The clinical use of enriched bone marrow stem cells combined with porous beta-tricalcium phosphate in posterior spinal fusion

Yaokai Gan, Kerong Dai, Pu Zhang, Tingting Tang, Zhenan Zhu, Jianxi Lu

Abstract
Cytotherapy for bone regeneration has not been widely used clinically. A new method based on enriched bone marrow-derived mesenchymal stem cells (MSCs) combined with porous beta-tricalcium phosphate (β-TCP) was used for posterior spinal fusion in 41 patients. The aim of the present study was to assess the clinical feasibility of peri-operative bone marrow stem cell enrichment and their combination with tricalcium phosphate. About 252 ml marrow per patient was harvested from bilateral iliac crest, the enriched MSCs were produced by a cell processor peri-operatively, then combined with porous β-TCP granules by a negative pressure and a short-time incubation in the meantime of conventional operation, which were finally implanted back into the patient. About 45 ml enriched MSC suspension was collected, and 78 ± 16% of MSCs were recovered. By enrichment technique, the number of colony-forming units (CFUs) was increased 4.3 times; the increasing folds of bone marrow nucleated cells (NCs) and MSCs had a positive correlation. The natural log (ln) of MSC number declined with age, and also, the MSC number of younger subjects (<40 years) was more than that of older ones (>40 years), but none for NCs. The number of NCs and MSCs was not different significantly between men and women. However, the patients with thoracic-lumbar fracture (TLF) had significantly more MSCs than those with degenerative disc disease (DDD), but not for NCs. On the other hand, enriched MSCs could adhere to the wall of porous β-TCP within 2 h combination, and proliferate well during culture in vitro. After 34.5 months, 95.1% cases had good spinal fusion results. None of the samples before grafting was positive in bacterial culture. Only four patients had a little exudation or moderate swelling in their wounds, and recovered with conservative treatment.

Keywords:
Enrichment technique
Bone tissue engineering
Mesenchymal stem cell
Ceramics

1. Introduction

With the progress of stem cell and tissue engineering techniques, much of the literature suggests that the effectiveness of any successful bone graft material generally can be attributed to one or more of three core properties: osteoconduction, osteoinduction, and osteogenic cells [1–4]. However, the efficacy of any osteoconductive material or osteoinductive stimulus depends entirely on the presence of a sufficient number of osteoprogenitors (OPs) in the graft or nearby the graft site. Meanwhile, many clinical settings are suboptimal, which is supported by a great deal of animal and clinical data [1,3,5–7]. Fortunately, bone marrow is a natural reservoir of mesenchymal stem cells (MSCs), which are pluripotent and can proliferate, subsequently differentiating into specific phenotypes, such as OPs [8–10]. MSCs represent a small (0.001–0.01%) fraction of the total population of the nucleated cells (NCs) in marrow [11,12]. To increase the concentration of MSCs, several techniques have been developed, especially cell ex vivo expansion, but many problems limited its clinical application, such as the sterility technique, long culture time, high cost and the mixture of human cell culture medium with fetal bovine serum. On the contrary, the centrifugation technique [13–15] of bone marrow MSCs may be a useful way for clinical bone repair without many limits. With the technique of density gradient centrifugation, a nucleated cell (NC) of the middle layer from about 300 ml of bone marrow could be separated and collected safely. This buffer coat contained not only MSCs but also other mononuclear cells and some of these other cells may be a source of angiogenic or osteogenic cytokines with a positive clinical effect [16]. This separating system provides a sterile, rapid, and non-culture process of cytotherapy, which facilitates clinical usage.
But, with injection alone, a part of concentrated marrow would constantly run off, thereby decreasing the therapeutic effects. On the other hand, there are few reports concerning the inherent influential factors about this technique. To avoid the implanted cells running off and to demonstrate the influential factors of the concentration–separation technique, we began this study, and in it, the term “enrichment technique” was used to describe the density gradient centrifugation of bone marrow which can supply the buffer coat enriched MSCs. A rapid technique to make a cell-material composite graft peri-operatively was developed. A porous β-tricalcium phosphate (β-TCP) was used as the scaffold. It is a degradable bioceramic with certain mechanical strength. A construct can be made peri-operatively. We chose the spinal fusion procedures to observe the results of the new composite graft implantation.

In this study, we used autologous enriched MSCs combined with porous β-TCP (enriched MSCs/β-TCP) as a composite graft to perform posterior spinal fusion. Three questions were expected to be answered: (1) The possibility of the fabrication of enriched MSCs/β-TCP constructs, including the feasibility of the peri-operative enrichment technique and the quick combination technique. (2) The inherent influential factors about this technique, including the changes of individual bone marrow NCs and MSCs, and the influences by manipulation. (3) The clinical safety and effectiveness of this technique in posterior spinal fusion.

