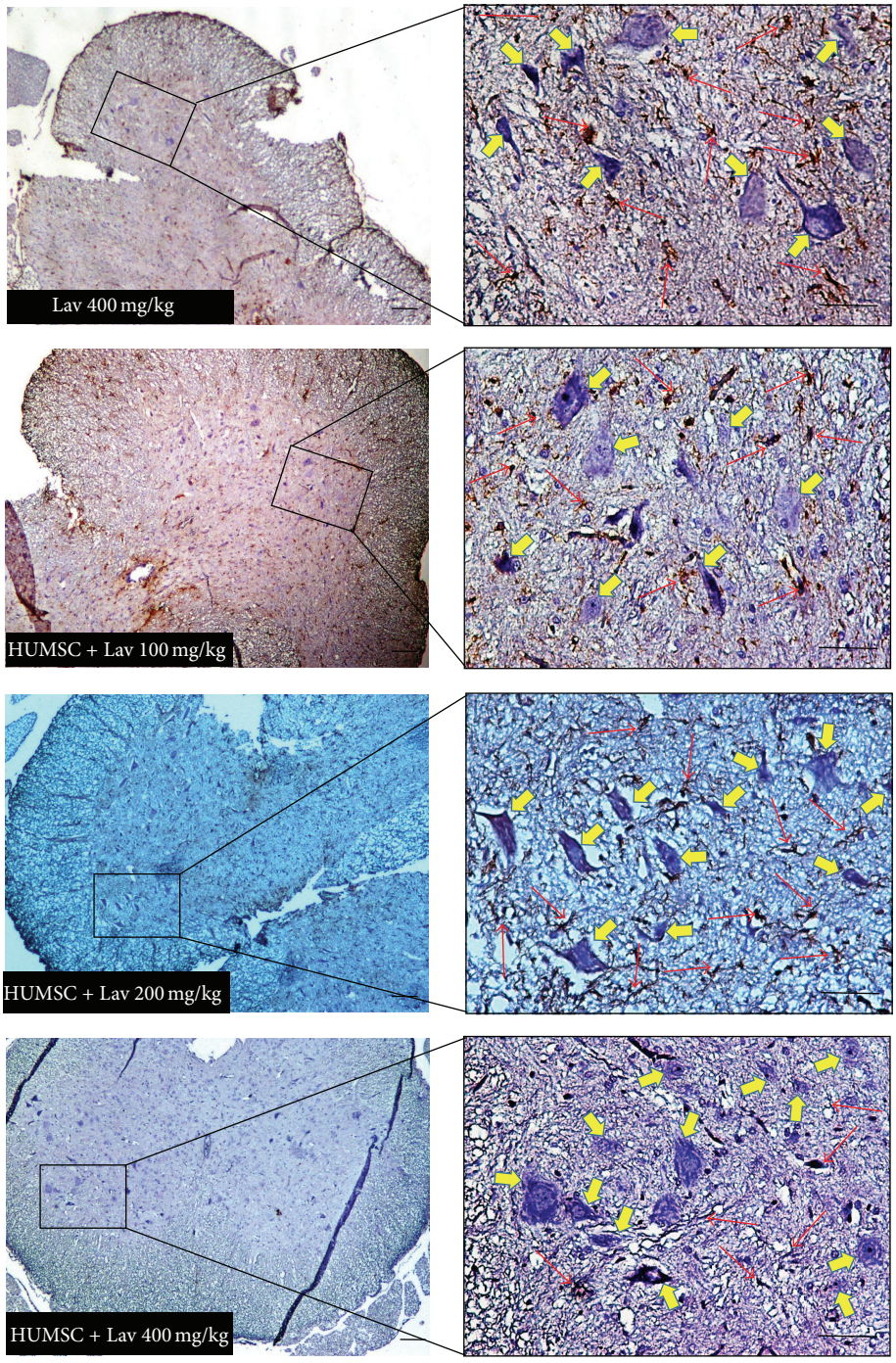


(b)

Figure 4: Administration of *L. angustifolia* decreased GFAP expression in the rat spinal cord contusion model transplanted HUMSCs. Intraperitoneal administration of *L. angustifolia* extract daily for 14 consecutive days postinjury significantly decreased the levels of GFAP expression in the rat spinal cord contusion model. Data are represented as the mean ± S.E.M. (a) *, **, and *** show significant differences between HUMSCs, HUMSCs + Lav 100, 200, and 400, and SCI (Control 1) ($P < 0.05$, $P < 0.0001$, and $P < 0.0001$, resp.). $^{\#\#\#}$ shows significant differences between HUMSCs and HUMSCs + Lav 400 ($P < 0.0001$) (Per 35625 μm^2). (b) Transverse section of spinal cord showing the ventral horn gray matter at the T12-L1 level for all groups on day 56 GFAP-stained images. Yellow arrows indicate VHMNs. Red arrows indicate the GFAP astrocytes. Decreased GFAP astrocytes and increased VHMNs are evident. Bar in 40X = 100 micrometer and bar in 200X = 50 micrometer. (ECLIPSE 5Oi microscope).

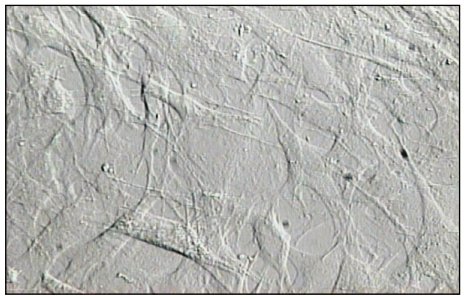


Figure 5: Mesenchymal Wharton's jelly stem cell. Dissociated mesenchymal Wharton's jelly stem cells were dispersed in 10% FBS-DMEM and counted under a microscope with the aid of a hemocytometer. The mesenchymal cells were then used directly for cultures or stored in liquid nitrogen for later use. 24 hours after injury marked HUMSCs (3×10^5 cells/μL) with BrdU in 9 μL of normal saline were sucked in to a Hamilton syringe and were injected slowly at a rate of 0.25 μL/min by microinjector to 3 separate places and transplanted into the three sites of lesion area (epicenter, distal, and proximal) at a depth of 1.2 mm.

cellular outcomes in a rat SCI model. In the present study, we evaluated the therapeutic potential of *L. angustifolia* for SCIs treated with HUMSCs in a conventional animal model. Intraperitoneal *L. angustifolia* improved motor function following contusion SCIs treated with HUMSCs. Thus, this is the first report demonstrating that administration of *L. angustifolia* extract itself potentiates behavioral and cellular outcomes in SCI rats treated with mesenchymal Wharton's jelly stem cells. Our findings are consistent with Yang's results, which successfully demonstrated improvements in locomotion and axonal regeneration in the corticospinal tract, even after complete transection of the rat spinal cord [33].

SCI involves a variety of neurochemical, cellular, and molecular events, including calcium overload [34], extracellular accumulation of glutamate [3], and induction of oxidative stress [5, 35]. Increased oxidative stress after spinal cord trauma can lead to secondary processes, such as impaired activity of membrane enzymes [35, 36] and overexpression of inflammatory mediators, which potentiates secondary injuries to the spinal cord via a variety of processes, such as activation of microglia and stimulation of astrocyte proliferation [6, 37].

It has been demonstrated that administration of *L. angustifolia* extract could alleviate the extracellular accumulation of glutamate [38] and could decrease oxidative stress [31, 32]. Protection against the progression of secondary injury to spinal cord neurons appears to be one of the most effective therapeutic strategies for limiting tissue injury and improving the outcome of spinal cord trauma [4]. As neuroprotection could preserve neurologic function by preventing cell death, one of the most important roles of *L. angustifolia* could be its neuroprotective effects. We believe that calcium-calmodulin may play an important role in the neuroprotective effects of *L. angustifolia* [24].

