Indian J Med Res 144, December 2016, pp 831-837
DOI: 10.4103/ijmr.IJMR_8_15

Quick Response Code:

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

Balasubramanian Balakumar[1], Karthikeyan Rajagopal[2] & Vrisha Madhuri[1,2]

[1]Paediatric Orthopaedics Unit, Department of Orthopaedics & [2]Centre for Stem Cell Research, Christian Medical College, Vellore, India

Received January 3, 2015

***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/cm^2, the average yield in monolayer cultured with FBS was 3.35 × 10^5, 5.9 × 10^5, 14.1 × 10^5 and BME was 0.66 × 10^5, 1.57 × 10^5 and 3.48 × 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

Application of tissue engineering has become a standard technique for the treatment of articular cartilage injury with chondrocytes[1]. Autologous chondrocyte transplantation involves harvesting, culturing and transplanting chondrocytes into the articular defects[2]. Current chondrocyte culture methods utilize foetal bovine serum (FBS) to grow the cells[3]. Issues associated with the use of FBS includes possible transmission of virus or prion infections, batch to batch variability of composition, ethical issues associated with harvesting of serum from an unborn calf and immunological reactions[4]. Alternatives such as human platelet lysate, insulin transferrin selenium (ITS), human serum and serum-free defined media are

being used to avoid FBS[4-6]. Human bone marrow has many essential growth factors required for chondrocyte growth such as insulin-like growth factor (IGF-1), transforming growth factor (TGF), bone morphogenetic proteins (BMPs), fibroblastic growth factor (FGF) and platelet-derived growth factor (PDGF)[7].

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[8]. 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[8]. Human iliac apophyseal chondrocyte is hence considered a potential source for several cartilage tissue engineering applications[8]. Bone marrow aspirate has many essential growth factors needed for chondrocyte growth, and its constituents have been evaluated earlier[7]. Human chondrocytes proliferate at a faster rate without dedifferentiation in the presence of such individual growth factors than FBS[9].

There has been no study comparing the FBS with human bone marrow extract (BME) for human apophyseal chondrocyte culture. Hence, this study was undertaken to evaluate the *in vitro* characteristics of human iliac apophyseal chondrocyte to determine the effectiveness of BME for chondrocyte culture and phenotype maintenance as compared to 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 an iliac osteotomy, requiring splitting of the iliac apophysis during surgery. An Institutional Review Board (IRB) clearance was obtained to do the study. Written informed consent was obtained from the parents/ guardians of children. This study was conducted between January 2010 and January 2012 at the Centre for Stem Cell Research, Christian Medical College, Vellore, Tamil Nadu, India.

Harvest of cartilage and bone marrow: The iliac crest was exposed up to the perichondrium after opening the skin, subcutaneous and deep fascia for hip surgeries. Slivers of cartilage (150-350 mg) were harvested from the iliac apophysis, and the specimens were transferred into a sterile 50 ml centrifuge tube with 15 ml of Dulbecco's Modified Eagle's Medium (DMEM)/F12 (Ham's) (Sigma, USA) at 4°C. The iliac crest marrow or the fresh oozing marrow from osteotomy of the pelvis was harvested with a Jamshidi (Care Fusion, USA) bone marrow aspiration needle and transferred into a 50 ml centrifuge tube containing 3 ml of Acid Citrate Dextrose (Terumo Penpol, India) at 4°C. The harvested cartilage and the bone marrow were transported to the cell culture facility in an icebox at 4°C.

Processing of the sample: The harvested cartilage was transferred into a petridish in a type A2 biosafety cabinet (1300 series A2, Thermo Scientific, USA). The specimen was washed twice with DMEM/F12 medium, and the perichondrium was removed using a size 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 CO_2 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/cm^2 in a 25 cm^2 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 CO_2 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 cm^2/well). Seeding density was 2500 cells/cm^2 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 CO_2 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[10]. 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[11]. 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*[12]. 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[13].

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 4.96×10^5 cells/100 mg of cartilage ($3.5 \times 10^5 - 6.3 \times 10^5$ cells/100 mg of cartilage) with a mean cell yield of 1.317×10^6 cells ($1.145 - 1.673 \times 10^6$ cells). The mean yield of chondrocytes after expansion in a monolayer was 1.57×10^7 cells ($9.26 \times 10^6 - 2.24 \times 10^7$ cells). The cell yield, morphology and phenotype were assessed after harvesting the four samples (Table I).