2. Materials and methods

2.1. Participants

Between March 2004 and October 2007, 41 patients scheduled for posterior spinal fusion with transpedicular spinal instrumentation and enriched MSCs/β-TCP implantation were enrolled in this prospective, institutionally reviewed, hospital board-approved study. Local ethical committee provided ethics approval, and patients gave written informed consent before being included in the study. Inclusion criteria were segmental posterior spinal fusion with pedicle screw fixation for the treatment of degenerative disc disease (with lumbar instability or with spondylolysis), or instable thoracolumbar fracture. Exclusion criteria were previous spinal surgery with instrumentation, previous iliac crest harvest, history of spine infection, history of myeloproliferative disorder, previous irradiation of either the spine or the pelvis, a systemic inflammatory disease, and/or a coagulation disorder. Patients were also excluded from the study if they were being managed with chronic steroid therapy. Patients averaged 44.0 years of age (range, 14–66 years) and included 30 men and 11 women. Preoperative symptoms and signs were well documented, and radiological studies included radiographs, computerized tomography (CT) scan, and magnetic resonance image (MRI) studies. Among them, a total of 22 patients were enrolled as either having a degenerative disc disease with lumbar instability (n = 17 patients), or having Grade I or Grade II spondylolisthesis (n = 5); 19 patients had thoracolumbar fractures, of which four patients had neurological deficit (Table 1).

2.2. Harvest of autologous bone marrow

After the patient was prepared under general anesthesia and draped in the supine position, the superior aspect of the anterio-superior iliac spine was palpated 2.2 cm along the same trajectory to continue a new turn of aspiration. Every aspirate much as possible[17]. After one full turn, the needle and its plunger was advanced 2 cm along the same trajectory to continue a new turn of aspiration. Every aspirate was rapidly pooled in a plastic bag containing an anticoagulant (1000 U heparin for each 10 ml of bone marrow). The time during which bone marrow stayed in the syringe was less than 10 s. The needle generally can be advanced approximately 5–6 cm parallel to the line of the iliac crest. As soon as this point is reached, the needle must be removed, and redirected in a different path (2 cm up or down the iliac crest). Alternatively, the needle may be reoriented into a fan shape through the same skin opening in order to harvest additional marrow. For each iliac side, 120–130 ml marrow was obtained, and then pooled marrow was filtered to eliminate the cellular aggregates and miniature bone fragments. When the harvested marrow was sent to the preparation in a nearby operating room, spine surgery began, which means the cells’ enrichment and cell–scaffold combination steps were accomplished in the same time as the surgery step in the operating room.

2.3. Enrichment of bone marrow

In the preparation room, the bone marrow was enriched using a cell separator (COBE 2991™ Cell Processor, GAMBRIO BCT, Inc.). A 7-min centrifugation at 400g (where g—one gravity) forced the different elements of marrow to demix with different specific density. The middle layer containing not only MSCs, but also other nucleated cells, was collected in a semi-automatic manner. The internal layer consisted of plasma without cells, whereas the external layer contained red blood cells which could be collected after centrifugation. The volume of enriched MSCs recovered (Vb, in ml) had a relationship with two parameters: the velocity of flow rate (FR, in ml/s) and the recovery time (RT, in s), where Vb = FR × RT. Then, if FR was 100 ml/60 s and RT was 18–30 s, Vb would be 30–50 ml. By artificial real-time control, the layer of nucleated cells (NCs) was recovered in which enriched MSCs could be used for implantation peri-operatively; 5–10 ml of marrow samples was extracted for bacteriological examination.

2.4. Graft preparation

β-TCP (Bio-Lu Bioceramics, Shanghai, China), whitish granules, 3–5 mm in diameter with a 75 ± 10% porosity (framework porosity of 500 ± 200 μm, interconnection pore size 150 ± 50 μm, fully interconnected geometry), was combined as scaffolds with enriched MSCs (Fig. 9). A 15–25 g sample (39.5–65.8 ml) of β-TCP granules was first loaded into a 50 ml syringe, using a cap at the tip of syringe to prevent passage of the contents from the syringe. Then the suspension of marrow was pooled into the syringe, and the β-TCP granules soaked completely in the suspension. After gently mixing this suspension with β-TCP granules, a negative pressure of 0.5–0.25 atmospheres was applied to the mixture, during which vacuum exhausted air from the granule pores, and facilitated the cells to enter inside of the

