Growth Kinetics of Rat Mesenchymal Stem Cells From 3 Potential Sources: Bone Marrow, Periosteum and Adipose Tissue

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Objective: *Mesenchymal stem cells (MSCs) have potential in orthopaedic applications as they are able to differentiate into bone and cartilage. These cells can be isolated from a variety of adult tissues. Three sources that are relevant for orthopaedic applications are bone marrow, periosteum and adipose tissue. The purpose of the present study was to compare the growth kinetics and colony forming potency of rat MSCs from these sources.*

Material and Method: *Bone marrow from the femur, periosteum from the femoral diaphysis and adipose tissue from the inguinal area of Wistar rats were harvested for MSC isolation. The cells from 2nd-4th passage from primary culture were selected for study of their growth curves, population doubling time and colony forming ability using the percentage of colony forming units and colony forming area as the outcome measure.*

Results: *The isolated cells from these 3 sources were capable of osteogenesis, chondrogenesis and adipogenesis. The growth kinetics were compared using the growth curve and the population doubling time (PDT): bone marrow derived cells (PDT = 3.99 days, SD = 1.19) and periosteum derived cells (PDT = 3.55 days, SD = 1.21) had faster growth kinetics than adipose derived cells (PDT = 4.65 days, SD = 1.53). The percentage of colony forming units and the colony forming area from bone marrow derived cells (% colony forming unit = 8.58, SD = 1.35 and % colony forming area = 25.12, SD = 7.31) and periosteum derived cells (% colony forming units = 9.92, SD = 2.06, % colony forming area = 32.45, SD = 10.74) were significantly greater ($p < 0.05$) than adipose derived cells (% colony forming units = 5.92, SD = 0.78, % colony forming area = 15.80, SD = 9.035).*

Conclusion: *The growth kinetics and colony forming potency of MSCs from bone marrow and periosteum were comparable. The bone marrow and periosteum should be a suitable source for MSC isolation. The growth kinetics of MSCs derived from adipose tissue was lower than the other sources. Adipose tissue can be used as an alternative source as it is readily available and dispensable.*

Keywords: *Mesenchymal stem cells, Bone marrow, Periosteum, Adipose tissue, Growth kinetics, Population doubling time, Colony forming units*

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Bone fractures and articular cartilage injuries are serious and common clinical problems for orthopaedic surgeons. These conditions cause significant patient morbidity and also consume a large amount of health care resources[1,2]. Mesenchymal stem cell therapy has several potential roles for tissue regeneration in the orthopaedic field, *e.g.*, repair of osteochondral and bone defects using MSCs for osteogenesis and chondrogenesis. The use of MSCs in musculoskeletal regeneration has been reported in several pre-clinical studies[3,4]. Clinical studies have reported that the application of MSCs for cartilage repair is safe and effective[5,6]. A study from Thailand, Kasemkijwattana et al[7] reported clinical improvement

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without complications in two patients with large traumatic cartilage defects of the knee using autologous bone marrow mesenchymal stem cell (BM-MSC) implantation. Thus, the application of MSCs might be an alternative intervention in orthopaedic treatment. One factor that needs to be considered when using MSCs is their source as this could have a major influence on the yield of MCSs from the preparation. The number of implanted MSCs has been reported to determine the success rate[8]. MSCs have been isolated from a variety of adult tissues[9]. Three sources that are relevant for orthopaedic applications are bone marrow, periosteum and adipose tissues. The goal of the present study was to compare and determine *in vitro* the growth kinetics using growth curve, population doubling time and clonogenic ability of MSCs from 3 different sources; namely bone marrow, periosteum and adipose tissue. The findings of the present study would be expected to have relevance for both research and therapeutic fields.

Material and Method

Animals

All experiments were conducted with cells isolated from 3-months old Wistar rats. They were obtained from a recognised biological service. Animals were humanely sacrificed using carbon dioxide overdose for cell isolation.

MSC isolation and cell culture technique

Bone marrow from the femur, periosteum from the femoral diaphysis and adipose tissue from the inguinal area of three Wistar rats were harvested for MSCs isolation. Cells were obtained from the femoral bone marrow by flushing with Dulbecco's Modified Eagle Medium (DMEM) using a sterile technique according to protocol reported by Lennon and Caplan[10]. Cells were isolated from adipose tissue and periosteum after digestion with collagenase solution as described by Zuk et al[11]. The isolated cells were placed into flasks containing DMEM with 10% Fetal Bovine Serum (FBS) from Gibco® and incubated at 37 degrees C° and 5% CO_2. Cells from passage 2-4 of primary culture were used for study of their growth curves, population doubling time (PDT) and colony forming ability.