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

Table I. Characteristics of monolayer expanded chondrocytes after harvest

Sample no.	Confluence (Day)	Viability (%)	Collagen expression	Yield (cells)
1	13	96.13	COL2+, COL1-	9.26×10^6
2	10	98.89	COL2+, COL1-	2.24×10^7
3	10	96.21	COL2+, COL 1-	1.38×10^7
4	10	97.64	COL2+, COL 1-	1.76×10^7

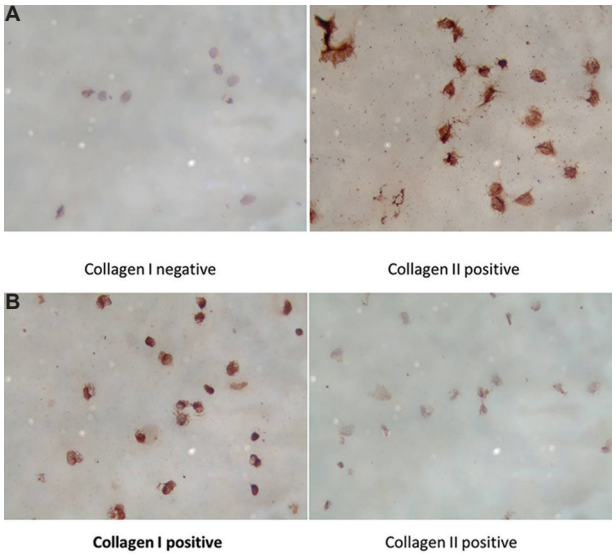

Fig. 1. (**A**) Light microscope image of growth plate chondrocyte from day 21 of culture. Immunocytochemistry image of growth plate chondrocyte grown with bone marrow extract from day 21 of culture. Collagen I negative and collagen II positive till day 21 (Light microscope, ×40). (**B**) Light microscope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

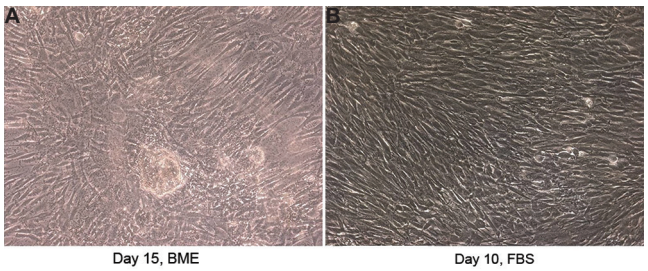

Fig. 2. (**A**) Phase contrast microscopic image of growth plate chondrocyte cultured with bone marrow extract. Confluence at day 15 (×10). (**B**) Phase contrast microscopic image of growth plate chondrocytes cultured with foetal bovine serum. Confluence at 10th day (×10).

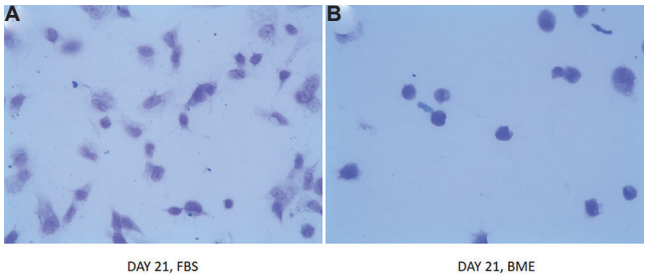

Fig. 3. Light microscope images of growth plate chondrocytes (**A**) feotal bovine serum group, (**B**) BME group on day 21 of culture (×40). Toluidine blue which stains the sulphated glycosaminoglycan in the cells and extracellular matrix. The cells in the BME group (**B**) have stained brighter as compared to the foetal bovine serum group (**A**).

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].