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Distribution of baseline diseases of patients</th>
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<tbody>
<tr>
<td>Patients</td>
<td></td>
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<tr>
<td>Degenerative disc disease (DDD)</td>
<td>17 (41%)</td>
</tr>
<tr>
<td>Lumbar instability</td>
<td>17 (41%)</td>
</tr>
<tr>
<td>Spondylolisthesis with stenosis</td>
<td>5 (12%)</td>
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<tr>
<td>Thoracolumbar fracture (TLF)</td>
<td>15 (37%)</td>
</tr>
<tr>
<td>Non-neurological deficit</td>
<td>4 (10%)</td>
</tr>
<tr>
<td>With neurological deficit</td>
<td>4 (10%)</td>
</tr>
</tbody>
</table>

Fig. 1. Porous β-tricalcium phosphate (β-TCP) with a 75 ± 10% porosity, framework porosity of 500 ± 200 μm, interconnection pore size 150 ± 50 μm, fully interconnected geometry (SEM: 100×).
2.5. Spinal surgery

Soon after the bone marrow was harvested, the patient was changed to prone position for operative procedures. Laminec- tomies were performed over an average of 1.4 levels (1 level for 14 patients, 2 levels for 8 patients) for a one- or two-segment fusion in instable lumbar segment or spondylolisthesis with stenosis cases (n = 22). Transpedicular screw/rod instrumentation was then placed over one or two motion segments where spondylolisthesis was present. For thoracolumbar fracture patients (n = 19), laminec- tomies were performed among those who had neurological deficits (n = 4). In the neurologically intact patients (n = 15), decompression by laminec- tomies was not performed. The reduction of fracture vertebrae was mainly done by body posture and instrumentation before fixation under biplane fluoroscopic control. About 2.2 levels (2 levels for 15 patients and 3 levels for 4 patients) for two- or three-segment fusion were done in the patients with thoracolumbar fractures. Transpedicular screw/rod instrumentation was then placed over two or three motion segments.

2.6. Implantation of materials

For decompression cases, locally harvested bone from the decompression site was stripped off all soft tissue before being morselized into small bone pieces with a rongeur forceps, and combined with the autologous enriched MSCs/β-TCP com-posite grafts. For non-decompression cases, only the autologous enriched MSCs/β- TCP composite grafts served as fusion material. None of the patients needed the extra autograft. No drainage was used in wound.

2.7. The NC and MSC counts

The number of nucleated cells (the number of nucleated cells per 1.0 ml of marrow aspirate) was estimated immediately using a hemocytometer (Couter HMX). Also the number of MSCs in a sample can be estimated by counting the number of colony-forming units (CFUs) expressing alkaline phosphatase activity in culture (CFUs-ALP + )[18]. Alkaline phosphatase is an early marker for osteoblastic differentiat- ion of these pluripotent MSCs. Each sample was placed into cultures in six- well plates and each was suspended in α-MEM (alpha-Minimum Essential Medium) containing 10% fetal bovine serum (Hyclone, USA), 50 mg/ml sodium ascorbate (Sigma, USA), antibiotic/antimycotic, and 10 μM dexamethasone (Sigma, USA). Cultures were maintained at 37 °C in a humidified atmosphere of 5% CO2 in air. Medium was exchanged after 48 h and then again every 2 or 3 days. Alkaline phosphatase staining was carried out on Day 10. The number of CFUs (the alkaline phosphatase-positive clusters which were 2 mm in diameter or larger) were counted using confocal microscopy. Meanwhile, several com- posite particle samples were cultured in six-well plates for up to 2 weeks. Then cell adhesion and growth morphology on the three-dimensional (3D) construct were monitored using confocal microscopy.

2.10. Clinical observations

Clinical observations were performed in an outpatient clinic. Radiographic and clinical assessments were made after 2 weeks. Thereafter, additional examinations were made after 1 month, 3, 6, 12 and 24 months. Following that, more assessments were conducted using computerized tomography (CT) scanning. Two independent orthopedic surgeons assessed the follow-up radiographs to evaluate the fusion segments. The surgical site was considered fused when both observers found no radiographic evidence of nonunion. Fusion of each side was defined as conclusive evidence of continuous bony bridging in operating levels. The presence of any apparent gaps in the fusion mass on the posterior spine fusion site was classified as nonunion.