Tri-lineage differentiation

To demonstrate the differentiation potential of MSCs from the 3 sources, passage 3-4 MSCs were cultured *in vitro* under (a) osteogenic, (b) chondrogenic and (c) adipogenic conditions. (a) Osteogenic potential: to induce osteogenesis, the cells were cultured in basal medium until 60-70% confluent and then changed to the osteogenic differentiation medium (basal medium supplemented of 100 nM dexamethasone, 10 mM β-glycerophosphate and 50 µg/ml L-ascorbic acids). Osteogenic medium was changed every 3 days for 2 weeks. The cells were stained with Alkaline Phosphatase (ALP) assay kit from Sigma-Aldrich® using the manufacturer's protocol. (b) Chondrogenic potential: to induce chondrogenesis using the micro mass culture technique, 4 x 10^5 cells were resuspened in polypropylene tubes, centrifuged gently to form a micro mass, and cultured in serum-free medium containing high-glucose DMEM supplemented with 100 nM dexamethasone, 1x Insulin-transferrin-selenium plus premix (ITS Premix, from BD®); final concentration: 6.25 µg/mL bovine insulin, 6.25 µg/mL transferrin, 6.25 µg/mL selenous acid, 5.33 µg/mL linoleic acid and 1.25 µg/mL bovine serum albumin), 50 µg/mL ascorbic acid, 100 µg/mL sodium pyruvate, 50 µg/mL proline and 20 ng/mL transforming growth factor-β3 (TGF-β3). The medium was changed every 3 days. After 3 weeks, pellets from the culture were frozen, sectioned (6 µm thick) and stained with Alcian blue. (c) Adipogenic potential: to induce adipogenesis, the cells were cultured in basal medium until 60-70% confluence and then changed to theadipogenic differentiation medium (basal medium supplemented with 1 µM dexamethasone, 10 µg/mL insulin, 0.5 mM isobutyl-methylxanthine (IBMX) and 0.5 mM indomethacin). Adipogenic medium was changed every three days for two weeks. Oil Red O staining was used to assess lipid accumulation in the cells.

The growth curve

To investigate the growth pattern of MSCs from the 3 sources, 3-4 passage cells from each source; bone marrow, periosteum and adipose tissue were seeded at 5 x 10^4 cells/well in 6-well culture plates and culture medium was changed every three days until the end of experiment. Cells were trypsinized and counted using a haemocytometer every second day. Growth curves were plotted from these data (in replicate).

Determination of population doublings time

To determine the population doubling time, cells at passage two from each source; bone marrow, periosteum and adipose tissue were seeded in T25 flasks at a density of 1.5 x 10^5 cells. When the cells were

approximately 80% confluent (average 1 week after seeding), the cells were counted and reseeded at 1.5 x 10^5 cells. The numbers of the cells were counted from the third to the fifth passage. Population doubling number (PDN) and population doubling time (PDT) were calculated according to the equations: (1) PDN =log (N/N_0) x 3.31; PDN the population doubling number, N = the number at the end of the period (which was seven days), N_0= the initial number of cell which was 1.5 x 10^5 cells and (2) PDT = CT/PDN; PDT = population doubling time, CT = the duration of culture which was 7 days.

Colony forming unit assay

The 2nd-4th passaged MSCs from the 3 sources were evaluated for their clonogenic ability by using colony forming assays. The cells were seeded in low density (20 cells per cm^2) and they were cultured in 10% FBS DMEM with 1% penicillin/streptomycin for 2 weeks without changing of medium. After two weeks, the colonies were stained with Giemsa stain. The colonies that had > 50 cells were counted. The numbers of colonies were counted manually under a light microscope. The colony forming ability was compared by calculating the percentage of cells that formed colonies [(Number of colonies/Number of cells seeded) x 100] Six regions of each well were randomly captured under 4x microscope and the images were analysed using the image J programme[12] to obtain the percentage of the surface area of the well covered by colonies.

