Bone marrow extract as a growth supplement for human iliac apophyseal chondrocyte culture

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Background & objectives: Human bone marrow is rich in various growth factors which may support the chondrocyte growth. This study was conducted to compare the culture characteristics of human growth plate chondrocyte in foetal bovine serum (FBS) and human autologous bone marrow extract (BME) in monolayer culture.

Methods: Iliac crest apophyseal cartilage was harvested from four donors, aged between two and nine years, undergoing hip surgery. Chondrocytes were propagated under two culture conditions, with 10 per cent FBS and 10 per cent autologous BME harvested from the same donors. Cells were harvested at 7, 14 and 21 days to assess viability, morphology, cell count and immunocytochemistry.

Results: With an initial seeding density of 2500 cells/cm², the average yield in monolayer cultured with FBS was $3.35 \times 10^5$, $5.9 \times 10^5$, $14.1 \times 10^5$ and BME was $0.66 \times 10^5$, $1.57 \times 10^5$ and $3.48 \times 10^5$ at 7, 14 and 21 days, respectively. Viability was 98.21 per cent with FBS and 97.45 per cent with BME at 21 days. In BME supplemented cultures, hyaline phenotype was maintained up to 21 days. The yield was higher in the FBS supplemented group; however, the phenotype could not be maintained by the FBS group as long as BME group.

Interpretation & conclusions: Autologous BME was found to be a safer alternative to FBS for human studies. BME could maintain the hyaline phenotype for a longer time. Ways to enhance the cell yield needs to be explored in future studies.

Key words Bone marrow extract - chondrocytes - culture - foetal bovine serum - monolayer - phenotype
Slivers of cartilage (150-350 mg) were harvested from skin, subcutaneous and deep fascia for hip surgeries. Cartilage harvest from the articular surface is generally associated with donor site morbidity. Iliac crest apophysis is an alternative source for cartilage harvest from children. The growth and culture characteristics of this iliac apophyseal cartilage have been studied earlier in a goat model. This study has revealed that the iliac apophysis has a rich supply of chondrocytes which have a high growth rate and a greater ability to retain the phenotype compared to articular chondrocytes. Human iliac apophyseal chondrocyte is hence considered a potential source for several cartilage tissue engineering applications. Bone marrow aspirate has many essential growth factors needed for chondrocyte growth, and its constituents have been evaluated earlier. Human chondrocytes proliferate at a faster rate without dedifferentiation in the presence of such individual growth factors than FBS.

Material & Methods

Growth plate cartilage samples were harvested from the iliac crest in children undergoing hip surgery for dysplasia of hips. As a pilot study, four iliac crest apophysis cartilage samples were collected from children undergoing open reduction of the hip and the perichondrium was removed using a 22 surgical blade. After weighing, the cartilage was minced into fine pieces and digested with Type 2 collagenase (0.8 mg/ml) (Worthington, Lakewood, New Jersey) in the culture flask and incubated overnight in CO₂ incubator. The collagenase was diluted the next day with twice the volume of medium. Undigested debris was removed using a 100 µm cell strainer. Cells were pelleted by centrifuging the culture medium at 800 × g for 10 min at 25°C, and the pellet was washed twice with DMEM/F12 by centrifuging at 800 × g for 10 min at 25°C. The pellet was re-suspended in 3-5 ml of DMEM/F12 medium.

Bone marrow extract (BME): The harvested bone marrow was converted into one millilitre aliquots at 4°C and centrifuged at 1,000 × g for 10 min at 4°C. The supernatant was removed and filtered through 0.2 µm syringe filter and stored at -20°C for this study.

Primary expansion of harvested chondrocytes: The isolated chondrocytes were expanded into a monolayer. The cells were seeded at a density of 10,000 cells/cm² in a 25 cm² tissue culture flask with DMEM/F12, 10 per cent FBS (Gibco, United Kingdom) and 62 µg/ml of ascorbic acid and subsequently maintained in a CO₂ incubator. The cultured chondrocytes reached confluence on day 10, and were harvested on day 13 using trypsin digestion. The harvested chondrocytes were washed by centrifuging twice at 800 × g for 10 min at 25°C. The pellet was digested with collagenase (1 mg/ml) for 15-20 min in a shaking water bath at 37°C to disengage the cells and centrifuged twice at 800 × g for 10 min at 25°C. The pellet thus obtained was suspended in 5 ml of DMEM/F12 and used for experimentation.
Experimental culture using foetal bovine serum (FBS) versus bone marrow extract (BME): Cells were seeded in a six-well plate (9.86 cm²/well). Seeding density was 2500 cells/cm² with DMEM/F12 medium. Two sets of culture were commenced simultaneously with two different growth media. Both groups had similar culture conditions except for the addition of FBS or BME as growth supplement. DMEM/F12 with 10 per cent FBS or 10 per cent autologous BME and ascorbic acid (62 µg/ml) was added to all groups. The cultures were maintained in a 5 per cent CO₂ incubator at 37°C. The medium was changed twice every week. Cells were harvested by trypsinization at three different time points, i.e., 7, 14 and 21 days of culture. Cell count and viability were assessed using trypan blue dye exclusion test. The formulae used to calculate cell yield, and viability were as follows:

Cell yield:  
Total cell yield = Number of cells/ml × Total volume of cell suspension

Number of cells/ml = Number of viable cells/Number of square counted × dilution factor × 10⁻⁴

Viability:  Percentage of viable cells = Number of viable cells (unstained)/Total number of cells (both stained and unstained) × 100

Doubling time was determined using online doubling time calculator. Cells were checked for morphology and growth pattern by phase contrast microscopy and phenotype by immunocytochemistry at three different time points.