2.11. Statistics

Depending on the distribution properties, each outcome value (the number of nucleated cells and the prevalence of MSCs) was summarized as a mean ± standard deviation (SD) or as medians with ranges. These data were analyzed relative to gender, age, and disease. For the normal distribution data, Student’s t-test was used to compare differences in means of two samples, and paired t-test was used for paired means of two samples. Nevertheless, for non-normal distribution data, signed rank-sum test was used to compare differences in means. A natural logarithm transformation was applied to normalize the data and to eliminate the positive distribution skew in linear regression analysis, which describes the relationship between nucleated cells and age, the prevalence of MSCs (CFUs-ALP + ) and age. The resulting variables have a normal distribution and were assessed with analysis of variance methods. The Pearson correlation coefficients were calculated for the en- richment folds of NCs and those of MSCs (CFUs-ALP + ). A P value < 0.05 was con- sidered to indicate statistical significance. Statistical analyses were done with SAS version 8.0.

3. Results

Forty-one patients underwent the implantation of enriched MSCs/β-TCP in spinal fusion. All of the patients were harvested of their bone marrow in operating room, taking 15–30 min per procedure. None of the patients had complications during the harvesting procedures: no decrease in oxygen saturation, no change in pulse rate nor blood pressure. An average of 252 ± 29 ml of marrow was aspirated from the two iliac crests of each patient. Then a mean of 45 ± 13 ml enriched MSCs’ suspension (V0) was obtained by enrichment technique. The volume of initial marrow reduced to one-fifth of the original value, a mean of 18 ± 4%.

3.1. The effect and assessment of enrichment technique

The number of NCs had a mean value of (161.6 ± 4.1) × 106/ml in pre-enrichment marrow, and (445.1 ± 5.1) × 106/ml in post-enrichment suspension, M (Sign) = 20.5, P = 0.0001. The prevalence of CFUs-ALP + was (213.7 ± 246.3) /ml (median, 146.0 CFUs-ALP+ /ml; interquartile range, 66.7–256.7 CFUs-ALP+ /ml) in pre-enrichment marrow, and (865.6 ± 875.9) /ml (median, 540.0 CFUs-ALP+ /ml; interquartile range, 310.0–1033.3 CFUs-ALP+ /ml) in post-enrichment suspension, M (Sign) = 20.5, P = 0.0001. On an average, the recovery of MSCs (CFUS-ALP+ ) was 78 ± 16% (range: 53–98%) after the cell-enrichment process, and the number of CFUs-ALP+ was enriched 4.3 ± 1.2 times over the original.

Fig. 2 illustrates the relationship between the increased folds of the number of CFUs-ALP+ (M) and those of NCs (N). There was a positive correlation between M and N, r = 0.3490, P = 0.0253. The linear regression analysis model gives M = 0.5818N + 2.7043, R² = 0.1218, adjust R² = 0.0993, P = 0.0253, slope (m) = 0.5818.

3.2. The factors relating to individual bone marrow NCs and MSCs

The statistical results (Figs. 3 and 4) showed: (1) the number of nucleated cells (NCs) was not significantly different between men and women (normal distribution), t = 0.7163, P = 0.4780; and the prevalence of MSCs (CFUs-ALP+ ) was not significantly different between the two groups (non-normal distribution), χ² = 1.0611, P = 0.3030. However, (2) for younger (<40 years old) and older (>40 years old) subjects, although the number of NCs was not significantly different (normal distribution, t = 0.3383, P = 0.7370), the number of MSCs (CFUs-ALP+ ) of the younger subjects was significantly more than that of older subjects (non-normal distri- bution), χ² = 4.4837, P = 0.0342. (3) For disease-related changes, the number of NCs between degenerative disc disease (DDD) sub- jects and those with thoracolumbar fractures (TLFs) was not signif- icantly different (normal distribution t = 0.1222, P = 0.9033), whereas the number of MSCs (CFUs-ALP+ ) was significantly more in TLF subjects than those with DDD (non-normal distribution, χ² = 9.8471, P = 0.0017).
of NCs had no significant differences between men and women, between the patients with degenerative disc disease (DDD) and those with thoracolumbar fractures (TLFs), recovered

\[
\frac{\ln (\text{NCs})}{C_0} = C_1 + C_2 \cdot x
\]

0.6712, \(P = 0.0004\). There was no significant relationship between the natural logarithm of CFUs-ALP+ and age. There was no significant relationship between the natural number of NCs and CFUs-ALP+

Fig. 2. The correlation between the increased folds of the number of CFUs-ALP+ (M) and those of NCs (N). Regression line between M and N is shown.