Our findings are consistent with Yang's results, which showed improvement in locomotion and axonal regeneration in the corticospinal tract even after complete transection of the rat spinal cord [33]. However, our results showed that Lav 400 mg/kg can also potentiate the curative effects of stem cell transplantation in SCI rats.

Due to *L. angustifolia*'s antimicrobial, anti-inflammatory, and analgesic properties, it seems that it could prevent wound infections and play a role in reducing pain by lowering inflammation [39]. This effect of *L. angustifolia* should make it easier to induce the therapeutic effects of HUMSCs.

Certain growth factors, such as epidermal growth factor (EGF), insulin-like growth factor (IGF), platelet-derived growth factor (PDGF), and fibroblast growth factor (FGF), regulate cellular proliferation, differentiation and migration, and the synthesis of extracellular matrix proteins, as well as angiogenesis during wound healing [40, 41]. There are some reports that lavender oil reduces scar tissue in wound healing [42]; although this needs further investigation, it may reduce axonal scarring in SCI.

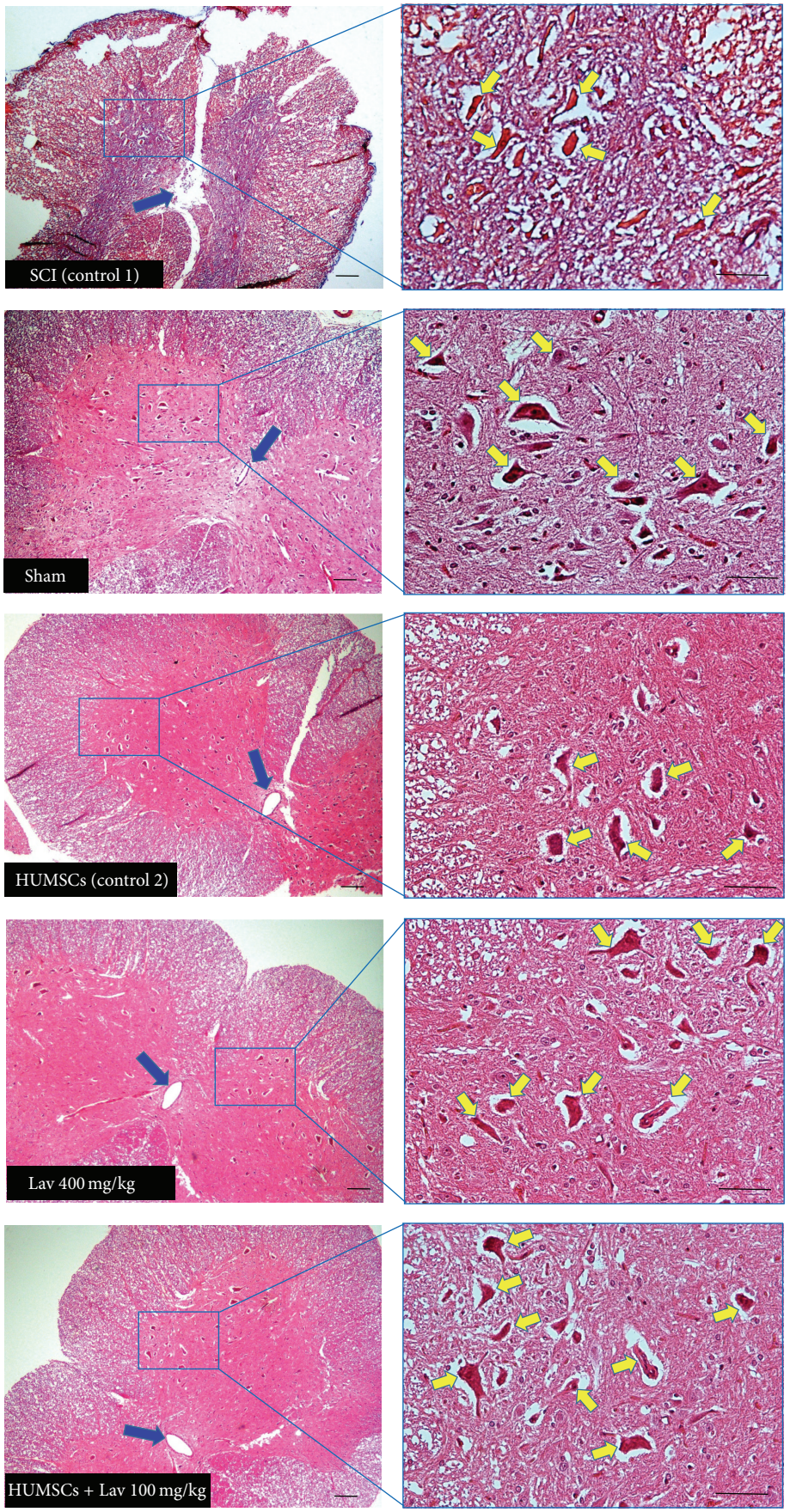
According to another study, *L. angustifolia* oil resulted in the highest EGF and FGF-2 reactions, from which it was concluded that *L. angustifolia* was effective in the stimulation of reepithelialization and granulation for tissue formation [43]. HUMSCs have been used in animal models and clinical trials for the treatment of many diseases, such as myocardial infarction, graft-versus-host disease, stroke, and SCI [44], and have demonstrated paracrine, immunomodulatory, anti-inflammatory, and antiapoptotic effects [45]. HUMSCs can migrate and secrete a variety of cytokines in injured tissues, including IGF, brain-derived neurotrophic factor (BDNF), vascular EGF, granulocyte-macrophage colony stimulating factor (GM-CSF), FGF-2, and transforming growth factor (TGF) [46]. Therefore, it is clear that the potentiation of the effects of HUMSCs in SCI animals is relevant to *L. angustifolia*, and the synergic effects of these two separate and inexpensive treatment protocols potentiate each other.

5. Conclusion

In conclusion, HUMSCs from Wharton's jelly improved motor function and promoted morphological improvement in a rat SCI contusion model. Due to its neuroprotective properties, *L. angustifolia* extract potentiated the motor, sensory, and cellular improvements associated with HUMSC transplantation for SCI. While further studies are needed to clarify the mechanism of action of *L. angustifolia* in SCI models, the present results suggest that *L. angustifolia* extract can potentiate the therapeutic effects of Wharton's jelly HUMSCs for treating patients with SCI.

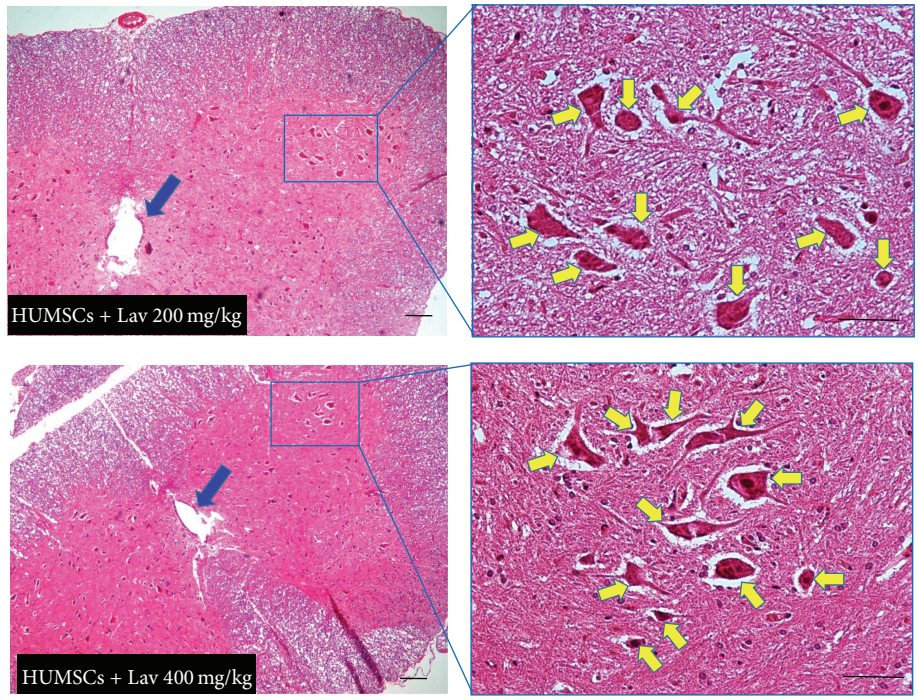
Conflict of Interests

All of the authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interests.



