Introduction

It has been my greatest pleasure and honour to accept this, the 11th Yahya Cohen Memorial Lectureship on the anniversary of a half century of the Academy of Medicine.

I did not have the privilege to work under Dr Cohen, but know well how keenly he emphasised the total care of surgical patients. A robust unification of 3 steps with great pedagogic significance: Pre, intra and postoperative patient care.

Preoperative procedure was designed around increasing both the safety and optimisation of the patient before surgery. Intraoperatively, he emphasised that the surgeon is not merely a technician but also should appreciate the underlying rationale in carrying out a safe and not just speedy surgery. He also fervently believed that operations extended beyond the time in the operating theatre. Postoperative care was equally important in ensuring the success of any procedure.

Mesenchymal stem cells (MSCs) have been shown to have promising potential in musculoskeletal tissue engineering. The MSCs isolated from bone marrow, in mammalian organisms, are heterogeneous. Different separation techniques were used in various laboratories to liberate MSCs from bone marrow. However, these MSCs shared 2 features; by adhering to the culture container walls while growing with a finite lifespan, and by displaying multi-lineage differentiation potential into osteoblasts,1-8 chondrocytes,1-4,9-11 or adipocytes.1,2,4,12

In 2002, Zuk et al13,14 reported that human adipose tissue can be a source of multi-potent stem cells with mesenchymal origin, possessing low levels of contaminants and are pluripotent, meaning that they can be differentiated in vitro into adipogenic, chondrogenic and osteogenic cells. Since then, there have been several publications reporting the multi-lineage differentiation capabilities of adipose cells.15,16 Erickson et al15 demonstrated that the human adipose tissue-derived MSCs can produce characteristic cartilage matrix molecules in nude mice. Our group found that fat-derived cells perform better in the repair of the...
osteochondral defects compared to periosteum-derived MSCs in both histological appearance and the biomechanical property in the rabbit femoral condyle. The periosteum is another alternative source of stem cells that demonstrate osteogenesis and chondrogenesis potentials.

To date, there has been no publication using these MSCs in the repair of physeal injury in any animal models except 1 from our group using periosteal-derived MSCs. Physeal injury in a growing child can lead to growth arrest with varying degrees of angular deformity and leg-length discrepancy and presents a challenge during treatment. Our current study is to investigate the efficacy and to compare the ability of MSCs derived from bone marrow, periosteum and fat in correcting growth arrest in immature rabbits.

**Materials and Methods**

The study was carried out in 3 stages. The first stage was aimed at developing a means of separating and culturing the MSCs and subsequently characterising the cells in terms of morphological and functional criteria in vitro. The second stage was to induce growth arrest in immature rabbits and to transplant the MSCs into the rabbits while the third stage was to assess the gross reconstituted physis, using radiographical and histological methods.

In the first stage, 5~10 mL of bone marrow was aspirated from the posterior iliac crests of a 6-week-old NZW rabbit. The bone marrow, mixed with heparin to prevent coagulation, was diluted with 2 times the bone marrow volume of Hank’s balanced salt solution (HBSS). The nucleated cell layer was obtained using a Ficoll-Paque density gradient centrifugator (Sigma, USA) at 450 x g for 20 minutes. The nucleated cells were then harvested using centrifugation at 250 x g for 10 minutes twice and rinsed with HBSS. The pellet was re-suspended in a complete Dulbecco’s modified Eagle’s medium (DMEM) (Gibco, USA), also containing 10% fetal bovine serum (FBS), penicillin 100 unit/mL, streptomycin 100 μg/mL (Gibco, USA), 10 mM HEPES buffer, and centrifuged at 250 x g for 10 minutes.

The periosteum was harvested from both tibiae of an immature NZW rabbit through an anteromedial incision. The periosteum was washed 3 times with normal saline solution containing 200 units/mL penicillin and 200 μg/mL streptomycin. The periosteum was cut into smaller pieces and digested for 2 hours with 0.2% collagenase (Gibco, USA) supplemented with 0.3% albumin (Sigma, USA) for 2.5 hours at a 37°C water bath with a magnetic stir bar for agitation. MSCs were collected by centrifugation at 450 x g for 10 minutes. The pellet was washed twice with a complete DMEM culture medium.