Immunocytochemistry: Phenotype of cultured chondrocytes on days 7, 14 and 21 was assessed using anti-collagen Type I and anti-collagen Type II antibodies following protocol by Marlovits et al. All antibodies used in this study were purchased from Abcam, United Kingdom. Briefly, cells were fixed using 4 per cent paraformaldehyde. The primary antibody at a dilution 1:100 was used. The secondary antibody (horseradish peroxidase conjugated, dilution 1:250) was used to detect the primary antibody, and then the chromogen 3, 3’-diaminobenzidine with hydrogen peroxide was used to develop the colour. Finally, the cells were counterstained with haematoxylin and observed under light microscope. The cells without primary antibody served as a negative control.

Toluidine blue staining: Toluidine blue staining was performed on day 21 culture to qualitatively assess the glycosaminoglycan secretion as described earlier.

Statistical analysis: Two groups were compared for the difference in the observed results using statistical analysis software - SPSS version 16.0 (SPSS Inc., Chicago, IL, USA). Mann–Whitney U-test for non-parametric data and Student’s t test for parametric data were used for analyzing the experimental results between the two groups.

Results

The mean age of the four donors was 5.1 yr (2-9 yr). The mean volume of bone marrow harvested was 7 ml (7-8 ml). The mean weight of the cartilage harvested was 279 mg (range 186-340 mg). The average time to transport to the laboratory was 30 min (25-40 min). The average amount of chondrocyte harvested was 4.96 × 10⁵ cells/100 mg of cartilage (3.5 × 10⁵ - 6.3 × 10⁵ cells/100 mg of cartilage) with a mean cell yield of 1.317 × 10⁶ cells (1.145 − 1.673 × 10⁶ cells). The mean yield of chondrocytes after expansion in a monolayer was 1.57 × 10⁷ cells (9.26 × 10⁶ − 2.24 × 10⁷ cells). The cell yield, morphology and phenotype were assessed after harvesting the four samples (Table I).

| Sample no. | Confluence (Day) | Viability (%) | Collagen expression | Yield (cells) |
|------------|-----------------|---------------|---------------------|--------------|
| 1          | 13              | 96.13         | COL2+, COL1-        | 9.26×10⁶    |
| 2          | 10              | 98.89         | COL2+, COL1-        | 2.24×10⁷    |
| 3          | 10              | 96.21         | COL2+, COL1-        | 1.38×10⁷    |
| 4          | 10              | 97.64         | COL2+, COL1-        | 1.76×10⁷    |

Immunocytochemistry: Immunocytochemistry for the phenotype demonstrated that BME group maintained the hyaline phenotype (Type II collagen-positive) for 21 days, however, the FBS group became collagen I positive (fibrocartilage) by day 14 in all the samples (Fig. 1A & B). The chondrocytes reached confluence on day 10 of culture in the FBS supplemented medium and on day 15 in the BME supplemented medium (Fig. 2A & B).

Toluidine blue staining: The cells from both groups were stained positive for toluidine blue. The cells in the BME group had stained brighter than the FBS group.
indicating a better phenotype of the chondrocytes in the BME group (Fig. 3).

There was a significant difference in the yield on days 7, 14 and 21. The FBS group had a higher yield compared to the BME group ($P=0.005$) (Fig. 3). The doubling time was slower in the BME group on days 7, 14 and 21 compared to the FBS group. The difference in doubling time between the FBS and BME was significant ($P=0.001$). There was no significant difference in the viability of the cells in both groups on days 7, 14 and 21 (Table II).

**Discussion**

Our results showed that monolayer apophyseal chondrocyte culture with BME supplementation maintained the chondrocyte phenotype up to 21 days of culture. Even though the yield and viability were high in FBS, the phenotype was fibroblastic. The yield from BME was significantly lower than the FBS-supplemented medium. The fine tuning of the BME concentration is required to get optimal results. Factors in the BME which could induce quiescence in a portion of cells need to be identified. This would account for a good phenotype, a high viability but a slow proliferation rate as seen in our results with BME as nutrients.

A literature search shows that human apophyseal chondrocyte has not been used as a source of chondrocytes for clinical use. Normal joint cartilage has been a major source for autologous chondrocyte implantation$^{14}$. The morbidity associated with the harvest and long-term consequences of articular cartilage harvest can be avoided with an apophyseal source. There are very few human *in vitro* studies on foetal epiphyseal chondrocytes which have looked at the hormonal influence on growth plate$^{15-20}$. None of the studies evaluated apophyseal chondrocyte expansion with BME for clinical translational purposes.