(a)

Figure 6: Continued.



(b)

FIGURE 6: Stem cell therapy and administration of *L. angustifolia* improved histomorphological evaluation results in the rat spinal cord contusion model. Transverse section of spinal cord showing the ventral horn gray matter from spinal cord at the level of T12-L1 of all groups which evaluated in this study at day 56. H&E staining showed shrinkage and decrease of ventral horn motor neurons of HUMSCs in compare with control 1 (SCI) ($P < 0.0001$) and HUMSCs + Lav 100, 200, and 400 mg/Kg/day in comparison with HUMSCs. Yellow arrows illustrating the ventral horn motor neurons and blue arrows show central canal. HUMSCs therapy improved the number and shape of central canal and *L. angustifolia* extract potentiated this effect. Bar in 40X = 100 micrometer and bar in 200X = 50 micrometer. (ECLIPSE 5Oi microscope).

Authors' Contribution

Dr. Gholamreza Kaka and Kayvan Yaghoobi developed the original idea and the protocol, abstracted and analyzed the data, and wrote the paper. Gholamreza Kaka, Kayvan Yaghoobi, Ruhollah Hosseini, Shaghayegh Davoodi, Seyed Homayoon Sadraie, and Korosh Mansouri contributed to the development of the protocol and abstracting data. Dr. Kaka supervised all of the protocol.

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Review Article

Cartilage Defect Treatments: With or without Cells? Mesenchymal Stem Cells or Chondrocytes? Traditional or Matrix-Assisted? A Systematic Review and Meta-Analyses

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Articular cartilage defects have been addressed by using multiple strategies. In the last two decades, promising new strategies by using assorted scaffolds and cell sources to induce tissue regeneration have emerged, such as autologous chondrocyte implantation (ACI) and mesenchymal stem cell implantation (MSCI). However, it is still controversial in the clinical strategies when to choose these treatments. Thus, we conducted a systematic review and meta-analyses to compare the efficacy and safety of different cartilage treatments. In our study, 17 studies were selected to compare different treatments for cartilage defects. The results of meta-analyses indicated that cell-based cartilage treatments showed significant better efficacy than cell-free treatments did (OR: 4.27, 95% CI: 2.19–8.34; WMD: 10.11, 95% CI: 2.69–16.53). Another result indicated that MACT had significant better efficacy than traditional ACI did (OR: 0.49, 95% CI: 0.30–0.82). Besides, the incidence of graft hypertrophy of MACT was slightly lower than that of traditional ACI (OR: 2.43, 95% CI: 1.00–5.94). Current data showed that the cell-based treatments and MACT are better options for cartilage treatments, but more well-designed comparative studies are still needed to enhance our understanding of different treatments for cartilage defects.

1. Introduction

Articular cartilage lines the surface of diarthrodial joints, distributes forces to underlying subchondral bone, and provides a low-friction interface for motion. Articular cartilage defects are common intractable clinical problems because they cannot heal spontaneously. It has been confirmed that cartilage defects often progress to higher grade and larger lesions without proper treatments. They can also lead to the development of osteoarthritis over time [1, 2]. Articular cartilage defects have been addressed by using Pridie drilling, microfracture, mosaicplasty, and abrasion chondroplasty. Pridie drilling involves surgical access to bone marrow space, which promotes blood clot formation, a crude scaffold for fibrocartilaginous repair tissue produced by extravasated bone marrow stem cells. In 1960s, Pridie was the first to advance this concept; subsequent iterations resulted in modern-day microfracture. Another paradigm, mosaicplasty or osteochondral autograft transfer, involves the surgical transfer of mature autologous tissue from a nonloadbearing region to a cartilage defect or transfer of mature allograft tissue from a cadaveric specimen. Arthroplasty is the definitive treatment for end-stage osteoarthritis, but it is only suitable for old patients because of limited durability [3]. However, none of these treatments can generate tissue that adequately recapitulates native cartilage. In the last two decades, promising new strategies by using assorted scaffolds and cell sources to induce chondrocyte regeneration have emerged. As a potential therapeutic option which can regenerate tissues, more and more preclinical and clinical studies were conducted to evaluate the efficacy and safety of scaffold-based cartilage treatments [4].

Biodegradable scaffolds, tissue forming cells, and growth factors are the three principle components of tissue engineering [5, 6]. The rationale for using a scaffold is to have a temporary 3-dimensional structure of biodegradable

Table 1: Short description of major treatments for cartilage defects.

Technique	Stage	Scaffold	Procedure	Major disadvantages
Pridie drilling	1 stage	None	Open procedure	(a) 2 to 2.5 mm drill holes to access bone marrow; (b) inconsistent results; (c) long recovery; (d) high complication rate.
Microfracture	1 stage	None	Arthroscopic procedure	(a) 0.5 to 1 mm drill holes to access bone marrow; (b) same major disadvantages as Pridie drilling and less impact than Pridie drilling on biomechanics of underlying subchondral.
Abrasion chondroplasty	1 stage	None	Arthroscopic procedure	(a) Irreproducible, unreliable; (b) loss of underlying subchondral mechanical support.
Mosaicplasty	1 stage	None	Arthroscopic procedure	(a) Morbidity at harvest site; (b) osteochondral plugs 15–20 mm deep; (c) blood clot in interspace.
Traditional ACI	2 stages	None	Open/arthroscopic procedure	(a) Periosteal patch or collagen membrane; (b) secured by sutures and/or fibrin glue; (c) greatest clinical experience.
MACT	1 stage or 2 stages	Hydrogel, fibrous scaffold, decellularized ECM, or composite	Open/arthroscopic procedure	Cells expanded and seeded in scaffold or matrix.

polymer to permit the growth of living cells, mimicking the highly organized zonal architecture of articular cartilages [7, 8]. Recent efforts are focused on forming structures that allow bone-cartilage interface that is similar to the native osteochondral interface [9–12]. On the other hand, for cartilage defect treatment, cell sources will greatly affect the overall outcomes. The milieu required to arrest mesenchymal stem cells (MSC) differentiation and prevent chondrocyte to fibroblast differentiation has been indicated [13–15]. And demonstrating which type of cells has better ability to regenerate tissues is controversial. Besides, in order to enhance the cell performance and tissue regeneration, one or more growth factors should be used [16–18].

Autologous chondrocyte implantation with periosteal flap (ACI-P), as the first-generation ACI, covers cartilage defects with the help of a periosteal flap removed from the tibia [19, 20]. And, as the second generation, autologous chondrocyte implantation with a flap made of collagen (ACI-C) has similar clinical outcomes to ACI-P and in avoiding the removal of periosteum from the tibia [21, 22]. Despite good clinical results of the first and second generations, which were defined as traditional ACI, they have evident surgical and biological limitations [23–25]. In order to achieve better redifferentiation, more homogeneous distribution, better protection, easier handling for surgical implantation, and matrix-assisted autologous chondrocyte transplantation (MACT) emerged. The cells of MACT were harvested and cultured in vitro and then put on the 3-dimensional biomaterial [26]. Although MACT seems to have many advantages, it is still controversial whether MACT has better efficacy and safety than traditional ACI, especially in clinical trials.