Fig. 5 presents the natural logarithmic transformation of the number of NCs and CFUs-ALP+/ml for the entire cohort distributed by age. There was no significant relationship between the natural logarithm (ln) of NCs and age, between which the Pearson correlation coefficient \(r = -0.04162\), \(P = 0.7961\). However, there was a negative correlation between ln (CFUs-ALP+) and age, \(r = -0.5301\), \(P = 0.0004\). The linear regression analysis model yields

\[
\ln (\text{NCs}) = -0.0009 \cdot x + 2.7856, R^2 = 0.0017, \text{adjusted } R^2 = -0.0239, \quad P = 0.7961; \quad \ln (\text{CFUs-ALP+}) = -0.0397 \cdot x + 6.6852, \quad R^2 = 0.2809, \quad \text{adjusted } R^2 = 0.2625, \quad P = 0.0004.
\]

3.3. The relationship between cell recovery rate and the volume recovered

The recovery of the number of NCs and MSCs (CFUs-ALP+), respectively, had relationships with the volume recovered (x) by

\[
Y = -0.4364 \cdot x + 2.7856, \quad R^2 = 0.0017, \quad \text{adjusted } R^2 = -0.0239, \quad P = 0.7961; \quad Y = -0.3557 \cdot x + 6.6852, \quad R^2 = 0.2809, \quad \text{adjusted } R^2 = 0.2625, \quad P = 0.0004.
\]

4. Combination of MSCs/β-TCP and its proliferation in vitro

The porous β-TCP granules were combined with enriched MSCs’ marrow by means of the vacuum process primarily and incubation secondarily at 37°C in 5% CO2 (air) (Fig. 8).

Some samples of the composite were examined by SEM which showed that some MSCs entered the inside of scaffolds through inter-pore connection, and adhered to the wall of inside porous β-TCP within 2 h of incubation (Figs. 9 and 10).

By being cultured for 2 weeks and then examined the constructs with a confocal microscope, it was found that the composite was covered by proliferating spindle cells on the surface, as well as the inside of the scaffolds (Figs. 11 and 12). Cells then migrated into and colonized the inside of porous scaffold granules by an inter-pore connection, which caused effective cell proliferation.

3.5. Clinical observations

All patients who underwent the cytotherapy for spinal fusion were followed up in the outpatient clinic, in 34.5 ± 6.2 months, median 36.5 months. None of the patients presented pain in their bone marrow donor sites of iliac crests after 3 months postoperatively. The X-ray and CT scans were assessed by two independent orthopedic surgeons. The fusion rate was 95.1%. Demography of local grafts and spinal fusion levels are shown in Table 2. None of the patients needed extra autograft. The patients who underwent decompressed laminectomy, including 22 patients with degenerative disc disease (DDD) and 4 thoracolumbar fracture (TLF) patients with neurological deficit, had 6.7 ± 2.7 ml bone chips grafted from laminectomy decompression procedures, which were mixed with enriched MSCs/β-TCP materials for grafting in fusion sites. Fifteen TLF patients without neurological deficit had only the autologous enriched MSCs/β-TCP composites as fusion grafts without using any other bone grafts. The volume of

Fig. 4. The number of MSCs (CFUs-ALP+) of marrow pre-enrichment in different groups. The average number of MSCs of the entire cohort was 213.7/ml. The number of MSCs had no significant differences between men and women, \(P > 0.05\). But the number of MSCs was more in the younger patients (<40 years) than those in the older ones (>40 years), \(P < 0.05\). The number of MSCs was more in patients with thoracolumbar fractures (TLFs) than those with degenerative disc disease (DDD), **\(P < 0.005\).

Fig. 3. The number of nucleated cells (NCs) of marrow pre-enrichment in different groups. The average number of NCs of the entire cohort was \(16.1 \times 10^6/ml\). The number of NCs had no significant differences between men and women, between the patients <40 years and those >40 years, between the patients with degenerative disc disease (DDD) and those with thoracolumbar fractures (TLFs), \(P > 0.05\).
constructs (enriched MSCs/β-TCP) implanted was 51.4 ± 7.0 ml. None of the bacteriological examinations before grafting was positive for bacterial culture. Only four patients had a transient exudation or moderate swelling in their wounds, which were all negative for the presence of bacterial cultures, recovered under medical treatment. Two patients who underwent two-level fusion had a side nonunion of one segment, but they presented little discomfort during their follow-up examination. Another two patients had back pain in follow-up, but neither of them presented these conditions as a result of the cytotherapy. One patient's pain was a result of bursa synovialis caused by the end extremity of the implanted screw, and the other had a progressive instability of the adjoined supra-vertebra. Both of them were relieved by physical therapy.