A piece of fat (3 x 3 x 1 cm³) was harvested from the abdominal subcutaneous tissue of a 6-week-old NZW rabbit. The fat was washed 3 times with normal saline solution containing 200 units/mL penicillin and 200 μg/mL streptomycin. The fat was further cut into smaller pieces and digested for 2 hours with 0.2% collagenase supplemented with 0.2% albumin. The cells were isolated by centrifugation at 250 x g for 10 minutes. The cell pellet was washed twice with the complete DMEM culture medium and re-suspended again with the complete DMEM.

10⁴ nucleated cells were plated on each of the 4-chamber slide and their mesenchymal origin was assessed using the vimentin antibody. On the fifth day, the chambers were removed. The cells on the slide were washed with phosphate-buffered saline (PBS, pH 7.4) and fixed with acetone at 4°C for 10 minutes. According to the Ultra Vision Detection System (Lab Vision Corporation, USA), the slide was incubated in a hydrogen peroxide block for 10 to 15 minutes, washed thrice with PBS containing 0.1% triton X. The slide was incubated with 1:100 primary antibody (vimentin, DAKO, USA) for 1 hour and rinsed 4 times with washing buffer, then incubated with biotinylated-goat antirabbit monoclonal antibody for 10 minutes and rinsed for 4 times again using the same mixture as above. The slide was incubated with streptavidin peroxidase for 10 minutes and rinsed 4 times with washing buffer, then incubated with freshly prepared DAB solution for 5 to 15 minutes. The slide was then mounted using a permanent mounting media and observed under the microscope. All the above procedures were carried out at room temperature.

1.6 x 10⁴ nucleated cells were plated into a T-75 flask (10⁴ cells/mL) and cultured at 37°C, 5% CO₂ in a humidified tissue culture incubator. The medium was changed every 3 to 4 days. For bone marrow-derived MSCs, the first change of the medium was carried out after 3 days, and every 3 to 4 days thereafter. The cells were observed daily with a phase contrast microscope and photographed on day 3, 7 and 11. When the cells were 75% to 80% confluent at around 10 to 14 days for the primary culture, adherent colonies of cells were trypsinised, counted and passaged.

Cells at passage 1 were analysed for their chondrogenic and osteogenic differentiation potentials. Aggregate culture was adopted in chondrogenic differentiation. 2.5 x10⁴ first passaged cell aliquots were spun down at 500 x g in 15 mL polypropylene conical tubes. The medium containing FBS was then replaced with a defined medium, consisting of DMEM-HG with 1% of ITS + Premix (BD, USA), 1 mM of pyruvate, 37.5 mg/mL of ascorbate 2-phosphate, 10⁻³ M of dexamethasone and 1 μg/mL of TGF-β1 (recombinant human, D-NAmix Biotechnology). The cells were cultured without TGF-β1 serving as a control. The ITS + Premix contains insulin (6.25 mg/mL), transferrin (6.25 mg/mL),...
selenous acid (6.25 mg/mL), linoleic acid (5.35 mg/ml) and bovine serum albumin (1.25 mg/mL). The pelleted cells were cultured at 37°C, 5% CO₂ incubator. By day 1, a roughly spherical pellet was formed. The tube was taped or the medium was pipetted to make sure that the pellet was not adhering to the wall of the tube. Medium changes were carried out at 2–3 day intervals and aggregates were fixed with 10 mL of 10% buffered formalin phosphate for 20 minutes at room temperature on day 14, day 21 and 28.10 The pellets were then carefully harvested and transferred using a large orifice micropipette tip and embedded in a OCT medium (Sigma, USA). The frozen pellets were cut into 5 μm slices and processed using immunohistological staining of collagen type II. Using the same kit procedure as Vimentin (Ultra Vision Detection System), the expression of type II collagen in chondrogenic differentiated pellets was processed. The monoclonal antibody used for collagen type II staining was purchased from ISN (USA).

For osteogenic differentiation, 1 mL of first passaged cell suspension (1x10⁴ cells) was plated into the wells of a 12-well plate and allowed to attach overnight using a complete DMEM medium containing 10% FBS. On the second day, the medium was replaced with an osteogenic supplement medium, which contains DMEM-LG with 100 mM of dexamethasone, 50 μM of ascorbic acid-2-phosphate and 100 mM of β-glycerophosphate. For control, the medium was replaced by DMEM-LG without dexamethasone. The medium was changed every 3–4 days, up to 40 days. In order to qualitatively assay the mineralisation of the osteogenic differentiated cells, the cells were processed using the von Kossa staining procedure. The cultures were rinsed twice with cold HBSS and fixed with 10% buffered formalin phosphate for 30 minutes. The cells were then rinsed with distilled water twice. 5% silver nitrate was added to cover the cells and the plate was placed under UV light for 1 hour. The wells were washed with distilled water thrice. The cells were then treated with 5% sodium thiosulphate for 5 minutes. The cultures were rinsed thrice again with distilled water and dehydrated with 100% ethanol for 1 minute, before being air-dried for observation.