Because all these treatments have disadvantages and advantages, it is difficult to choose the most appropriate treatment when we are facing cartilage defects (Table 1). Consequently, we conducted this study to review the current comparative clinical trials of scaffold-based cartilage treatments. The aim of our study is to compare the efficacy and safety among cell-based and cell-free cartilage treatments, different cell sources, traditional ACI, and MACT. We hope our study could indicate a new direction for future studies.

2. Methods

2.1. Search Strategy. We conducted a computer-assisted systematic search of PubMed databases from their commencement to July 2015, attempting to find all publications on clinical trials of scaffold-based cartilage defect treatments. Key words and medical subject heading (Mesh) terms for the search of PubMed were as follows: ("cartilage" [Mesh]) AND ("tissue scaffold" [Mesh]) AND "ACI" OR "MACT" OR "mesenchymal stem cell" OR "Microfracture" AND "Clinical trial". We also reviewed the bibliographies of relevant articles to identify additional studies that might have been missed (Figure 1).

2.2. Selection Criteria. We screened titles and abstracts of identified papers to exclude studies that clearly did not meet the inclusion criteria. Full texts of those selected for further review were retrieved and evaluated. To make sure of the comparability of all the studies, we made some criteria to study selection. The criteria were as follows: (1) They were comparative studies of scaffold-based cartilage treatments;

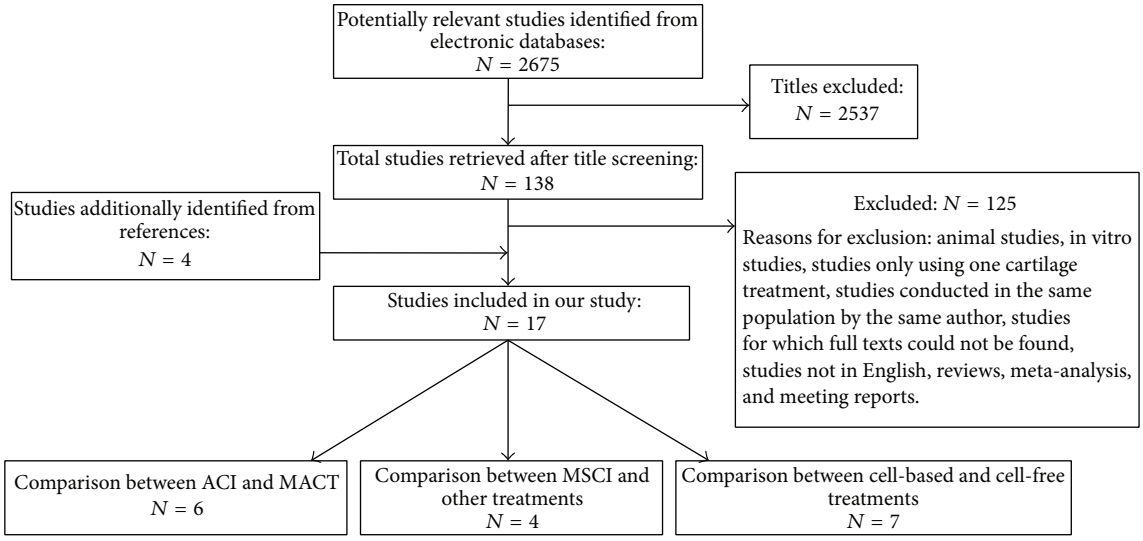

Figure 1: Scheme of research methodology.

case series were excluded. (2) The studies must test on human; the in vitro experiments and animal trials were excluded. (3) Reviews, meta-analysis, and meeting reports were excluded. (4) Studies from same authors with same patients were excluded. But two studies conducted by the same author were included in our study because they researched on totally different population. (5) Other criteria were publications being in English; full texts could be found and followed-up for over 1 year.

2.3. Methodological Quality Evaluation. We evaluated the methodological quality of all randomized controlled trials (RCT) by using 7-point modified Jadad scoring system [42]. Meanwhile, observational studies, including case-control studies (CCS) and cohort studies (CS), were evaluated based on the 9-star Newcastle-Ottawa Scale [43]. 4–7 points of Jadad scoring system and 6–9 stars of Newcastle-Ottawa Scale were defined as good quality of the studies.

2.4. Data Extraction. All data were extracted according to the criteria. Discrepancies were discussed and resolved by consensus. Data extracted from each study included the first author, year of publication, types of studies, regions of the population investigated, number of patients of different groups, follow-up, age, gender, locations of lesions, major assessment of efficacy, number of patients who achieved excellent, good, fair, and poor results, and other assessments in the studies. For studies which compared traditional ACI with MACT, graft hypertrophy and frequency of reoperation were extracted to assess the safety. For studies which compared cell-based and cell-free treatments, preoperation and postoperation scores were also extracted. For studies focusing on MSC, brief descriptions were summarized from the studies.

2.5. Meta-Analysis. Stata Statistical Software was used for all the analyses (version 12.0, Stata Corporation, College Station, TX, USA). The measure of estimated effect of interest was OR (odds ratio) or weighted mean difference (WMD) with 95% CI.

We used two models to calculate the pooled relative risk estimates: a fixed-effects model known as the Mantel-Haenszel method [44] and a random-effects model known as the DerSimonian-Laird method [45]. We used the Cochran Q test to evaluate the heterogeneity of the studies [46] and the quantity I^2 was also calculated [47, 48]. I^2 is the proportion of total variation contributed by between-study variation, and values of 25%, 50%, and 75% have been regarded as representing low, moderate, and high heterogeneity, respectively. When I^2 was over 50%, a random-effects model was used to calculate the pooled relative risk estimates. On the contrary, a fixed model was used.

Publication bias was evaluated to find whether the results of the studies were homogeneous. The funnel graph, the Egger regression asymmetry test [49], and the Begg-Mazumdar adjusted rank correlation test [50] were used. When the p value of Egger's test and Begg's test < 0.05, we considered obvious bias among the studies.

3. Results

3.1. Search Results. We found 2675 records in PubMed database, and 4 records were found from the reference lists. With our selection criteria, we identified 17 studies in our study, including 6 studies which compared ACI with MACT [20, 34–37, 51], 7 studies which compared cell-based with cell-free treatments [27–33], and 4 studies which were focused on MSCI [38–41] (Figure 1). Tables 2, 3, and 4 summarized the characteristics of all the included studies.

Table 2: Characteristics of studies that compared cell-based and cell-free cartilage treatments.