There was no neurological deterioration after operation, and there was no deep vein thrombosis or pulmonary embolism as well. None of the patients followed up needed re-operative procedures as a result of the removal of an improperly-placed screw or neurological deterioration in follow-up, and 95.5% (21/22) patients with degenerative disc disease had improved preoperative motor or sensory dexterity. Only one had residual numbness in the sole of the foot, but demonstrated no motor weakness. Three thoracicolumbar fracture patients with neurological deficits had marked improvement in both motor and sensory function. One who had preoperative paraplegia failed to improve after surgery and a series of physiotherapeutic procedures.

During operation, nine patients of this series consent underwent blood salvage, and the suspension of residual red cells was transfused back intravenously by a cell saver system. None of them had any side effects from the autologous transfusion.

Bony fusion interlaminar or intertransverse process sites began to be clear in 6 months post-operation, the bulk of composite graft was replaced gradually by newly formed bone. Positive bony fusion was observed in 12 months postoperatively by CT scanning and 3D reconstruction (Figs. 13 and 14) during follow-up.

![Fig. 5](image)

**Fig. 5.** Regression line for the natural log (ln) transformation of the number of NCs and the prevalence of CFUs-ALP+ with age.

![Fig. 6](image)

**Fig. 6.** The correlation between the volume recovered (x) by enrichment technique and the number of NCs (yN). Regression line for the correlation is shown.

![Fig. 7](image)

**Fig. 7.** The correlation between the volume recovered (x) by enrichment technique and the number of CFUs-ALP+ (yC). Regression line for the correlation is shown.
4. Discussion

In recent years, although numerous preclinical investigations of stem cell application techniques for promoting bone regeneration have been studied [1,4,11,19–24], there were few reports concerning human clinical cytotherapy [13,14,16,25,26]. Because of some ethical or technical limits, clinical cytotherapy has not been widely used with much success. By means of enrichment techniques, marrow MSCs could be concentrated, and by rapid combination with scaffolds, concentrated MSCs could adhere to the inner wall of porous β-TCP. Finally, a new cell-based composite graft could be produced peri-operatively. This procedure avoids many limited points, including easy contamination during culture, long culture time, high cost, the mixture of culture medium with animal serum, and the pre-operation waiting time, therefore it can be accepted clinically. The findings of this study not only demonstrate the effects and influential factors of the enrichment technique and the feasibility of combination technique, but their clinical safety and effectiveness in posterior spinal fusion as well.

4.1. Advantage of the cytotherapy method

There are five elements necessary to determine the actual clinical value of a cytotherapy: safety, effectiveness, minimal...
invasiveness, convenience and cost. Firstly, the procedures were safe, as the enrichment technique was performed in a sealed system in this study. With a single usage channel, and with all procedures done in the laminar flow operation room, it assured sterility, security and non-cross-contamination. None of the samples of this study was positive in bacterial culture. Secondly, the cytotherapy could promote bone repair and bone regeneration. Hernigou and his colleagues reported that 80–88% of atrophic nonunion fractures had healed by the injection of concentrated MSCs’ suspension, and they considered its efficacy was related to the number of osteoprogenitor cells in the graft [13,26]. There is little doubt that there is no new tissue formation except through the activity of living cells in the grafted site, particularly the small subset of stem cells and progenitor cells that are capable of generating new tissue. Notably, a factor not to be ignored is the environment of the cells. We used enriched MSCs/porous β-TCP to promote posterior spinal fusion. By the combination technique, cells could enter the inside of materials and adhere to their walls, which provided both a large and effective surface structure for the colony of cells. Small granule formation favored the interior cells with a nutritional exchange of interstitial fluid. The porous, bio-degradable, ceramic enlarged cells’ life space, and provided ions of Ca²⁺ and PO₄³⁻ to the local environment for the mineralization of extracellular matrix when the ceramic degraded in vivo. Thirdly, compared with autologous iliac crest bone donor, autologous bone marrow harvest was only slightly invasive, and caused little discomfort to the patient. As the most common site for harvesting autograft material, iliac crest donor sites often have the morbidity associated with bone graft harvesting, including deep infection, large hematomas, neurological and vascular injuries, iliac wing fractures, and even herniation of abdominal contents [27–29]. Notably, 30% of patients might experience chronic pain at the donor site [28]. In our study, none of the patients presented any pain on the bone marrow donor sites in 3 months postoperatively. Fourthly, although an enrichment technique could not provide the same number of MSCs as a cell expansion technique, further operation or injection under anesthesia for re-implantation is unnecessary after about 3 weeks of cell expansion in vitro. In addition, it avoids some ethical problems associated with cell expansion and becomes both less expensive and more convenient for clinical usage. And also, it avoids the inevitably physico-chemical influences in cell expansion in vitro. It was the fifth advantage that only 1000 yuan RMB (about 136 US dollars) was expended for this procedure, instead of the extra autologous bone graft or the other bone substitutes used.