At stage 2, using the same experimental model of growth arrest as published,10,11,19 the excision of the medial half of the proximal physis exceeded 50%. The bony bridge was excised 3 weeks later. 1.6 x 10⁶ second passaged cells were mixed with fibrin glue and transferred into each physis defect. Four groups of rabbits were involved in the study. Each group was injected separately with bone marrow-derived MSCs (group I), periosteum-derived MSCs (group II), fat-derived MSCs (group III), and fibrin glue alone (control, group IV). There were 20 rabbits in each group. All the rabbits were allowed to move freely in their cages and sacrificed at 8 and 16 weeks postoperatively, respectively.

All surgical procedures were done under approved anaesthetic methods comprising of tranquillisation with hypnorm at 0.3 mL/kg, induction with intravenous diazepam at 0.3 mL/kg and maintenance with 1% halothane.

The ability of MSCs in correcting the partial growth arrest was evaluated in stage 3. The tibiofemoral angle and length of the medial and lateral tibiae on both operated and non-operated sides were measured on gross specimen to evaluate the growth disturbance. Radiographs were taken from both legs and histological analyses were performed. Student’s t-test was used to evaluate the differences, and the differences were deemed significant at the 5% level.

In order to understand the source of the cells in regenerated physis, the MSCs were labelled with green fluorescence protein (GFP) in vitro prior to transplantation. 1.2 x 10⁶ first passaged cells were plated into a T-25 flask the day before the transfection in order to get about 50–80% confluent culture on the day of transfection. The culture medium was replaced with 5 mL of fresh DMEM containing 10% FBS 2 hours prior to transfection. According to the instruction of the CalPhos™ Mammalian Transfection Kit (BD, USA), 600 μL of transfection solution containing 5 μg plasmid DNA (pEGFP-F Vector, BD, USA) was prepared and added drop-wise to the culture flask medium. The flask was gently agitated to distribute the transfection solution evenly and incubated at 37°C for 6 hours in a 5% CO₂ incubator. The medium was then removed, the cells washed with DMEM medium and refilled with 5 mL of fresh complete DMEM medium and grown in a culture incubator. MSCs incubated with no plasmid DNA transfection solution served as a control. About 48 hours post-transfection, the medium was changed into medium supplemented with 0.5 g/L of neomycin (BD, USA). The medium was changed every day for 7–10 days. The non-transfected cells detached and were washed away during the medium exchange. Once all cells have died in the dish of the negative control (non-transfected), the transfected cells were refilled with fresh neomycin-medium and changed every 3 to 4 days until a further assay or enough cells for transplantation into the defects was obtained. The total times of the passages were less than 4 times. A small amount of MSCs were plated onto a sterile chamber slide for detection of stable GFP-transfected MSCs using a confocal laser at 488 nm. There were 2 rabbit legs in each group at every time point and were transferred with GFP-transfected MSCs. When the rabbits were sacrificed at 8 and 16 weeks post-transplantation, the physis were processed and the usual histology protocol and the sections were analysed with a confocal microscope at 488 nm.
Results

Proliferation Rate

In vitro studies demonstrated similar proliferation rate in all 3 kinds of cells at day 4, 7 and 11 of primary cell culture. However, bone marrow and periosteum derived MSCs are more homogenous than the fat-derived ones.

Characterisation

All the cultured cells isolated from the 3 sources demonstrated typical fibroblast-like mesenchymal stem cells appearance. Almost 100% of the cultured MSCs cells stained with vimentin, an intermediate filament protein found predominantly in cells of mesenchymal origin,13,14 suggested that our techniques of isolation and culture MSCs from these 3 kinds of origin are workable.

Multi-lineage Differentiation

Positive collagen type II staining showed that 3 kinds of MSCs have the potential to be induced into chondrogenic lineage cells in vitro on day 21 and 28. Furthermore, these 3 kinds of MSCs also have the potential to be induced into osteogenic lineage cells. There was calcium formation in the MSCs derived from bone marrow induced by osteogenic differentiation cocktail medium on day 8, not in the control group. On the induction day 26, all MSCs derived from bone marrow, periosteum and adipose were induced to undergo osteogenic differentiation, with the Von Kossa assay showing the black brown precipitation, but not in the respective controls.