Study	Year	Study design	Regions	Number of patients	FU	Age Mean, range (yr)	Gender Male/female	Locations	Major assessments of efficacy	Excellent	Good	Fair	Poor	Preoperation score (mean ± SD)	Postoperation score (mean ± SD)	Other assessments
Gudas et al. (athletes) [27]	2005	RCT	Lithuania	OAT: 28 MF: 29	Mean, 37.1 mo.	24.3, 15–40	NR	Knee	ICRS	Excellent + good: 27 Excellent + good: 15		Fair + poor: 1 Fair + poor: 14		50.67 ± 4.05 50.84 ± 4.07	85.88 ± 4.69 75.59 ± 4.65	HSS, MRI
Kon et al. [28]	2009	CS	Italy	MACT: 40 MF: 40	5 yr	29.0 30.6	33/7 27/13	Knee	IKDC	29 6	6 24	5 7	0 3	40.5 ± 15.2 41 ± 12.3	80.2 ± 19.1 70.2 ± 14.7	TAS
Gudas et al. (children) [29]	2009	RCT	Lithuania	OAT: 25 MF: 22	Mean, 4.2 yr	5–15	NR	Femoral condyles	ICRS	Excellent + good: 19 Excellent + good: 12		Fair + poor: 6 Fair + poor: 10		NR NR	NR NR	X-ray, MRI
Basad et al. [30]	2010	RCT	Germany	MACT: 40 MF: 20	2 yr	33.0 37.5	25/15 17/3	Knee	ICRS	14 2	14 4	2 3	0 1	51 ± 26 55 ± 25	92 ± 9 69 ± 26	TAS, MRI, and Lysholm
Cole et al. [31]	2011	RCT	USA	CAI: 20 MF: 9	2 yr	32.7 33.0	14/6 5/4	Knee	IKDC	NR	NR	NR	NR	NR NR	82.95 ± 14.88 59.5 ± 13.44	MRI, SF-36, and KOOS
Kon et al. (soccer players) [32]	2011	CS	Italy	MACT: 21 MF: 20	2 yr	23.7, 16–37 26.5, 18–35	21/0 20/0	Knee	IKDC	16 13	4 5	1 2	0 0	43.2 ± 13.7 47.3 ± 8.5	90.5 ± 12.8 86.8 ± 9.7	TAS, ICRS, and recovery time
Crawford et al. [33]	2012	RCT	USA	NeoCart: 21 MF: 9	2 yr	41 39	19/2 6/3	Distal femoral cartilage	IKDC	NR	NR	NR	NR	44 ± 13 52 ± 12	65 ± 12 73 ± 16	SF-36, KOOS

FU: follow-up; RCT: randomized clinical trial; CS: cohort study; MF: microfracture; MACT: matrix-assisted autologous chondrocyte transplantation; ICRS: International Cartilage Repair score; HSS: Hospital for Special Surgery score; IKDC: International Knee Documentation Committee; MRI: magnetic resonance imaging; SF-36: Short-Form-36 Health Survey; TAS: Tegner Activity Score; CAI: cartilage autologous implantation; KOOS: Knee Injury and Osteoarthritis Outcome Score; NeoCart: an autologous cartilage tissue implant; OAT: osteochondral autologous transplantation.

Table 3: Characteristics of studies that compared ACI with MACT.

Study	Year	Study design	Regions	Number of patients	FU	Age Mean, range (yr)	Gender Male/female	Locations	Major assessments of efficacy	Excellent	Good	Fair	Poor	Graft hypertrophy	Frequency of reoperation	Other assessments
Bartlett et al. [21]	2005	RCT	England	ACI-C: 44 MACT: 47	1 yr	33.7, 15–49 33.4, 17–47	54/37	Knee	MCS	10 15	16 19	10 7	8 6	4/44 3/47	4/44 4/47	ICRS, biopsy
Manfredini et al. [34]	2007	CCS	Italy	ACI-C: 17 MACT: 15	Mean, 48.5 mo.	32.3, 17–51 35.2, 20–55	15/2 13/2	Knee	HSS	9 6	6 3	2 1	0 0	NR NR	NR NR	ICRS, MRI, and biopsy
Ferruzzi et al. [35]	2008	CCS	Italy	ACI-P: 48 MACT: 50	5 yr	32.3 35.2	30/18 36/14	Knee	IKDC	7 7	10 23	23 11	8 9	4/48 2/50	6/48 2/50	MRI, biopsy
Zeifang et al. [20]	2010	RCT	Germany	ACI-P: 10 MACT: 11	2 yr	29.5 29.1	16/5	Femoral condyle	IKDC	NR NR	NR NR	NR NR	NR NR	7/9 1/8	1/10 3/11	Lysholm, SF-36, TAS, and MOCART
Macmull et al. [36]	2012	CCS	England	ACI-C: 25 MACT: 23	Mean, 40.3 mo.	34.6, 17–50 35, 21–46	16/9 14/9	Patellae	MCS	4 6	6 7	6 8	9 2	NR NR	NR NR	VAS, Bentley score
Panagopoulos et al. [37]	2012	CCS	England	ACI-P: 11 MACT: 8	Mean, 37.5 mo.	32.2, 18–43	8/3 7/1	Knee	Lysholm	0 0	2 1	4 6	5 1	3/11 2/8	NR NR	IKDC, TAS

FU: follow-up; RCT: randomized controlled trial; CCS: case-control study; ACI-C: autologous chondrocyte implantation with a flap made of collagen; ACI-P: autologous chondrocyte implantation with periosteal flap MACT: matrix-assisted autologous chondrocyte transplantation; MCS: Modified Cincinnati Rating System; ICRS: International Cartilage Repair score; HSS: Hospital for Special Surgery score; IKDC: International Knee Documentation Committee; MRI: magnetic resonance imaging; SF-36: Short-Form-36 Health Survey; MOCART: Magnetic Resonance Observation of Cartilage Repair; VAS: Visual Analogue Scale; TAS: Tegner Activity Score.

Table 4: Characteristics of studies that compared MSCI with other cartilage treatments.

Study	Year	Study design	Area	Number of patients	FU	Age Mean, range (yr)	Gender Male/female	Locations	Assessments	Brief description
Nejadnik et al. [38]	2010	CS	USA	MSCI: 36 ACI: 36	2 yr	44 42.5	18/18 20/16	Knee	IKDC, ICRS, TAS SF-36, and Lysholm	There was significant improvement in the patients' quality of life after cartilage repair in both groups. However, there was no difference between the MSCI and the ACI group in terms of clinical outcomes except for Physical Role Functioning, with a greater improvement over time in the MSCI group.
Lee et al. [39]	2012	CS	Singapore	MSCI: 35 MF: 35	Mean, 24.5 mo.	44 44	16/19 20/15	Knee	ICRS, IKDC, MRI, VAS, and Lysholm	There were no clinically significant adverse events reported through the course of the study. Both groups showed significant improvement in all scores. No significant difference in improvement between the two groups.
Yamasaki et al. [40]	2014	CS	Japan	MSCI: 12 Cell-free control: 12	Mean, 16 mo.	63, 49–70	15/9	Knee	HSS	Both groups showed significant improvement in HSS scores. The difference in clinical improvement between the groups was not significant, the arthroscopic and histological grading score was better in the cell-transplanted group than in the cell-free control group.
Gobbi et al. [41]	2015	CS	Italy	MCSI: 18 MACT: 19	3 yr	45.5 43.1	10/8 9/10	Patellae	IKDC, KOOS, TAS, and VAS	Both groups showed significant improvement in all scores, but there was no significant difference in improvement between the two groups, except for the IKDC subjective score, which favored the MSCI group.

FU: follow-up; CS: cohort study; ICRS: International Cartilage Repair score; HSS: Hospital for Special Surgery score; IKDC: International Knee Documentation Committee; SF-36: Short-Form-36 Health Survey; VAS: Visual Analogue scale; TAS: Tegner Activity Score; MF: microfracture; KOOS: Knee injury and Osteoarthritis Outcome Score; MSCI: mesenchymal stem cell implantation.