### 4.2. Influential factors of the number of enriched MSCs

Three main factors affected the number of enriched MSCs: marrow harvest method, individual constitution, and efficiency of enrichment. To avoid a large volume of aspirations which would decrease the concentration of osteoprogenitor cells because of dilution by peripheral blood [17], we designed a fan-shaped, layering solution by peripheral blood [17], we designed a fan-shaped, layering.

<table>
<thead>
<tr>
<th>Patient number</th>
<th>Disease Local bone graft (ml)</th>
<th>Iliac crest bone graft</th>
<th>Enriched MSCs/β-TCP (ml)</th>
<th>Fusion levels</th>
<th>Fusion outcome</th>
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TLF: thoracolumbar fracture. DDD: degenerative disc disease.
constitution might have more effects on the number of MSCs. In this study, there are no significant differences between men and women in both the number of NCs and the prevalence of MSCs, although the mean prevalence of MSCs seemed to be higher in men than in women. Muschler et al. reported that the prevalence of MSCs might be higher in men than women, but not for NCs [7]. He inferred that this disparity might be relevant to the pathophysiology of age-related bone loss and post-menopausal osteoporosis of women. As to age-related variation, we found the number of MSCs in younger subjects (<40 years old) was more than that of older ones (>40 years old). Moreover, a linear negative correlation existed between the natural logarithm of the prevalence of MSCs (CFUs-ALP+) and age, but not for the number of NCs. This phenomenon inferred that the prevalence of MSCs in younger subjects was higher than that in older ones, and the prevalence of MSCs declined with age. Our results illustrated that it was not so much a linear age-related decline in MSCs over the entire age range of 14–66 years, but rather a sharp drop after the rapid juvenile bone growth period to a lower rate in mature adults. It was a logarithmic relation (Fig. 5). The mechanism of the age-related changes in MSCs’ population in bone marrow was mainly determined by the condition of skeletal growth [18,30,32,33]. The majority of bone growth is formed in the youth period of development. When the bone tissue grows as height increases, MSCs are relatively more prevalent in bone marrow recesses. Bone remodeling has a minor role after juvenile phase, and the MSCs of bone marrow decrease quickly. In particular, women experience menopause, so bone turnover increases as well as the quantity and activity of osteoclasts, then followed by the relative augmentation of the quantity...
and activity of osteoprogenitor cells, which postpones the declined tendency of the number of osteoprogenitor cells in bone marrow. Otherwise, there were generally two kinds of patients in this study: degenerative disc disease (DDD) and thoracolumbar fracture (TLF) injury. The prevalence of CFUs-ALP+ was significantly increased in the TLF group than that in the DDD group, which was a result of the mobilization of bone marrow by acute blood loss. The animal experiment demonstrated that the prevalence of osteoprogenitor cells could be increased notably by a 1% loss of blood volume [31]. Blood loss could stimulate the increase of osteogenic peptide in circulation, which was a bone stimulating peptide, similar to erythropoietin that stimulates hemopoietic cell production in bone marrow [34]. The osteogenic peptide stimulated the differentiation and proliferation of MSCs and increased the prevalence of osteoprogenitor cells, which favored the matrix formation and constructed the hematopoiesis surrounding.

The efficiency of enrichment technique was related to several factors. Generally, a 5-min centrifugation at 400g forced the nucleated cells to aggregate in the middle layer and separate from the remainder of the marrow. We used 7 min of centrifugation to gather MSCs as much as possible in the middle layer. One reason was because there was often larger volume of marrow or higher density of the cells to deal with, and the other was a desire to decrease the bulk volume of suspension. The current study showed the number of MSCs was enriched 4.3 times, and the recovery rate of MSCs was about 78%. These data were a little different from the results of Hernigou, who reported that MSCs increased about 3.9 times and 84% cells could be recovered [13,35]. The differences could be ascribed to the volume recovered of enriched MSCs (Vc). At a flow rate (FR) of 100 ml/min, Hernigou collected the middle layer for 40–50 s of recovery time (RT), but we reduced RT to between 18 and 30 s. Therefore, we obtained a lesser volume of the nucleated cell suspension. Accordingly, the number of MSCs recovered had a positive correlation with the volume recovered in certain circumscription (Figs. 6 and 7); we recovered less of the MSCs. And because the middle layer was precisely selected, less suspension volume was recovered, the MSCs were concentrated about 4.3 times more than the results of Hernigou. In addition, the increased folds of the number of CFUs-ALP+ and those of NCs by enrichment technique could be related to each other by a linear regression equation (Fig. 2). This relationship could be used as an easy reference for the estimation of clinical enrichment effect, which means to estimate the increasing folds of MSCs after enrichment, only comparing the number of NCs in pre- and post-enrichment samples is needed, instead of 10 days culture in vitro. The linear regression model could be useful in certain circumstances, but depends on the skill of manipulator, and his or her judgment of the middle layer (the nucleated cell layer) and the recovered volume.

