Shaking improves the whole bone marrow adherent method of purification

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Abstract. The aim of the present study was to investigate the potential effects of mechanical shaking on the purity, activity, differentiation and possible apoptosis of rat bone marrow mesenchymal stem cells (BMSCs). Based on the whole bone marrow adhesion method, different durations and frequencies of mechanical shaking were used on primary cells. The biomarkers, CD29, CD90, CD45 and CD31, in addition to the apoptosis labels, annexin V-FITC and PI, were investigated using flow-cytometric analysis. The differentiation capability following purification was evaluated. Following shaking treatment, the purity of adherent cells increased, in particular there was an increase in CD29 and CD90 positive cells, with the majority of the detached cells negative for these two markers. In addition, the apoptotic rates increased with the increasing shaking duration and frequency. Furthermore, cells following shaking were induced to differentiate into osteoblasts and adipocytes. The shaking method allows for mesenchymal stem cells at to be obtained with higher positive rates of CD29 and CD90. In addition, horizontal shaking has little influence on cell activity or differentiation, with low levels of apoptosis occurring as a result of shaking.

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Introduction

Bone marrow mesenchymal stem cells (BMSCs) are a more easily accessible type of mesenchymal stem cells and have received increasing attention in induced cellular differentiation (1,2), tissue engineering (3), cell therapy (4,5) and additional research areas. However, the number of BMSCs in bone marrow is relatively low (~0.001-0.01% of nucleated cells), and BMSCs easily differentiate and age in *in vitro* culture (6). Thus, a suitable method for acquiring sufficient levels of high-purity, high-activity and low-differentiation state mesenchymal stem cells is required for associated research.

Currently, the purification of BMSCs from bone marrow predominantly employs the following four methods: Plastic adherence (7), density gradient centrifugation (8), the flow cytometry separation method (9,10) and the immunomagnetic separation method (11). The plastic adherence method isolates mesenchymal stem cells according to the differences in the adhesion capacity of different cell types. This method is simple, however cell purity is reduced. The density gradient centrifugation method is more complicated. Although the extracted cells are of a higher purity, the cells grow slowly and have low activity, with a lengthy first fusion time for primary cells (12). The flow cytometry separation method and the immunomagnetic separation method use specific antibodies to isolate BMSCs. These methods are costly and are burdened by a lack of accessibility, and only specific stem cells may be achieved (9,11). Therefore, a simple and highly efficient method for isolating BMSCs is required.

The isolation and purification of mesenchymal stem cells from bone marrow is contaminated by two cell types: Hematopoietic stem cells and fibroblasts. Of these cells, the fibroblasts have the strongest adhesion capacity, followed by BMSCs, and then the hematopoietic stem cells (7). A previous study described a purification method for microglial and oligodendrocyte precursor cells (OPCs) which involved vigorous agitation (13). Therefore the current study hypothesized that the mechanical shaking of cells for different durations and frequencies may aid in the purification of BMSCs due to hematopoietic stem cells being swept away.

The whole bone marrow adherent method may be used to purify BMSCs, however it is a lengthy process. In the current

study, the addition of shaking to the whole bone marrow adherent method was observed to improve the purity of the cells obtained and reduce the duration of the purification process.

Materials and methods

Animals. Sprague Dawley (SD) rats, 100±10 g in weight, were provided by the Animal Experimental Center of The Second Military Medical University (Shanghai, China). The experimental procedures for the current study were approved by The Second Military Medical University Animal Ethics Committee.