Statistical analysis

The growth curves from the 3 sources were compared using available online statistic software (http://bioinf.wehi.edu.au/software/compareCurves/index.html). All values from population doubling time study and clonogenic study were reported as mean $\pm$ standard error (SD). These data were analysed using parametric test. One-way ANOVA (analysis of variance) was used to compare the data from three sources and bonferroni's multiple comparison test was used to test between each source using the statistical package of GraphPad Prism. A p-value < 0.05 was considered to be statistically significant.

Results

The morphology of MSC from 3 sources

The cells derived from these three sources showed no obvious differences in their morphology (Fig. 1). They attached to plastic flasks and showed similar heterogeneous morphology at initial plating and then became a homogenous monolayer of fibroblast-like cells with passaging. Cells from each source could be passaged at least 12 times; however, they exhibited slightly large and flatted shape in later passages.

Differentiation ability

Due to the lack of a single specific marker of MSCs, the isolated cells from three were validated using physical, phenotypic and functional properties. The Tri-linage differentiation potential including (a) osteogenesis, (b) chondrogenesis and (c) adipogenesis was demonstrated. Cells from the three sources were able to differentiate into the direction of bone, cartilage and adipose tissue (Fig. 2-4). The cells under osteogenic condition showed the presence of ALP activity, the cells within the micromass culture under chondrogeneic condition demonstrated positive staining with Alcain blue and the cells under adipogenic condition formed lipid droplets intracellular which were revealed using Oil-red-O staining. Fibroblasts cells which were used as controls were not able to differentiate under osteogenic, chondrogeneic or adipogenic conditions (Fig. 5).

Growth kinetics of MSCs from three sources

Growth curve

The growth curves from the cells from the three sources revealed the same 'exponential' growth pattern which had three phases. Initially they were in the lag phase of 0-2 days' duration following which there was a log phase of rapid cell proliferation from day 4th to day 6th. Finally, there was a plateau phase after day 8th (Fig. 6). The growth of MSCs from bone

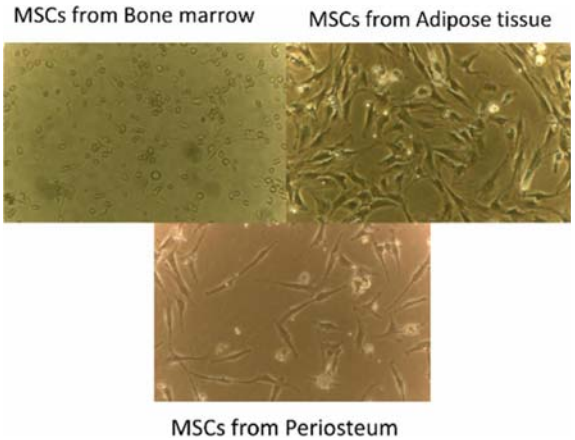

Fig. 1 MSCs from 3 sources; bone marrow, adipose tissue and periosteum (magnification x40)

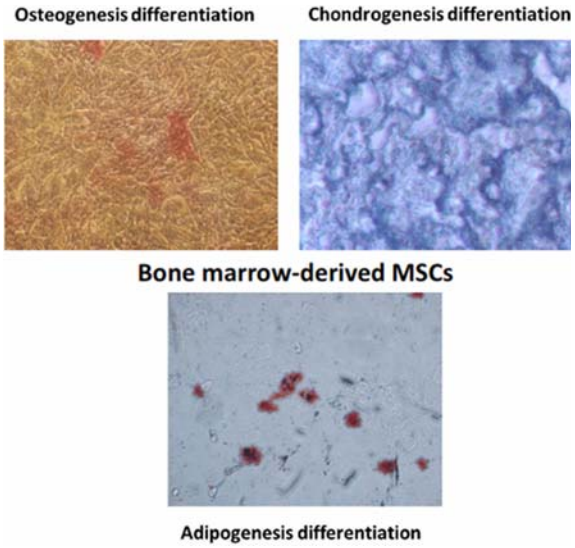

Fig. 2 Osteogenic (ALP staining), chondrogenic (Alcian blue staining) and adipogenic (Oil red o staining) differentiation of MSCs from bone marrow (magnification x40)