4.3. Combination of MSCs/β-TCP

Injection of autologous marrow for bone repair is one of conventional clinical method [13,25,26,36]. Besides the number of MSCs by injection, the clinical effects were relevant to many factors, including the precise injecting site, the effective remainders of injected cells, the surrounding vascularity, as well as the local biomechanics. We used the porous β-TCP granules as scaffolds, in which MSCs could be forced to migrate by a negative-pressure combination technique which can prevent cells from running off. There were two factors holding the cells in place: physical absorption of porous scaffolds and cell adhesion to the wall of β-TCP by incubation. Some MSCs adhering to the walls of β-TCP were found by SEM (Fig. 10) even within 2 h of incubation. With 75 ± 10% porosity and fully interconnected geometry of β-TCP granules, cells could enter the inside of scaffolds. The experiment in vitro (Figs. 11 and 12) also showed that MSCs could grow in place and proliferate well in the presence of manifold scaffold granules.

4.4. Clinical application and effects

In our study, we chose the posterior spinal fusion as a subject of cytotherapy. Spinal fusion is a common means to treat vertebral instability, and sometimes need considerable bone graft. Approximately 200,000 bone grafting procedures are done for spinal fusion each year in United States [2]. Posterior spinal fusion using autologous bone graft is a frequently used method for achieving lumbar intersegmental arthrodesis. However, pseudarthrosis and donor-site morbidities remained problematic [2,37]. These problems have led to the search for graft alternatives. A variety of materials have been investigated in spinal fusion, such as hydroxyapatite [19], tricalcium phosphate [38], demineralized bone matrix (DBM) [39], and type I collagen [19]. These materials may be served as bone graft extenders but not as bone graft substitutes in spinal fusion. We used enriched MSCs/β-TCP as fusion material to perform spinal fusion peri-operatively. By this means, no extra iliac crest bone graft needed, no donor sites’ complications existed, no further operation needed due to the removal of an improperly-placed fusion material or neurological deterioration. In addition, all the procedures herein described were performed in a clean environment which assured the safety, otherwise 95.1% patients had a successful spinal fusion. Among them, 15 thoracolumbar fracture patients without laminectomy decompresion having only the enriched MSCs/β-TCP composites as fusion grafts had good fusion results. Compared with the 65–95% success rate of autologous bone graft used as fusion material in other previous studies [2,37,40], the results of this study paralleled others equally successful.

By combining the autologous enriched MSCs and porous β-TCP granules, a new technique was developed, which produced a bio-material in which enriched bone marrow provides a rich source of cells ranging from MSCs to cells that have already committed down the osteogenic lineage and were destined to become osteoblasts. The autologous marrow was osteogenic and potentially osteoinductive. The porous β-TCP granules provided an osteoconductive scaffold, which favored the internal growth of cells and vessels. Therefore, with the three core properties, the new biomaterial would serve as a bone substitute in bone regeneration.

5. Conclusions

The enrichment of autologous marrow MSCs combined with porous β-TCP peri-operatively is as effective as autologous bone for the purposes of grafting for instrumented posterior spinal fusion surgery. Thus, the morbidity and potential complications associated with the harvesting of bone graft from the iliac crest may be avoided. As a clinical cytotherapy in orthopaedics, it provided a rapid, simple and safe method, which can provide an alternative approach to promote bone repair and regeneration. Notably, the younger patients or the patients with thoracolumbar fractures may have more MSCs in the pool of bone marrow.

Acknowledgments

We wish to thank Dr. Zhang Lei for SEM examinations, Dr. Guo Qiangou for the confocal microscopic analysis, and Professor He Qingpo for statistical analysis support. We are grateful for the kind help of Sun Xiaojiang, Ph.D., Xie Youzhuang M.D. Ph D. and BIAN Zhenyu, Ph.D. for cell analysis. We also gratefully acknowledge the support of the Shanghai Science Committee funding support (Grant No. 045407008) and the Key Project of Science and Technology Committee of Shanghai (Grant No. 05DJ14005).
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