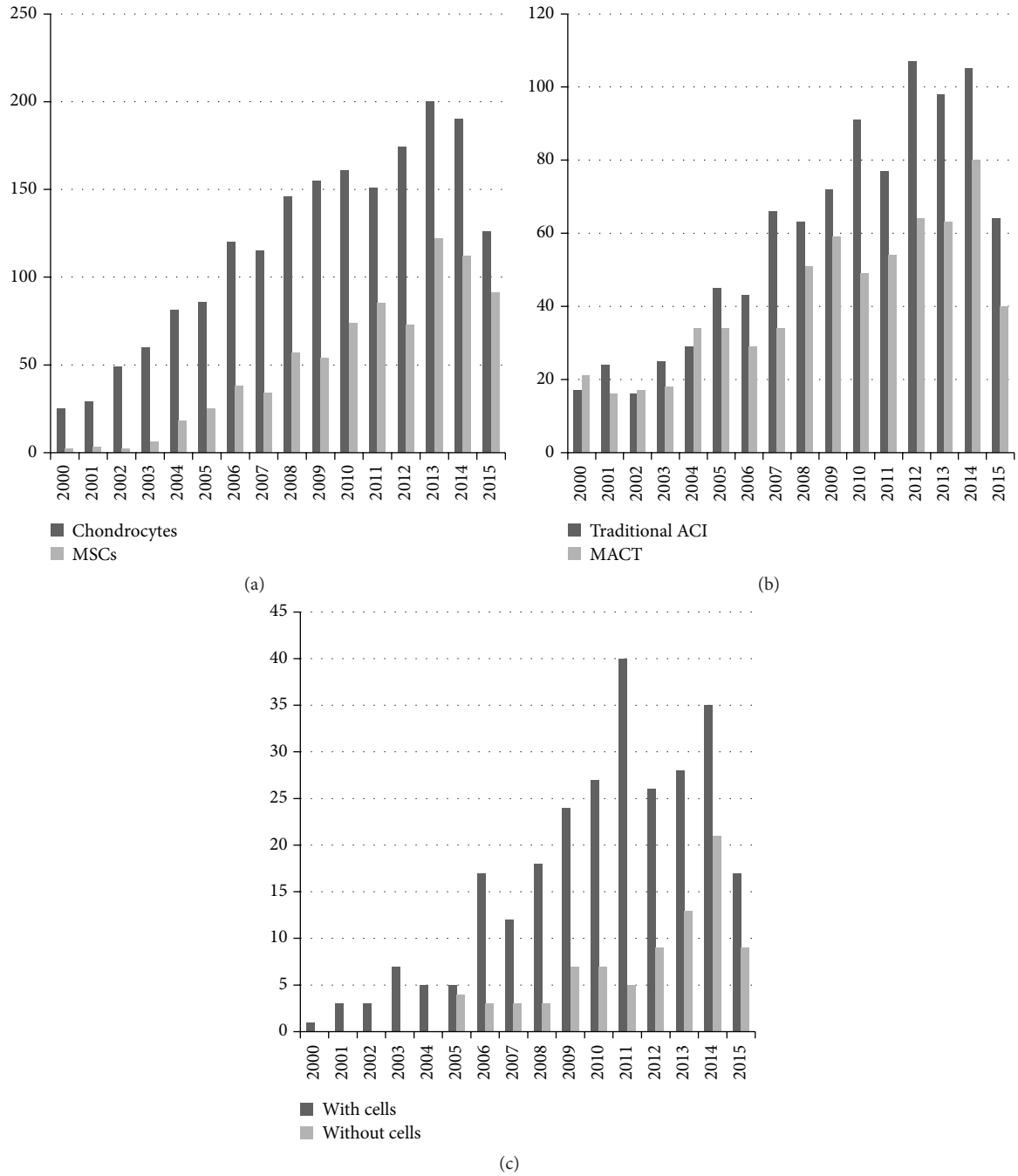

Figure 2: The number of published studies on cartilage treatments during the last 15 years. (a)–(c) The number of published studies on chondrocytes and MSCs, traditional ACI and MACT, and treatments with or without cells. MSCs: mesenchymal stem cells; ACI: autologous chondrocyte implantation; MACT: matrix-assisted autologous chondrocyte transplantation.

Besides, the number of published studies among the last 15 years increased progressively for both ACI and MACT, MSCs and chondrocytes, and treatments with cells and without cells. Although traditional ACI is still a hot spot for research, the number of studies on MACT has become closer to traditional ACI. On the other hand, MSCs, as a cell source, has the greatest potential, also widely concerned by many researchers. So far, it is still controversial that cartilage treatments with cells is better than treatments without cells; even the number of published studies on treatments with cells is more than treatments without cells, but the publications of both treatments are increasing with similar tendency in recent years (Figure 2(c)).

3.2. Methodological Quality Evaluation Results. For RCTs, only 2 of 7 studies were defined as good quality (4–7 points) because it was difficult to conduct a double-blind trial between two surgical procedures (Table 5). On the other

Table 5: Assessment of methodological quality of RCTs by using 7-point modified Jadad scoring system.

Study	Randomization	Allocation concealment	Blinding (observer)	Blinding (patient)	Withdrawals and dropouts	Jadad score
Bartlett et al. [21]	2	0	0	0	1	3
Zeifang et al. [20]	2	0	0	0	1	3
Gudas et al. (athletes) [27]	1	0	0	0	1	2
Gudas et al. (children) [29]	1	0	0	0	1	2
Basad et al. [30]	1	0	0	0	1	2
Cole et al. [31]	2	2	0	0	1	5
Crawford et al. [33]	2	2	0	0	1	5