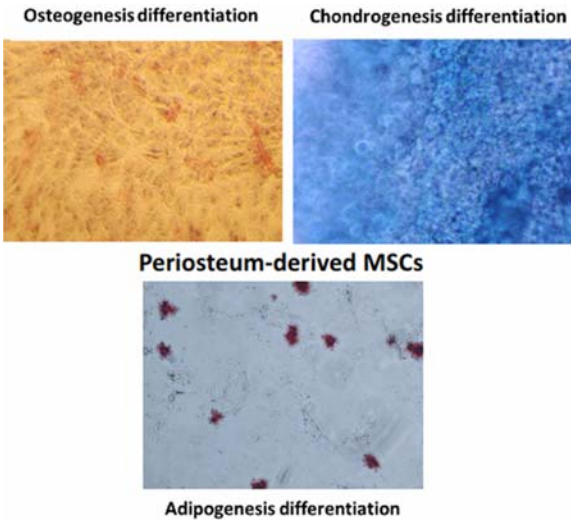

Fig. 3 Osteogenic (ALP staining), chondrogenic (Alcian blue staining), and adipogenic (Oil red O staining) differentiation of MSCs from periosteum (magnification x40)

marrow and periosteum were comparable and both of them were significantly faster than MSCs from adipose tissue ($p < 0.05$).

Population doubling time

The growth kinetics of cells from the 3 sources were compared using the population doubling time (Fig. 7). The mean PDT of MSCs from bone marrow from

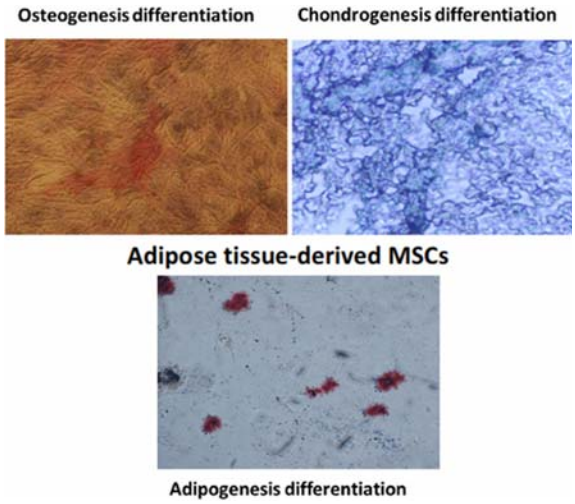

Fig. 4 Osteogenic (ALP staining), chondrogenic (Alcian blue staining), and adipogenic (Oil red O staining) differentiation of MSCs from adipose tissue (magnification x40)

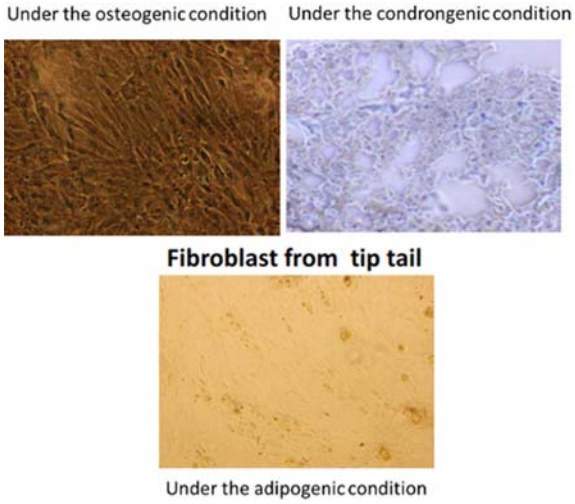

Fig. 5 Fibroblast culture under the osteogenic (ALP staining), chodrongenic (Alcian blue staining), and adiposgenic (Oil red O staining) condition for control (magnification x40)

was 4.00 days (SD = 1.2), from periosteum was 3.6 days (SD = 1.2) and from adipose tissue was 4.7 days (SD = 1.5). The bone marrow derived cells and the periosteum derived cells had faster growth kinetics than the adipose derived cells, but this difference did not reach statistical significance ($p > 0.05$)

Percentages of colony forming units and area

The percentages of colony forming units from bone marrow, periosteum and adipose tissue derived

cells were 8.6 (SD = 1.4), 9.9 (SD = 2.0) and 5.9 (SD = 0.8), respectively. The percentages of the area covered with colonies from bone marrow, periosteum and adipose tissue derived cells were 25.1 (SD = 7.3), 32.5 (SD = 10.7) and 15.8 (SD = 9.0), respectively. These results indicated that the clonogenic abilty of MSCs from bone marrow and periosteum were significantly higher than for adipose derived cells ($p < 0.05$) (Fig. 8, 9).