Isolation and culture of primary BMSCs. As previously described (14), a total of 36 SD rats were sacrificed by CO_2 asphyxia, and then placed in 75% ethanol for 5 min. Under sterile conditions, bilateral femurs and tibias were surgically separated and cleaned. Subsequently, both ends of the epiphysis were cut and the bone marrow cavity was flushed from the end of the long bone with Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) using a 5 ml syringe. Following centrifugation (200 x g, 20˚C, 5 min), cells were resuspended in complete medium containing 10% fetal bovine serum (ScienCell Research Laboratories, San Diego, CA, USA), DF-12 (Gibco; Thermo Fisher Scientific, Inc.) and 1% streptomycin and penicillin (Gibco; Thermo Fisher Scientific, Inc.). Cell suspensions were grown in T75 flasks (Corning Incorporated, Corning, NY, USA) in 10 ml culture medium, with the cells in each derived from an average of 2 rats, then incubated at 37˚C in 5% CO_2. The media was refreshed 24 h later and then every other day. The cells were cultured for 7 days, following which cell colony formations were observed, and the flask randomly selected for the subsequent experiments. The medium was refreshed 30 min prior to the shaking.

Verification indices and treatment methods. Flow cytometry (FAC500; Beckman Coulter, Inc., Brea, CA, USA) was used to detect the presence of the following cellular markers: CD29-fluorescein isothiocyanate (FITC), CD90-R-phycoerythrin (PE), CD45-allophycocyanin, CD31-PE) and the apoptotic rate using annexin V FITC, propidium iodide (PI) all supplied by BD Biosciences (Franklin Lakes, NJ, USA) (15-18).

All staining was performed according to the manufacturer's instructions. Briefly, the attached cells in the flask were gently washed with 15 ml of phosphate-buffered saline (PBS). Cells were digested with 0.25% trypsin (pre-warmed to 37˚C) (Gibco; Thermo Fisher Scientific, Inc.) at 37˚C for 2-3 min. Following this, the majority of cells became round in shape and were free-floating, and 5 ml complete medium was added to terminate digestion. Following sufficient pipetting to dissociate the cells, the cells in suspension were centrifuged at 150 x g for 5 min at 20˚C, and then the supernatant was discarded. Following three washes with PBS, the cell pellet was resuspended in PBS, ($1x10^7$cells/ml for verification and $1x10^6$cells/ml for apoptotic tests). Subsequently the antibodies or PI were added and mixed and incubated for 15 min. All flow cytometry analyses were complete within 1 h.

Verification of the effects of mechanical shaking on cell purity. The culture flasks were divided into two groups. One group was shaken at 37˚C on a horizontal shaker at 180 rpm for 1 h and the second group served as a control. The supernatant was removed immediately following shaking and then the purity of the attached cells was detected by flow cytometry (FAC500; Beckman Coulter, Inc.,).

Selection of the frequency of mechanical shaking. The culture flasks were shaken at 140, 180 and 220 rpm following the media replacement, with 3 flasks used for each group. Following 1 h of mechanical shaking, the supernatant and attached cells were collected for purity analysis. Sufficient numbers of attached cells were obtained to perform apoptotic analysis and cell counting (Countess Automated Cell Counter, Invitrogen; Thermo Fisher Scientific, Inc.).

Determination of the duration of mechanical shaking. A total of 2 flasks from the same batch were used for each group, and were shaken at 180 rpm for 0, 0.5, 1, 2, 4, 6 and 8 h. Immediately following mechanical shaking, the supernatant and attached cells were collected for purity analysis, apoptotic analysis and cell counting.

Long-term effects of mechanical shaking. A total of 2 flasks from the same batch were used for each group, and were shaken at 180 rpm for 0, 0.5, 1, 2, 4, 6 and 8 h. The media was refreshed immediately following shaking and 3 days later. The attached cells were analyzed for purity.

Evaluating the effects of multiple short-duration shakes. A total of 8 flasks of cells from the same batch were used. Of these, 4 flasks were mechanically shaken at 180 rpm for 0.5 h each day for 2 days, and the media replaced with fresh media following each shake. The remaining 4 flasks were shaken at 180 rpm for 4 h. The duration of shaking was precisely controlled so that the two groups finished their treatment at the same time. Subsequently, the attached cells from all 8 flasks were analyzed for purity.