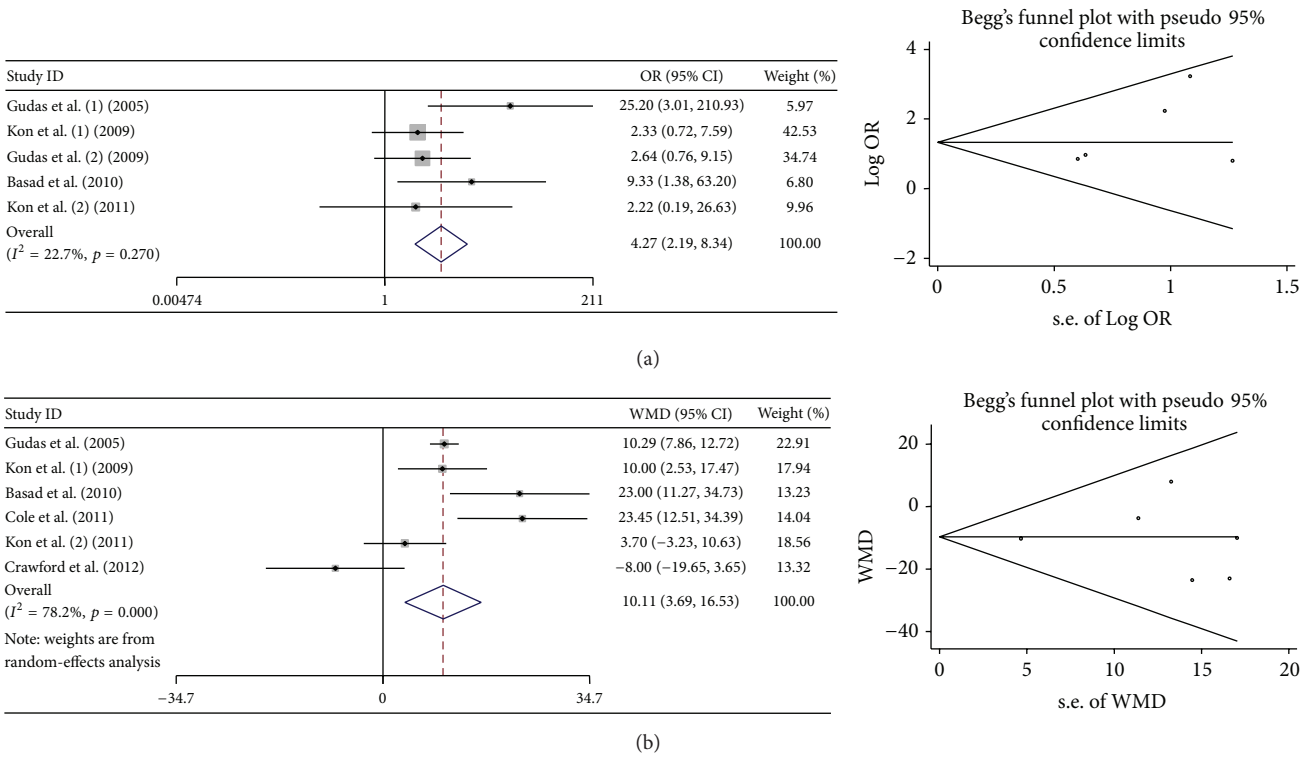

Figure 3: Forest plots and Begg's funnel plots of studies comparing cell-based with the cell-free cartilage treatments. (a) Forest plots and Begg's funnel plots conducted by using the number of patients achieved excellent and good results. (b) Forest plots and Begg's funnel plots conducted by using mean scores and standard deviations.

hand, for observational studies, 7 of 10 studies were defined as good quality (6–9 stars) because they were easier to conduct than RCTs (Table 6).

3.3. Comparison of Efficacy between Cell-Based and Cell-Free Cartilage Treatments. Seven studies were included to compare the efficacy between cell-based and cell-free cartilage treatments. The numbers of patients that achieved excellent and good results, mean scores, and standard deviations were extracted to evaluate the efficacy. Both meta-analyses indicated that cell-based cartilage treatments showed significant better efficacy than cell-free treatments. When meta-analysis was conducted by using the amount of patients who achieved excellent and good results, heterogeneity was considered low. When meta-analysis was conducted by using mean scores and standard deviations, heterogeneity was considered high. No obvious bias was found (Table 7, Figure 3).

3.4. Comparison of Efficacy and Safety between First-Generation ACI and MACT. Six studies were included to compare the efficacy and safety between traditional ACI and MACT. The numbers of patients that achieved excellent and good results were extracted to evaluate the efficacy. As the most common graft-related complication, graft hypertrophy and frequency of reoperation were extracted to evaluate the safety. The results of meta-analyses indicated that MACT showed significant better efficacy than traditional ACI did. Besides, the incidence of graft hypertrophy of MACT was slightly

Table 6: Methodological quality of included observational studies based on 9-star Newcastle-Ottawa Scale.

(a)

CS	Selection			Comparability	Outcome			Total score
	Representativeness of the exposed cohort	Selection of the nonexposed cohort	Ascertainment of exposure	Outcome of interest was not present at start of study	Control for important factor or additional factor	Assessment of outcome	Follow-up long enough for outcome to occur[†]	Adequacy of follow-up of cohort	
Kon et al. [28]	*	*	*	*	*	*	*	*	8
Kon et al. (soccer players) [32]	*	*	*	*	*	*		*	7
Nejadnik et al. [38]	*	*	*	*	*	*		*	7
Lee et al. [39]	*	*	*	*	*	*		*	7
Yamasaki et al. [40]	*	*	*	*		*		*	6
Gobbi et al. [41]	*	*	*	*	*	*		*	7

(b)

CCS	Selection				Comparability	Exposure			Total score
	Adequate definition of cases	Representativeness of cases	Selection of controls	Definition of controls	Control for important factor or additional factor	Ascertainment of exposure	Same method of ascertainment for cases and controls	Nonresponse rate	
Manfredini et al. [34]	*	*	*	*			*		5
Ferruzzi et al. [35]	*	*	*	*	*		*		6
Macmull et al. [36]		*	*		*		*		4
Panagopoulos et al. [37]	*	*	*	*			*		5

[†]Follow-up > 4 years. CS: cohort study; CCS: case-control study.

Table 7: Results of meta-analyses in our study.

	Number of studies	Assessment	Number of studies	Model, pooled relative risk estimates (95% CI)	Heterogeneity			Publication bias	
					χ^2	I^2%	p	Begg's p	Egger's p
Cell-based versus cell-free	7	Excellent and good results	5	Fixed, OR, 4.27 (2.19–8.34)	5.17	22.7	0.27	0.221	0.269
		Mean score and standard deviation	6	Random, WMD, 10.11 (2.69–16.53)	22.93	78.2	0	1	0.953
Traditional ACI versus MACT	6	Excellent and good results	5	Fixed, OR, 0.49 (0.30–0.82)	1.50	0	0.83	0.086	0.088
		Graft hypertrophy	4	Fixed, OR, 2.43 (1.00–5.94)	3.94	23.8	0.27	0.734	0.241
		Frequency of reoperation	3	Fixed, OR, 1.34 (0.53–3.37)	2.78	28.1	0.25	1	0.593

OR: odd ratio; CI: confidence interval; WMD: weighted mean difference; ACI: autologous chondrocyte implantation; MACT: matrix-assisted autologous chondrocyte transplantation.

lower than that of traditional ACI. For frequency of reoperation, no significant difference was found between traditional ACI and MACT. Heterogeneity was considered low when meta-analyses was conducted by using the number of patients who achieved excellent and good results and the incidence of graft hypertrophy. When meta-analysis was conducted by using the frequency of reoperation, heterogeneity was considered moderate. No obvious bias was found (Table 7, Figure 4).

3.5. Comparison between MSCI and Other Treatments. So far, although the researches of MSCs in cartilage repair have already increased year by year, few comparative studies were conducted to evaluate the efficacy and safety of MSCI. In our study, 4 comparative studies focusing on MSCI were included. However, because the data were not enough, no meta-analysis was conducted to evaluate the efficacy and safety of MSCI. But, from the brief descriptions in Table 4, we could easily see that although MSCI showed significant improvement in most of the scoring system, the differences between MSCI groups and control groups were not significant, no matter comparing with MACT, traditional ACI, or cell-free treatments.

4. Discussion

Articular cartilage defects have been addressed by using multiple strategies and the scaffold-based cartilage treatments have become a fascinating treatment option. The traditional ACI, MACT, MSCI, and other scaffold-based cartilage treatments have showed significant improvement in the processes of cartilage repair [24, 52]. The scaffold provides a structural basis for cartilage repair and stimulates the healing processes of damaged tissues. The roles of scaffold have been recognized by most of the researchers or physicians. On the other hand, cells play a controversial role in the scenario. Kon et al. also reviewed the preclinical and clinical studies of scaffold-based cartilage treatments and concluded that scaffold and cells combination were the most investigated option in the preclinical setting, showing generally superior results [4]. This conclusion was similar to our study, but since both strategies remain used clinically, cell-free treatments have the obvious advantages in avoiding cell manipulation and regulatory obstacles with good clinical results. On the other hand, there is still no study which directly compares the outcome of the same scaffold used alone or with cells. These studies usually made comparison with microfracture or other standard cartilage treatments, not with the scaffold alone. Thus, although our study indicated that positive effects of cells were in the healing processes, it is still difficult to clarify the real roles of cells in the healing processes of cartilage defects. More well-designed studies comparing cell-based scaffold with same scaffold alone are needed to clarify the efficiency and safety of cell-based treatments.