FBS has many essential nutrients and metabolites such as essential minerals, trace elements, cell adhesion factors such as fibronectin, epidermal and basic FGFs, insulin and steroid hormones. These factors stimulate cell growth and proliferation, promote differentiated functions and provide transport proteins and factors needed to maintain pH. This complex mixture is more optimal compared to human serum due to its lesser gamma globulin content. An animal serum free medium for chondrocyte culture is desirable, although ethical and disease transmission issues with FBS have resulted in a search for alternatives in human translational studies$^{21}$. 

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**Fig. 1.** (A) Light microscope image of growth plate chondrocyte from day 21 of culture. Immunocytochemistry image of growth plate chondrocyte grown with bone marrow extract from day 21 of culture. Collagen I negative and collagen II positive till day 21 (Light microscope, ×40). (B) Light microscope image of growth plate chondrocyte from day 21 of culture. Immunocytochemistry showing iliac crest chondrocyte cultured with foetal bovine serum. Collagen I positive and collagen II negative (×40).

**Fig. 2.** (A) Phase contrast microscopic image of growth plate chondrocyte cultured with bone marrow extract. Confluence at day 15 (×10). (B) Phase contrast microscopic image of growth plate chondrocytes cultured with foetal bovine serum. Confluence at 10th day (×10).

**Fig. 3.** Light microscope images of growth plate chondrocytes (A) foetal bovine serum group, (B) BME group on day 21 of culture (×40). Toluidine blue which stains the sulphated glycosaminoglycan in the cells and extracellular matrix. The cells in the BME group (B) have stained brighter as compared to the foetal bovine serum group (A).
Bone marrow is a niche for mesenchymal stem cells. A study on bone marrow reamer irrigator aspirator, platelet rich plasma (PRP) and iliac crest had concluded that the marrow aspirate was rich in FGFa, PDGF, IGF-1, BMP-2, TGF-β1, FGFb and which are the same constituents in FBS and PRP, both of which support chondrocytes. BME as a supplement for chondrocyte culture has not been described. Serum free substitutes to FBS include ITS and PRP. ITS has shown an increase in the growth rate and decreased dedifferentiation among nasal septal chondrocytes. However, ITS needs other growth factors to be added to prevent dedifferentiation and for long term cultures. PRP is another substitute for FBS with promising results in preclinical articular cartilage studies. However, its harvest is a separate procedure from cartilage harvest and commonly taken from a pooled allogeneic source. The two major advantages of BME are that it is autologous and can be aspirated under the same anaesthetic agent at the time of cartilage harvest and there is no disease transmission risk.

There was de-differentiation of chondrocytes in both the groups from day 14 onwards. Our own experience (unpublished observation) of using autologous human serum showed variable response due to batch to batch variability. Allogenic or pooled human serum though more standardized, risks immune reaction and disease transmission. Munirah et al indicated that human serum addition decreased the waiting period for implantation by shortening the population doubling time. However, addition of growth factors is required to enhance proliferation. Repeated passages, however, lead to dedifferentiation into fibroblasts. It was also noted that the alteration in the shape of chondrocytes from polygonal to spindle could be due to changes in the cytoskeleton following repeated passages. The use of passaged cells could be one of the reasons for earlier expression of collagen I in FBS supplemented cultures in our study.

The possible reasons why the BME yields did not exceed those of FBS may be due to the limited amount of BME available in young subjects (about 7 ml). The current standard technique for harvest of BME is from the posterior iliac crest and includes multiple aspirations to get a volume of 200 ml. The average duration for the harvest of BME for transplantation purposes could last up to an hour. In this study, the BME harvest was not the primary procedure as this was done in children who underwent pelvic osteotomy from the surgical site and ethical reasons prevented the harvest of larger amounts of marrow or doing a slow and careful BME harvest as per the standard protocol. This was a major limitation of this study.

Our results showed wide variations in the proliferation rate between various samples grown with BME supplementation. This emphasizes the need to standardize the BME harvest. One other issue is the standardization of various micronutrients and growth factors in BME. Further studies need to be carried out to quantify the constituents of the BME. The composition of the bone marrow in a child varies based on the demands in a child. Analysis of the proteomics of bone marrow constituents might reveal the key molecules which may affect the chondrocyte growth cycle and the proliferation rate.

Treatment of human growth plate injuries with cultured physeal chondrocyte need large quantities of cells. FBS in monolayer had good proliferation
rates but poor phenotype maintenance. Autologous BME could be used without losing the phenotype. BME, however, needs enrichment with additional nutrients and further studies to understand factors which enhance the yield. As chondrocyte transplant is an emerging field, there is a role for autologous BME in clinical translational studies. Future research in gene targeting for growth factor stimulation will be of clinical use in this area.

In conclusion, our study has shown that the monolayer culture with BME is better at maintaining the phenotype compared to FBS. There was a variable yield with BME and this needs to be explored further to arrive at a clinically useful protocol.

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Conflicts of Interest: None.

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