Discussion

Mesenchymal stem cell research is an extremely active tropic field both in regenerative medicine and in orthopaedics. MSCs are able to differentiate into bone, cartilage and fat tissue *in vitro* condition and retain the ability for self-renewal capacity or "stemness[13]". Therefore, MSCs have potential to be expanded and to regenerate bone and cartilage for use in bone defects, fracture non-unions, or cartilage injuries. MSCs have been identified in a variety of adult human tissues including bone trabeculae, teeth, skin, muscle, synovial, umbilical blood cord[9]. However, three potential sources which are relevant to orthopaedic surgeons are bone marrow, periosteum and adipose tissue. In the present study, the authors have confirmed that MSCs can be isolated from bone marrow, perisosteum and adipose tissue. The cells attached to plastic flasks and demonstrated heterogeneous morphology on the initial plating and then became a homogenous monolayer of fibroblast-like cells with passaging. The cells from each source could be passaged at least 12 times and they formed colonies under low-cell density. The isolated cells from these 3 sources were capable of osteogenesis, chondro genesis and adipogenesis. Thus, cells from these three sources were confined as MSCs which should have potential for musculoskeletal regeneration.

It has been reported that MSCs could repair bone in several animal bone defect models[14-16].

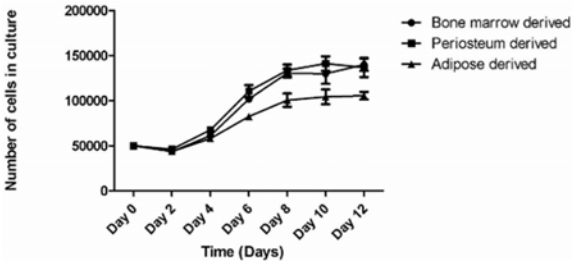

Fig. 6 The growth curve of MSCs from bone marrow, periosteum and adipose tissue under 2 weeks culture; the growth of MSCs from bone marrow and periosteum are comparable and both of them are significantly faster than MSCs from adipose tissue ($p < 0.05$)

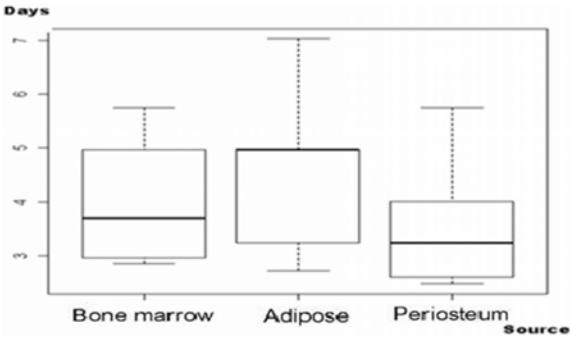

Fig. 7 The population doubling time (PDT) of MSCs from bone marrow, adipose tissue and periosteum; the PDT of bone marrow derived cells and the periosteum derived cells are more than than the adipose derived cells

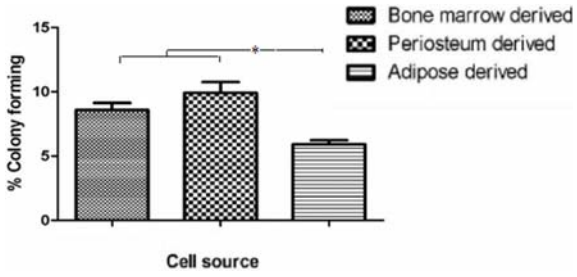

Fig. 8 The percentage of colony forming units of MSCs from bone marrow, periosteum and adipose tissue; mean percentage of colony forming of bone marrow derived and periosteum derived MSCs are more than adipose derived MSCs (*p-value < 0.05)

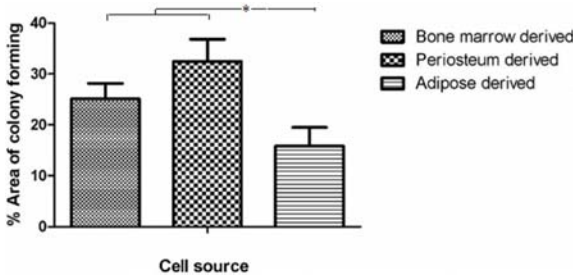

Fig. 9 The percentage of colony forming area of MSCs from bone marrow, periosteum and adipose tissue; mean area of colony forming of bone marrow derived and periosteum derived MSCs are larger than adipose derived MSCs (*p-value < 0.05)