Since the first-generation ACI emerged for cartilage treatment, ACI have shown good clinical results for clinical applications. And then, the incorporation of a scaffold or substrate to promote chondrocyte expansion represented the next step in ACI evolution, also known as MACT [3]. MACT also showed good clinical results with multiple advantages, such as better redifferentiation, more homogeneous distribution, better protection, and easier handling for surgical implantation [13–15]. Compared with abrasive technique, the results have been promising. Višňa et al. compared MACT with abrasive technique in a trial of 50 patients and then, at the 1-year follow-up, the MACT group had significantly better outcomes [53]. Basad et al. compared MACT with microfracture in a trial with 60 patients; similar to the comparison with abrasive technique, the MACT group had significant improvement in cartilage repair clinical indices [30]. On the other hand, some researchers made comparisons between traditional ACI and MACT. Although our study indicated that MACT had significant improvement in clinical results compared to traditional ACI with the similar degree of safety, some studies indicated that traditional ACI and MACT were clinically equivalent. Zeifang et al. compared MACT with periosteal flap technique ACI in a trial of 21 patients. The results were equivocal at the 2-year follow-up

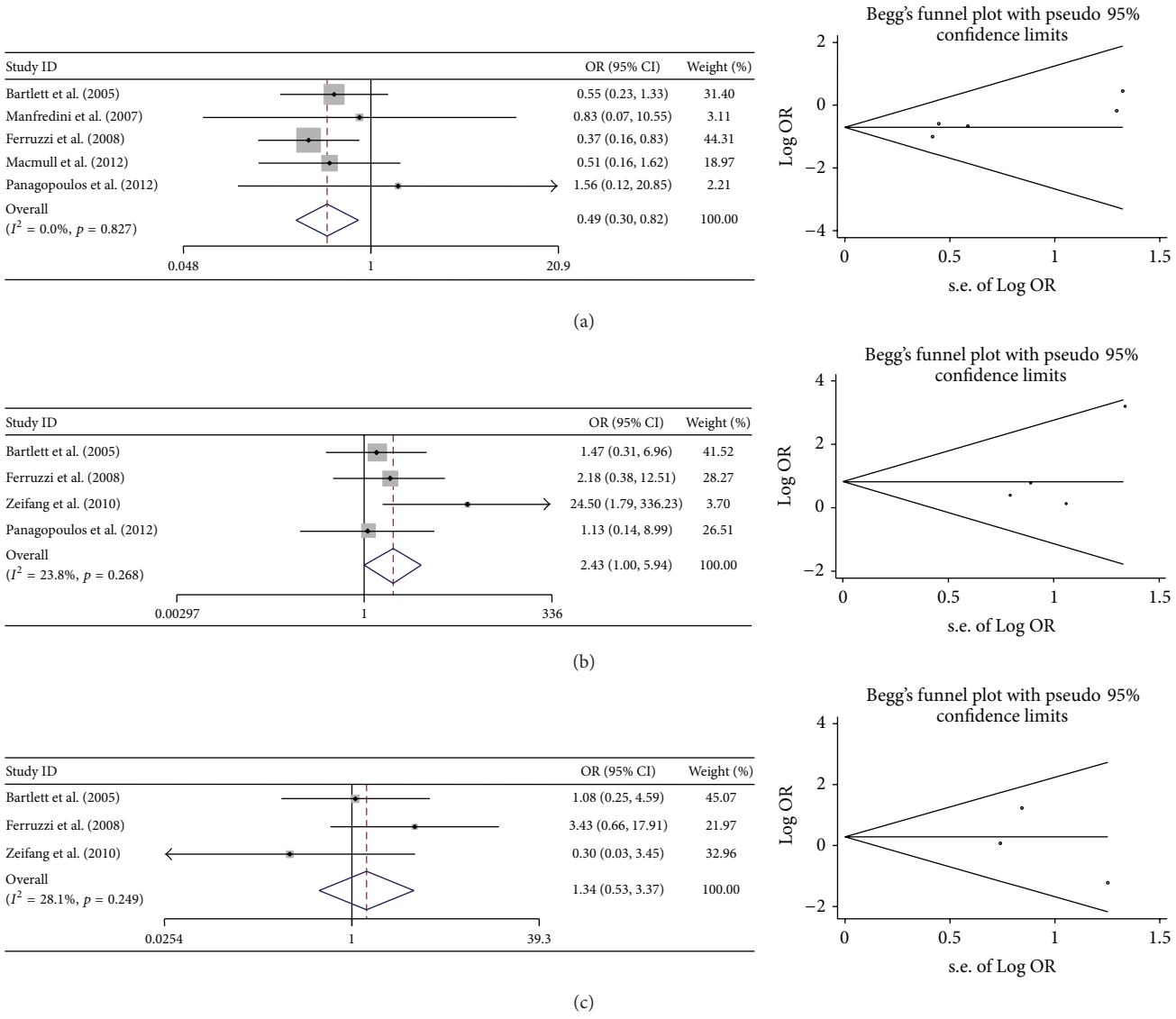

Figure 4: Forest plots and Begg's funnel plots of studies comparing the traditional ACI with MACT. (a) Forest plots and Begg's funnel plots conducted by using the number of patients achieved excellent and good results. (b) Forest plots and Begg's funnel plots conducted by using the incidence of graft hypertrophy. (c) Forest plots and Begg's funnel plots conducted by using the frequency of reoperation.

[20]. Bartlett et al. conducted a trial of 91 patients to compare MACT with collagen patch technique ACI. Then, at the 1-year follow-up, two groups reached the similar conclusion, which means that the two groups were clinically equivalent with similar histologic grades by biopsy and hypertrophy rates [51]. We believe that all these differences were caused by patient selection and prejudgments of cartilage defect as well as operations. Besides, in the studies which compared MACT with traditional ACI, only one study had a five-year follow-up. Few studies focused on the long-term efficacy and safety of these two techniques. Maybe the efficiency and safety of traditional ACI and MACT would be much clearer with the help of a long-term of follow-up. That is also one of the reasons why the ACI is still a hot spot in the current researches [24].

With the development of tissue engineering, cell sources have become another hot issue as one of the principle components of tissue engineering. The analysis of the cell sources proposed for the cell-based scaffold treatments indicated that, in preclinical research, MSCs have become the favorite cell type with an increase of studies year by year [24, 54]. However, chondrocyte was still the most common cell type used for cartilage repair in clinical studies (Figure 2). With the self-renewal characteristics, maintenance of "stemness" and potential for differentiation into cells forming multiple mesodermal tissues, MSCs have become an appealing tool for cartilage regeneration treatments. Despite the fact that the MSCs showed an exciting effect on cartilage regeneration in vitro, disappointingly, MSCs did not show great improvement in clinical trials when compared with ACI or

microfracture [38–41]. But it is still too early to give up on MSCs. As a cell source with so much potential, it is much more difficult to manipulate and regulate than chondrocytes. We believed that with an appropriate way of stimulation and regulation for MSCs, it could greatly improve the efficiency of cartilage treatments. There are some limitations in our study. Firstly, some factors that might affect the clinical results of different treatments were not discussed in our study, such as number of lesions and lesion size. Secondly, only one database was searched and only publications in English were included. Thirdly, in different studies, clinical results were evaluated by different scoring systems and the complications were recorded with different methods, which made the clinical results much more heterogeneous. Fourth, the studies included had different follow-ups and all the clinical results were extracted at the end of follow-ups. As a result, it was difficult to evaluate short-term, mid-term, and long-term efficacy and safety. Fifth, the number of comparative studies of MSC was too small, and the exact data could not be extracted from the publications. Instead, brief descriptions were summarized from the studies. Although there were so many limitations, we believe that the general understanding of cell-based and cell-free treatments, traditional ACI, and MACT could be achieved from our study.

Till now, although we are far from understanding which could be the best strategy for cartilage treatments, an increasing number of studies on this field showed huge research efforts. Cell source and scaffold properties are two of the most popular directions. More well-designed comparative studies are required to enhance our understanding of different cartilage treatments.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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