Evaluation of the in vitro differentiation potential of isolated BMSCs. Following a 2 h shake at 180 rpm, the attached cells were seeded on 24-well plates (Corning Incorporated) at a cell density of $1x10^5$cells/ml, 0.5 ml/well. Following 24 h incubation, osteogenic and adipogenic differentiation media (ScienCell Research Laboratories) were added individually. The media was replaced every 3 days. At 2 weeks later, the cells were fixed using 4% formaldehyde and were stained with alizarin red or oil red O (Sigma-Aldrich, St. Louis, MO, USA).

Statistical analysis. Values are presented as the mean ± standard error of the mean. All experiments were repeated with a minimum of 3 different batches. The differences between groups were analyzed using one way analysis of variance, and multiple comparisons were performed using Fisher's Least Significant Difference test or Student-Newman-Keuls method. Data were analyzed using SPSS software, version 21.0, (ISM SPSS, Armonk, NY, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Verification of the effects of mechanical shaking on cell purity. Following a 1 h shake at 180 rpm, the percentage of CD29 and CD90 positive cells was significantly increased (P<0.05; Fig. 1). The abundance of CD45 and CD31 positive cells was reduced, however, the percentage of these markers was lower than 1% and had minor effects on the results, therefore this data is not presented.

Selection of the frequency of mechanical shaking. The cell number was reduced following shaking at 220 rpm for 1 h, therefore only ~2x10^5 cells were cultured for subsequent tests.

Flow cytometric analyses revealed that with increasing shaking frequency, the number of attached cells was reduced and the cell purity was increased (Fig. 2A and B). The purity of the cells lost in the supernatant was increased, and in addition the purity increased with increasing frequency. However, the increase was of a smaller magnitude than that of the attached cells (Fig. 2C).

Apoptosis was observed to increase following shaking, with the level of apoptotic cells significantly greater at 140 rpm compared with the 180 and 220 rpm groups. However, there was no significant difference between 180 rpm and 220 rpm (P<0.05; Fig. 2D).

The determination of the duration of mechanical shaking. Following 8 h shaking, greater than 3x10^5 cells were present in culture. The percentage of CD29 and CD90 positive cells increased significantly with shaking durations up to 4 h, however, shaking durations longer than 4 h resulted in a reduction in CD29 and CD90 positive cells (Fig. 3B). In addition, significant differences were observed between the different shaking duration groups (P<0.01). The cells positive for CD29 and CD90 in the supernatant were increased with the increasing duration of shaking, with a large increase observed above 4 h shaking (Fig. 3C).

The level of apoptosis in the attached cells was observed to increase with increasing shaking duration, however, all values were lower than 12% (Fig. 3D).

Long-term effects of mechanical shaking. Following 3 days of mechanical shaking, the total number of cells obtained was high, with greater than 4x10^6 cells obtained from a single flask. The purity of the samples was greater compared with the unshaken control (P<0.01). However, the longer duration of shaking did not yield superior purity (Fig. 4).

Effect of multiple shorter duration shakes. Compared with a single 4 h shake at 180 rpm, multiple 0.5 h shakes prior to replacement of the media resulted in lower cell purity (P<0.01; Fig. 5).

Evaluation of the in vitro differentiation potential of isolated BMSCs. Following 2 weeks of differentiation induction, the purified BMSCs (passage 1) demonstrated osteogenic and adipogenic differentiation capacities (Fig. 6). BMSCs were cultured in osteogenic medium for 2 weeks, following which staining using Alizarin red was performed to detect the presence of bone nodules. The microscopy images indicated the

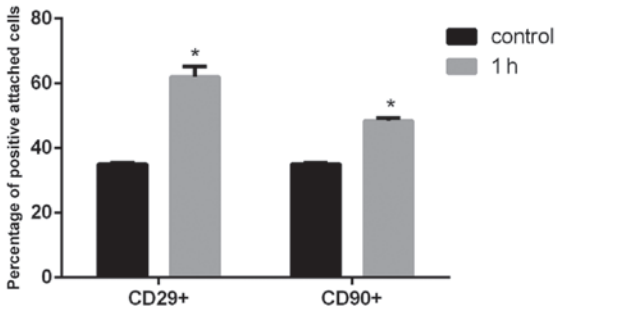

Figure 1. Cell marker abundances following a 1-h shake. *P<0.05 vs. control. CD, cluster of differentiation.