Assessment of the Ability of MSCs in the Correction of Growth Arrest in Vivo

In group I and group II, MSCs derived from bone marrow and periosteum were transplanted with fibrin glue into the physeal defect after the creation of growth arrest. There were only mild varus deformity (group I at 4 ± 5 degrees, group II at 9 ± 6 degrees) and small differences in the medial and lateral heights (group I was 0.14 ± 0.10 cm, group II was 0.25 ± 0.15 cm) of tibiae. The experimental leg grew almost as much as the normal leg (Fig. 1A & B). In group IV, which served as control group, only fibrin glue was transferred into the physeal defect after the creation of growth arrest. All the tibiae went into severe varus angulation from 20° to 45° with the mean value of 31 ± 9 degree. There were large differences in medial and lateral heights of the tibiae (1.04 ± 0.32 cm), as well as retardation of growth in overall length (Fig. 3). The retardation of growth, both varus deformity and longitudinal discrepancy, was significantly obvious than the tibia transferred with MSCs from the bone marrow, periosteum or adipose tissue.

Saffarin O staining showed that 3 kinds of MSCs have the chondrogenic potential to regenerate chondrocytes which could secrete cartilage matrix and correct the growth arrest in rabbits. The cells derived from bone marrow (group I, Fig. 4A) and periosteum (group II, Fig. 4B) took on a columnar arrangement similar to the normal physis. In these 2 groups, columnar chondrocytes were seen across the whole physis in the transplanted area at 8 weeks after transplantation. None of the specimens showed evidence of bony bridge formation. When the rabbits were followed-up for longer periods, the physeal area thinned out and disappeared by 16 weeks after transplantation. In group III rabbits injected with fat-derived MSCs, the chondrocytes in the matrix arranged in an irregular manner. However, no bony bridge appeared at 8 weeks postoperatively. In the control group, histological sections showed formation of the bony bridge except the irregular arrangement of fewer chondrocytes at 8 weeks postoperatively (data not shown). GFP labelling showed that the cells that regenerate the physis are from transplanted cells rather than the host cells of the rabbits. The transplanted cells can survive as late as 8 weeks after transplantation.

Discussion

The source of the cells and appropriate animal model are 2 very important issues that have to be considered in musculoskeletal tissue engineering. Our current study has chosen MSCs derived from bone marrow, periosteum and fat to regenerate physis in the immature rabbit model. We choose the physeal defect model as this physeal injury can lead to varying degrees of angular deformity and leg-length discrepancy in a growing child clinically. Although varying degrees of success have been achieved in the small physeal defect (<30%),23-30 the treatment of large physeal defect (50% of physis) remains challenging. With the development of cell-based strategies, there have been reports on the use of cultured chondrocytes to repair physeal defects.31-32 Our previous study showed that chondrocytes transferred with agarose into large physeal defect (50% of physis) could prevent the growth arrest with angular deformity.31 However, transfer of cultured chondrocytes from the iliac apophysis and articular cartilage involved the sacrifice of a significant amount of iliac apophysis and articular cartilage.

The therapeutic potential of multi-potent stem cells for applications in musculoskeletal tissue engineering and gene therapy is enormous. Although embryonic stem cells
(ESCs) have enormous multi-lineage potential theoretically, the practical use of ESCs is limited due to potential problems of cell regulation and ethical considerations. In contrast, the adult MSCs with its multi-potency to differentiate into different lineages and its mesodermal origin have proven experimentally promising in musculoskeletal tissue engineering with little ethical problems.

There have been several publications reporting the multi-potent ability of MSCs to differentiate into osteogenic, chondrogenic and adipogenic lineage from bone marrow and adipose in vitro, with some of the publications reporting the multi-potent ability in vivo as well, but mainly in nude mice. Our group is the first to apply MSCs derived from adipose tissue into the osteochondral tissue engineering in rabbits. To date, there are no publications that mention the use of MSCs to regenerate the growth plate in any animal except one of our own publications that tried to use periosteal-derived MSCs to repair large physeal defect. The present work represents the first published data on the use of MSCs derived from bone marrow and adipose tissue for the correction of physeal arrest. Furthermore, this publication compares the ability of MSCs from 3 different sources, bone marrow, periosteum and fat, to correct the growth arrest for the first time.