Table II. Results of chondrocyte culture in foetal bovine serum (FBS) and bone marrow extract (BME)

Day of culture	Analyzed parameters	FBS	BME
7	Yield (number of cells) ($\times 10^5$) (±SE)	3.35±0.3	0.66±0.13***
	Viability (%)	92.17	89.65
	Doubling time (days)	1.871	6.65***
14	Yield ($\times 10^5$) (±SE)	5.9±0.4	1.57±0.76***
	Viability (%)	90.80	89.90
	Doubling time (days)	3.062	11.14***
21	Yield ($\times 10^5$) (±SE)	14.1±2.1	3.48±2.01***
	Viability (%)	98.21	97.45
	Doubling time (days)	3.636	31.91***

***$P<0.001$ compared to FBS

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[22]. 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[23]. However, ITS needs other growth factors to be added to prevent dedifferentiation and for long term cultures[24]. PRP is another substitute for FBS with promising results in preclinical articular cartilage studies[25]. However, its harvest is a separate procedure from cartilage harvest and commonly taken from a pooled allogeneic source[3]. 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*[26] 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[27]. 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 1 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[28]. Analysis of the proteomics of bone marrow constituents might reveal the key molecules which may affect the chondrocyte growth cycle and the proliferation rate[29].

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[30,31].

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.

Acknowledgment

Authors thank all patients who participated in this study. The authors acknowledge the Centre for Stem Cell Research (a unit of inStem, Bengaluru), Christian Medical College for providing the laboratory facilities and Fluid research grants by the Institutional Review Board (IRB) for funding to carry out this study.

***Conflicts of Interest*:** None.

References

1. Nukavarapu SP, Dorcemus DL. Osteochondral tissue engineering: current strategies and challenges. *Biotechnol Adv* 2013; *31* : 706-21.
2. Zak L, Aldrian S, Wondrasch B, Albrecht C, Marlovits S. Ability to return to sports 5 years after matrix-associated autologous chondrocyte transplantation in an average population of active patients. *Am J Sports Med* 2012; *40* : 2815-21.
3. Rauch C, Feifel E, Amann EM, Spötl HP, Schennach H, Pfaller W, *et al.* Alternatives to the use of fetal bovine serum: human platelet lysates as a serum substitute in cell culture media. *ALTEX* 2011; *28* : 305-16.
4. Gstraunthaler G, Lindl T, van der Valk J. A plea to reduce or replace fetal bovine serum in cell culture media. *Cytotechnology* 2013; *65* : 791-3.
5. Zhang L, Song H, Zhao X. Optimum combination of insulin-transferrin-selenium and fetal bovine serum for culture of rabbit articular chondrocytes in three-dimensional alginate scaffolds. *Int J Cell Biol* 2009; *2009* : 747016.
6. Schallmoser K, Strunk D. Preparation of pooled human platelet lysate (pHPL) as an efficient supplement for animal serum-free human stem cell cultures. *J Vis Exp* 2009. pii: 1523.
7. Schmidmaier G, Herrmann S, Green J, Weber T, Scharfenberger A, Haas NP, *et al.* Quantitative assessment of growth factors in reaming aspirate, iliac crest, and platelet preparation. *Bone* 2006; *39* : 1156-63.
8. Rajagopal K, Dutt V, Manickam AS, Madhuri V. Chondrocyte source for cartilage regeneration in an immature animal: is iliac apophysis a good alternative? *Indian J Orthop* 2012; *46* : 402-6.
9. Yang YH, Barabino GA. Differential morphology and homogeneity of tissue-engineered cartilage in hydrodynamic cultivation with transient exposure to insulin-like growth factor-1 and transforming growth factor-ß1. *Tissue Eng Part A* 2013; *19* : 2349-60.
10. Strober W. Trypan blue exclusion test of cell viability. *Curr Protoc Immunol* 2001; Appendix 3: Appendix 3B.
11. Roth V. *Doubling time online calculator*; 2006. Available from: *http://www.doubling-time.com/compute.php*, accessed on October 3, 2015.
12. Marlovits S, Hombauer M, Truppe M, Vècsei V, Schlegel W. Changes in the ratio of type-I and type-II collagen expression during monolayer culture of human chondrocytes. *J Bone Joint Surg Br* 2004; *86* : 286-95.
13. Zhong ZM, Li T, Xu ZX, Meng TT, Zeng JH, Zheng S, *et al*. Effect of melatonin on the proliferation and differentiation of chondrocytes from rat vertebral body growth plate *in vitro*. *Int J Med Sci* 2013; *10* : 1392-8.
14. Harris JD, Siston RA, Pan X, Flanigan DC. Autologous chondrocyte implantation: a systematic review. *J Bone Joint Surg Am* 2010; *92* : 2220-33.
15. Ferrández MA, Carrascosa A, Audí L, Ballabriga A. Somatostatin effects on cultured human fetal epiphyseal chondrocytes. *Pediatr Res* 1992; *32* : 571-3.
16. Carrascosa A, Ferrández MA, Audi L, Ballabriga A. Effects of triiodothyronine (T3) and identification of specific nuclear T3-binding sites in cultured human fetal epiphyseal chondrocytes. *J Clin Endocrinol Metab* 1992; *75* : 140-4.
17. Carrascosa A, Audi L, Ferrandez MA, Ballabriga A. Biological effects of androgens and identification of specific dihydrotestosterone-binding sites in cultured human fetal epiphyseal chondrocytes. *J Clin Endocrinol Metab* 1990; *70* : 134-40.
18. Kawakita A, Sato K, Makino H, Ikegami H, Takayama S, Toyama Y, *et al.* Nicotine acts on growth plate chondrocytes to delay skeletal growth through the alpha7 neuronal nicotinic acetylcholine receptor. *PLoS One* 2008; *3* : e3945.
19. Olney RC, Wang J, Sylvester JE, Mougey EB. Growth factor regulation of human growth plate chondrocyte proliferation *in vitro*. *Biochem Biophys Res Commun* 2004; *317* : 1171-82.
20. Carrascosa A, Audí L, Ballabriga A. Morphologic and metabolic development of human fetal epiphyseal chondrocytes in primary culture. *Pediatr Res* 1985; *19* : 720-7.
21. Brunner D, Frank J, Appl H, Schöffl H, Pfaller W, Gstraunthaler G. Serum-free cell culture: the serum-free media interactive online database. *ALTEX* 2010; *27* : 53-62.
22. Akeda K, An HS, Okuma M, Attawia M, Miyamoto K, Thonar EJ, *et al*. Platelet-rich plasma stimulates porcine articular chondrocyte proliferation and matrix biosynthesis. *Osteoarthritis Cartilage* 2006 ; *14* : 1272-80.