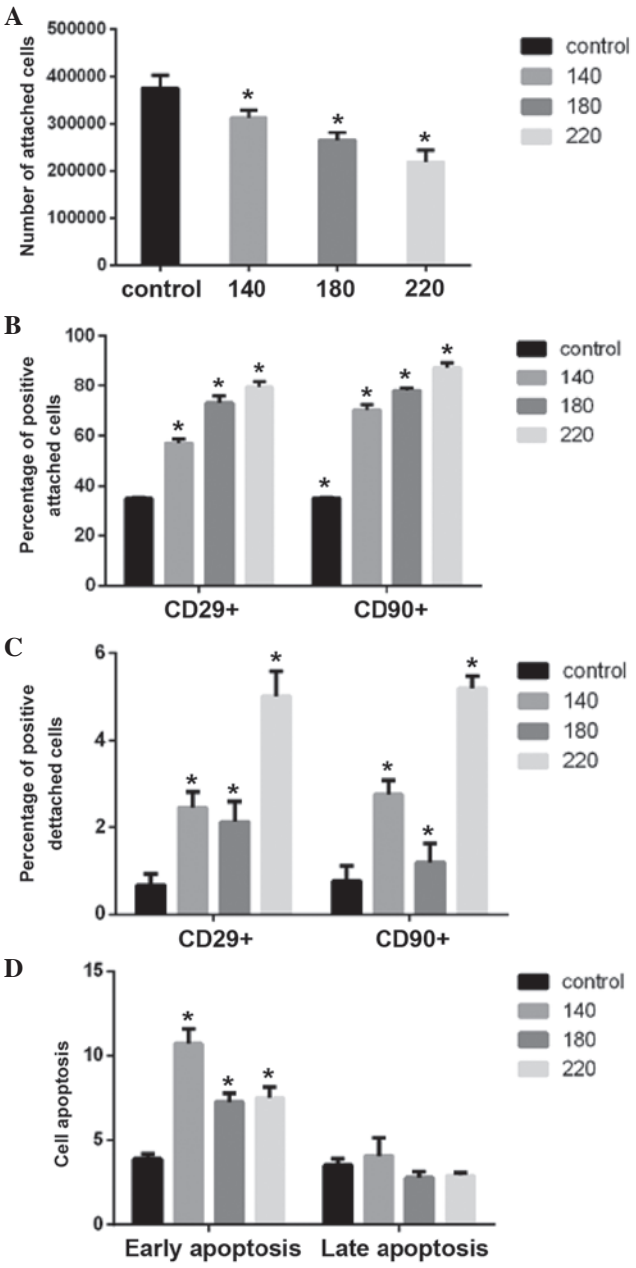

Figure 2. Effect of shaking frequency on cell purity. (A) The number of attached cells reduced with increasing shaking frequency. (B) Abundance of cell marker positive attached cells following a 1-h shake at different frequencies. (C) Abundance of cell marker positive detached cells following a 1-h shake at different frequencies. (D) Rate of apoptosis in attached cells shaken at different frequencies. Apoptosis includes the early and late stages. *P<0.05 vs. control. CD, cluster of differentiation.