Autologous bone marrow MSC transplanted with in ceramic cylinders successful treated rat segmental femoral defects[15] and in mice craniotomy defects[16]. MSCs from adipose tissue have been reported to improve healing in large critical size mouse calvarial defects[17]. There are only a few reports of the use of MSCs for bone repair in patients. Bajada et al[18] used 5 x 10^6 cells of expanded MSCs combined with calcium sulphate scaffold in a 9-year tibial non-union resistant to six previous surgical treatments. Non-union healed within two months of the MCS implantation procedure. MSCs from a periosteal source have been used to augment an atrophic non-union of the distal femur which was undergoing stabilisation with a 90 degree angle blade plate[19]. Apart from bony repair, MSCs also can be used for cartilage regeneration. Cartilage injuries are notoriouslydifficult to heal with hyaline cartilage[20]. Autologous chondrocyte implantation (ACI) has been used successfully to treat cartilage defects, however, the disadvantage of this method is the time taken to collect, expand and re-implant the chondrocytes, which is at least three weeks[21]. As MSCs have shown the potential for chondrogenesis under *in vitro* condition[22], it is possible that they may augment the cartilage regeneration in articular injuries. However, though there area number of successful studied demonstrating that MSCs can repair cartilage defects in animal models, the appropriated clinical studies of MSCs for cartilage repair are yet to be performed[23]. A case report from Thailand of two patients with large traumatic cartilage defects of the knee showed clinical improvement using autologous bone marrow mesenchymal stem cell (BM-MSCs) implantation without complications. Currently there is an increasing demand for the use of MSCs in orthopaedics[24]. As a result, alternative sources for MSCs should be considered in order to optimise the MSCs preparation. The yield of MSCs has been reported to be an important factor in determining a successful outcome[8]. Hernigou et al used concentrated autologous bone-marrow to treat atrophic tibial diaphyseal non-unions. The procedure was successful for 90% of the patients (n = 53). In the union group that had successfully united, the bone marrow that had been injected into the non-unions contained > 1,500 progenitors/cm^3 and an average total of 54,962 $\pm$ 17,431 progenitors. In the seven patients in whom bone union was not obtained significantly lower concentration (634 $\pm$ 187 progenitors/cm^3) and total number (19,324 $\pm$ 6,843) of progenitors had been injected. It is suggested that MSCs should be expanded in culture condition to obtain sufficient cells in an efficient manner. It is important to consider the growth kinetics of the cells from different sources. The results of the present study have been shown the bone marrow derived cells and the periosteum derived cells had faster growth kinetics than the adipose derived cells. The percentage of colony forming units and the colony forming area from the bone marrow derived cells and the periosteum derived cells were significantly greater than for adipose derived cells. The *in vitro* growth kinetics and the clonogenic ability of MSCs may also reflect their power of tissue regeneration *in vivo*. This conjecture is supported by Niemeyer et al who reported that an osteogenic potential of bone marrow derived mesenchymal stem cells (BMSC) and adipose-tissue derived stem cells (ASC) in a sheep with bone defect model. They found that the bone regeneration potential of BM-MSC was superior to ASC[25]. MCSs from periosteum have also been shown both *in vitro* and *in vivo* to have superior osteogenic differentiation potential in comparison with adipose tissue[26]. However, the periosteum is limited source and is difficult to harvest. Further, its removal will interfere with the blood supply of the bone. Thus, in clinical practice, the bone marrow should be still considered as an appropriate source.

Although the growth kinetics of MSCs derived from adipose tissue was lower than from the bone marrow or periosteum, adipose tissue is readily available and easy to harvest and thus, it can be used as an alternative source. Further sufficient numbers of stem cells can be obtained from adipose tissue without culture expansion[27]. Thus in the future the therapeutic role of adipose derived stem cells should be evaluated in patients with bone and cartilage defects.

As, a single surface marker of MSCs has not been identified so, isolation methods may differ in different units. Thus, in 2006, the mesenchymal and tissue stem cell committee of the International Society for Cellular Therapy (ISCT) has proposed the criteria necessary to define human MSCs. Firstly, MSCs must be plastic-adherent when maintained in standard culture conditions. Secondly, MSCs must express CD105, CD73 and CD90 and lack expression of CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA-DR surface molecules. Thirdly, MSCs must differentiate into osteoblasts, adipocytes and chondroblasts *in vitro*. However, in MSCs derived from animal tissues, they suggested that MSCs can be validated using several physical, phenotypic and functional properties[28]. The authors have used the classical assay to identify MSCs; *i.e.* the ability of adherent spindle shaped cells

toproliferate to form colonies that can be induced to differentiate into adipocytes, osteocytes and chondrocytes. In the future, if a MSC marker is identified the isolation, MSCs will be greatly simplified without the need for culture.