23. Chua KH, Aminuddin BS, Fuzina NH, Ruszymah BHI. Insulin-transferrin-selenium prevent human chondrocyte dedifferentiation and promote the formation of high quality tissue engineered human hyaline cartilage. *Eur Cell Mater* 2005; *9* : 58-67.

24. Liu X, Liu J, Kang N, Yan L, Wang Q, Fu X, *et al.* Role of insulin-transferrin-selenium in auricular chondrocyte proliferation and engineered cartilage formation *in vitro*. *Int J Mol Sci* 2014; *15* : 1525-37.

25. Ahmad Z, Howard D, Brooks RA, Wardale J, Henson FM, Getgood A, *et al.* The role of platelet rich plasma in musculoskeletal science. *JRSM Short Rep* 2012; *3* : 40.

26. Munirah S, Ruszymah BHI, Samsudin OC, Badrul AHMY, Azmi B, Aminuddin BS. Autologous versus pooled human serum for articular chondrocyte growth. *J Orthop Surg* (Hong Kong) 2008; *16* : 220-9.

27. Chua KH, Aminuddin BS, Fuzina NH, Ruszymah BHI. Basic fibroblast growth factor with human serum supplementation: enhancement of human chondrocyte proliferation and promotion of cartilage regeneration. *Singapore Med J* 2007; *48* : 324-32.

28. Proytcheva M. Bone marrow evaluation for pediatric patients. *Int J Lab Hematol* 2013; *35* : 283-9.

29. Kemptner J, Witzeneder K, Hennerbichler S, Gabriel C. Proteomic analysis of human-derived cell culture supplements. *Transfus Med Hemother* 2013; *40* : 413-5.

30. Baugé C, Cauvard O, Leclercq S, Galéra P, Boumédiene K. Modulation of transforming growth factor beta signalling pathway genes by transforming growth factor beta in human osteoarthritic chondrocytes: involvement of Sp1 in both early and late response cells to transforming growth factor beta. *Arthritis Res Ther* 2011; *13* : R23.

31. Murakami S, Kan M, McKeehan WL, de Crombrugghe B. Up-regulation of the chondrogenic Sox9 gene by fibroblast growth factors is mediated by the mitogen-activated protein kinase pathway. *Proc Natl Acad Sci U S A* 2000; *97* : 1113-8.

Reprint requests: Dr Vrisha Madhuri, Paediatric Orthopaedics Unit, Paul Brand Building, Christian Medical College, Vellore 632 004, Tamil Nadu, India
e-mail: madhuriwalter@cmcvellore.ac.in