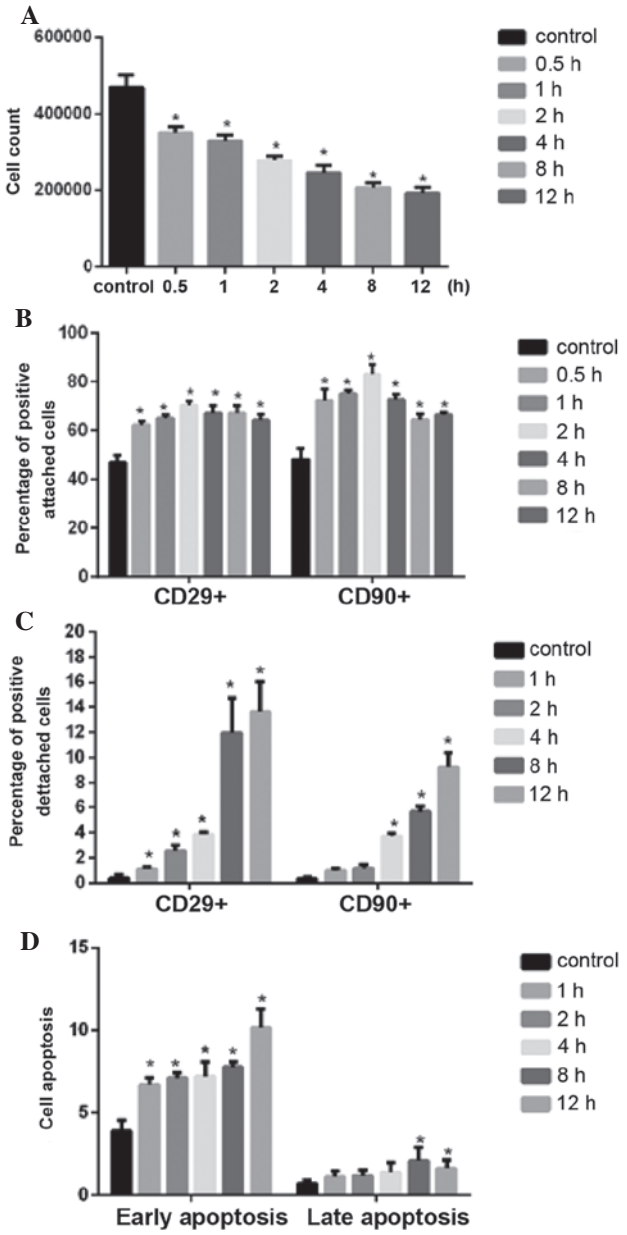

Figure 3. Effect of shaking duration on cell purity. (A) The number of attached cells was reduced when cells were shaken for different durations at 180 rpm. (B) Abundance of cell marker positive attached cells shaken for different durations at 180 rpm. (C) Abundance of cell marker positive detached cells shaken for different durations at 180 rpm. (D) Rate of apoptosis in attached cells shaken for different durations at 180 rpm. Apoptosis includes early and late stages. *P<0.05 vs. control. CD, cluster of differentiation.

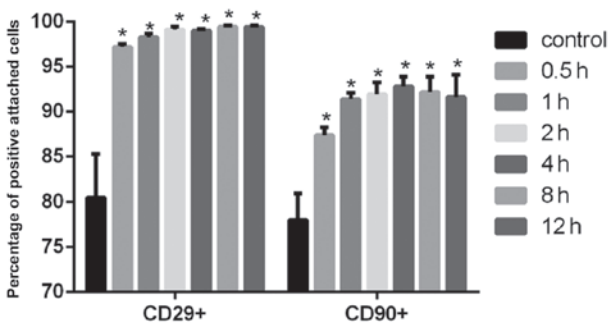

Figure 4. Cell purity analysis at 3 days following different shaking durations at 180 rpm. *P<0.05 vs. control. CD, cluster of differentiation.

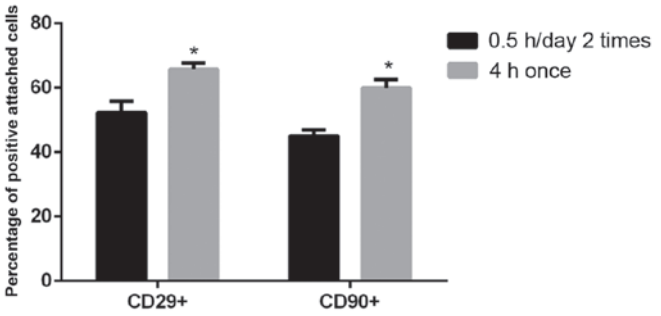

Figure 5. Effect of multiple short duration shakes on cell purity. Interval shaking and continuous shaking were performed, immediately followed by flow cytometry analysis. *P<0.05 vs. control. CD, cluster of differentiation.