Conclusion

The present study showed the growth kinetic of rat MSCs from three potential including bone marrow, periosteum and adipose tissue. Periosteum and adipose tissue can be used as the alternative source for MSCs isolation. However, the growth kinetics of MSCs should be taken to account. To investigate the therapeutic potential, the MSCs from these sources should be compared using a clinical relevant animal model for bone or cartilage regeneration.

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Potential conflicts of interest

None.

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การศึกษาและเปรียบเทียบ การเจริญเติบโตและจลนพลศาสตร์ ของเซลล์ต้นกำเนิดชนิดมีเซนไคม์ จากไขกระดูกเยื่อหุ้มกระดูกและไขมัน

ตุลยพฤกษ์ ถาวรสวัสดิ์รักษ์, อัลโตนิลโอ ซเปซี่โน, เอียร์น เมอร์เรย์, บูลโน เพียร,เฮมิส ซิมสัน

วัตถุประสงค์: *เพื่อทำการสกัดแยกเซลล์ต้นกำเนิดจากเนื้อเยื่อไขกระดูก เยื่อหุ้มกระดูก และเนื้อเยื่อไขมัน และศึกษาทดลองการแสดงศักยภาพในการเจริญเป็นเซลล์กระดูก, กระดูกอ่อน และไขมัน ของเซลล์ต้นกำเนิดจากการแยกสกัด อีกทั้งยังเปรียบเทียบจลนพลศาสตร์ การเจริญเติบโตและ การสร้างโคโลนี ของเซลล์ต้นกำเนิดจากเนื้อเยื่อทั้งสามชนิด*

วัสดุและวิธีการ: *การศึกษานี้ได้ทำการสกัดและเพาะเลี้ยงเซลล์ต้นกำเนิดจาก ไขกระดูก เยื่อหุ้มกระดูก และเนื้อเยื่อไขมันในหนูทดลอง โดยใช้เทคนิคปลอดเชื้อ เซลล์หลังจากการเปลี่ยนถ่ายครั้งสองถึงสี่ ได้นำมาใช้ในการศึกษาการเจริญเติบโต และเพิ่มจำนวณ ร่วมถึง คุณสมบัติการสร้างโคโลนีของเซลล์ที่สกัดจากเนื้อเยื่อทั้งสามชนิด*

ผลการศึกษา: *จากการทดลองแสดงให้เห็นว่าเซลล์ต้นกำเนิดที่ได้จากการแยกสกัดมี ลักษณะรูปร่าง ที่ไม่แตกต่างกัน และสามารถเปลี่ยนถ่ายในจานเพาะเลี้ยง อย่างน้อยสิบสองครั้ง โดยเซลล์ ที่เพาะเลี้ยงจากเนื้อเยื่อทั้งสามชนิด สามารถมีคุณสมบัติ osteogenesis, chondrogenesis และ adipogenesis จากการเปรียบเทียบ อัตราการเจริญเติบโตและเพิ่มจำนวณ ของ เซลล์ รวมถึงความสามารถในการสร้างโคโลนีของเซลล์ ที่สกัดจากเยื่อหุ้มกระดูก และ ไขกระดูกมากกว่า เซลล์ที่สกัดจากเนื้อเยื่อไขมัน อย่างมีนัยสำคัญ*

สรุป: *เซลล์ต้นกำเนิด ที่สกัดจากไขกระดูก และ เยื่อหุ้มกระดูก จะมี การเจริญเติบโตของ ที่เร็วกว่าจากเซลล์ที่ได้จากเนื้อเยื่อไขมัน อย่างไรก็ตาม เนื้อเยื่อไขมันสามารถ ใช้เป็นแหล่งทางเลือกที่เหมาะสม ทั้งนี้อาจจะต้องพิจารณาปรับปรุงและพัฒนาวิธีการแยกสกัดและเพาะเลี้ยงของเซลล์ต้นกำเนิด จากเนื้อเยื่อไขมันต่อไป*