Different laboratories adopted different techniques to isolate MSCs from different sources, such as bone marrow and fat. This makes it very difficult to compare the results from different laboratories. However, these MSCs shared 2 important features: adherent growth and multi-lineage differentiation potentials into adipogenic, osteogenic and chondrogenic cells. Our study showed that 3 kinds of MSCs derived from bone marrow, periosteum and fat demonstrated typical MSCs appearance, fibroblast-like morphology and grew by adhering to the container wall. Almost 100% of cells isolated from the bone marrow, periosteum and adipose
from our current methods showed mesenchymal origin, by possessing an intermediate filament protein found predominantly in cells of mesenchymal origin.\textsuperscript{13,14} All the cells demonstrated similar proliferative rate in the primary culture. However, MSCs derived from bone marrow and periosteum appeared to be more homogeneous than the MSCs derived from fat. Similar to most of the publications,\textsuperscript{4,6,11,13,14,33} our results showed that MSCs from bone marrow, fat and periosteum could differentiate into osteogenic and chondrogenic lineage under specific induction medium. These results have suggested the reliability of our isolation and purification techniques.

The purity of the cell source is one of the most important issues to be considered in cell-based therapy. Bone marrow is comprised of mixture populations of hematopoietic stem cells (HSCs) and mesenchymal stromal cells. The bone marrow stromal cells are heterogeneous in composition, containing several cell populations, including MSCs. MSCs have been characterised extensively. However, traditional bone marrow procurement procedures may be painful and the procurement has potential limitations. Meanwhile, very low numbers of MSCs are obtained upon processing (approximately 1 MSC per $10^5$ adherent stromal cells\textsuperscript{14}). Low MSCs yield necessitate an \textit{ex vivo} expansion step to obtain clinically significant cell numbers. As an alternative potential pure source of cells, the periosteum is considered as it has a relatively simple histological structure. The cells liberated from periosteum have both osteogenesis and chondrogenesis potentials,\textsuperscript{15,20-22,31,36} easily expanded in culture and are phenotypically stable\textsuperscript{22,31} with low-morbidity at the site of harvest. The disadvantages are that the availability of the donor material is limited and one extra operation is needed to obtain the periosteum. As an alternative source with the same embryonic mesoderm, the adipose tissue is considered because of its abundance, obtained with ease and low-morbidity of the harvest site. These cells can be maintained in vitro for extended periods with stable population doubling time and low level of senescence.\textsuperscript{14} They can also be differentiated into multi-lineage cells, such as adipocyte, osteocyte, chondrocytes and myocytes.\textsuperscript{13,14} However, like bone marrow cells, adipose tissue contains a heterogenous stromal cell population.\textsuperscript{13,14} Our study showed that mesenchymal cells isolated from bone marrow and periosteum using our current methods appeared more homogenous than the cells obtained from fat.

Wakitani et al\textsuperscript{36} reported that the periosteal and bone marrow liberated cells showed similar patterns of differentiation into articular cartilage and subchondral bone in the repair of large, full-thickness defects of articular cartilage in rabbits. Our results demonstrated the same tendency. The groups transferred with MSCs showed significant correction in both angular deformity and longitudinal discrepancy when compared with the control group ($P<0.01$). However, the correction in group III, which was transferred with MSCs from adipose, was less than that in group I and II ($P<0.05$). Histologically, the transplanted MSCs from bone marrow and periosteum were able to differentiate into chondrocytes that took on a columnar arrangement in the new physis at 8 weeks after implantation. Further studies need to be performed to elucidate whether it is the local factor or vascularity that influence the development of MSCs towards chondrogenesis in the physis. The group III and the control group, in which fibrin glue was transferred alone, showed chondrocytes in the matrix with an irregular arrangement. However, only the control group showed the formation of bony bridge at 8 weeks after implantation, but not in group III. By 16 weeks, most of the chondrocytes had disappeared, as fusion of the epiphyseal plate has occurred. The in vitro study showed that 3 kinds of MSCs from our current study shared the similarity in their proliferative rate as well as chondrogenic differentiation potential. There was no collagen type II staining during the 14 days induction period. However, on day 21 and 28, all 3 demonstrated quite weak collagen type II staining. The difference in the correction of the growth arrest might have resulted from the homogeneity of the MSCs population. Further study needs to be done to see the detailed difference among the 3 source MSCs in their chondrogenic process. Considering the availability and ease of obtaining adipose tissue as a source of stem cells, further purification techniques should be used, such as sorting of cells, in order to make use of MSCs derived from fat in musculoskeletal tissue engineering more practical.

In conclusion, the homogeneity of the MSCs population might play a role in the correction of the growth arrest.

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\textbf{REFERENCES}


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