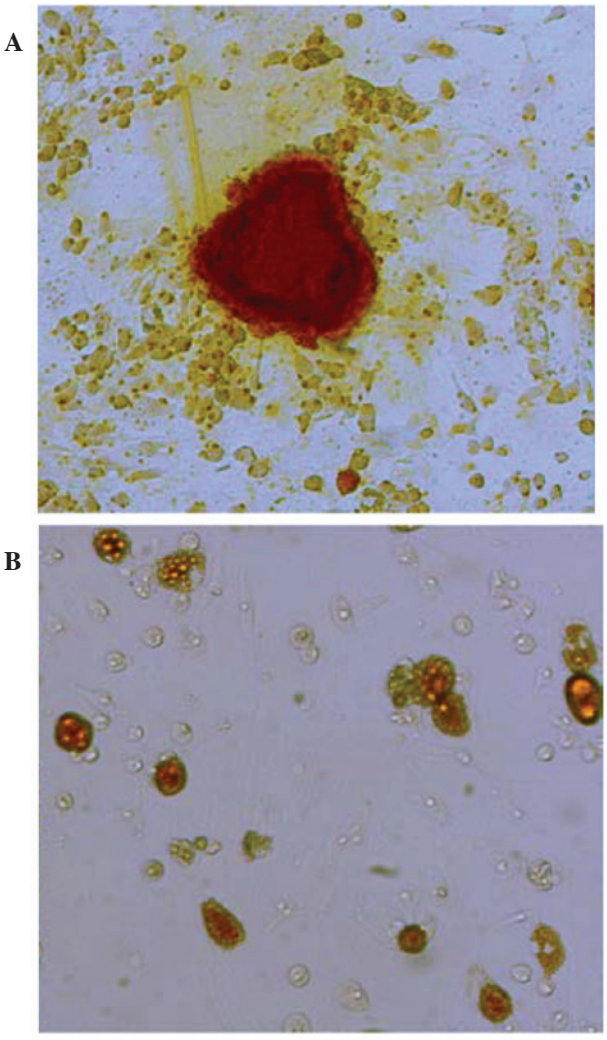

Figure 6. (A) BMSCs were cultured in osteogenic medium for 2 weeks, then bone nodules were stained using Alizarin red for 15 min. Purple calcium nodules were observed, and the cells are stained red. (B) BMSCs were cultured in adipogenic differentiation medium for 2 weeks, then lipid droplets were stained using oil red O for 15 min. The lipid droplets in cells were stained red by the oil red O. (Magnification, x400). BMSCs, bone marrow mesenchymal stem cells.

presence of calcium nodules. In addition, the BMSCs were cultured in adipogenic differentiation medium for 2 weeks and the presence of lipid droplets detected using oil red O.

As shown in Fig. 6B, red lipid droplets were observed in the differentiating BMSCs.

Discussion

During the cell culture process, simply increasing cell purity is not sufficient as cell numbers and activities must be taken into consideration. Therefore, a good isolation method must find a balance between cell purity, cell volume and cell activity. Thus, in the current study the purity of both the attached and detached cells was investigated. However, when evaluating the apoptotic indices, only apoptosis in attached cells was measured. This measure suggested that as the damage resulting from shaking accumulated, progressive apoptosis became an important factor for cell detachment.

A previous study of the isolation of microglial cells led to the selection of shaking at 180 rpm for 4 h as the initial testing condition (13). In the present study, different shaking frequencies were investigated for their effect on cell purity and apoptosis, and 180 rpm was selected as the experimental frequency. Based on multiple trials of shaking duration and the analysis of shaking-dependent cell apoptosis, 4 h was determined to be the most suitable time duration for isolation.

The present study indicated that shaking played a significant role in BMSC purification. BMSCs demonstrated a strong adhesion capacity, indicating that it is feasible and effective to isolate BMSCs using a physical method. Isolation using a horizontal shaker is achieved through liquid movement induced by the horizontal shaking to wash the flask bottom. Different shaking frequencies suggest different movement strengths of the liquid in the culture flasks, and thus, different amounts of shear stress on the cells (19). The present study indicated that isolation may be achieved through the selection of the appropriate shaking frequency and duration.

Within 1 h of shaking, cell purity increased with increased shaking frequency, however, the attached cell numbers were reduced and the apoptotic rate was altered with the different shaking frequencies. Although a shaking frequency of 220 rpm demonstrated superior effects on cell purity, the number of attached cells was markedly reduced compared with 180 rpm. Notably, the overall proportion of apoptotic cells was greatest for cells shaken at 140 rpm. This may be due to the lower level of physical force exerted on the cells, which is unable to detach the cells undergoing apoptosis. In general, shaking at 180 rpm for 1 h was advantageous, and resulted in a higher cell purity and reduced rate of apoptosis. Therefore, 180 rpm was selected as the shaking frequency.

The present study indicated that lengthy separation times may lead to reduced cell activity and even apoptosis. However, a shaking duration of 1 h was not necessarily the best option. In the preliminary testing, shaking for greater than 4 h did not result in a significant improvement in cell purity. In addition, the number of cells was greatly reduced following 12 h of shaking, resulting in a shortage in the number of cells available for flow cytometric analysis. Therefore, the shaking duration was limited to 4 h, and the results indicated that 2 h may be the optimal shaking duration.

Regarding cell apoptosis, the apoptosis of attached cells under fluid impact is a dynamic balance of continuous apoptosis and continuous detachment, and the cell populations maintain relatively stable proportions at different stages (20) Apoptotic cells, in particular those in the late stage, have a low adherence capacity and detach easily (21). In the present study, shaking led to apoptosis in the attached cells, which may result in the detachment of these cells from the flask wall and reduce the overall rate of apoptosis measured. However, with higher frequencies and the accumulation of damage from long shaking, there was a higher proportion of cells undergoing apoptosis. At the higher frequencies, the increased proportion of apoptotic cells and the subsequent detachment of apoptotic cells from the flask walls may result in reduced rates of apoptosis being measured in the 180 and 220 rpm groups.

The present study investigated the long-term effects of shaking following the isolation of cells, which indicated that the impact of isolation itself on cell apoptosis may not be significant. However, following the formation of cell colonies, the growth rate of the cells rapidly increased. Therefore, the cell purity indicated by the flow cytometry analysis was high. However, the high cell density may lead to stem cell differentiation (22,23). Therefore, although cell purity was high following cell growth for 3 days after shaking treatment, amplifying cells using this method is not recommended.

The results from the multiple short shakes indicated that shaking at 180 rpm for 0.5 h per day for 2 days was not sufficient to remove the loosely attached cells. However, it may interfere with fast-dividing BMSCs, leading to poor purity.

In summary, in a mixed culture system, increasing the purity of a certain cell type may be achieved because either the cell type's faster proliferation rate leads to a ‘‘relative’’ increase in purity or the reductions in the numbers of other cell types leads to an ‘‘absolute’’ increase in purity. The high proliferation capacity of stem cells is the foundation of the multiple-passage purification strategy in the whole bone marrow adhesion method. The mechanism of this physical separation method may occur as a result of the different physical attributes of the different cell types or by induced apoptosis due to differential resistance to physical stimulation. Therefore, the reason why BMSCs may be purified through physical separation may be due to their relatively strong adhesion capacity and their resistance to physical stimulation.

The addition of shaking to the whole bone marrow adhesion method is a simple and feasible method for BMSC purification. Following physical separation by shaking on a horizontal shaker, cell purity may be significantly increased. Compared with existing purification methods, this modified whole bone marrow adhesion method is simple, effective and inexpensive. This method may result in the enrichment of highly pure, highly active mesenchymal stem cells at low differentiation states and early stages.

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