ORIGINAL ARTICLE

Supplementation of TGF-Beta3 in Low Serum Media Promotes Chondrogenesis of BMSCs

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ABSTRAK

Kejuruteraan tisu merupakan satu bidang perubatan baru yang membolehkan tisu manusia dibentuk secara in vitro sebelum pemindahan tisu dilakukan. Sel stem mesenkima daripada sum sum tulang (BMSCs) adalah sumber sel yang sesuai dalam kejuruteraan tisu tulang rawan, ekoran kebolehannya untuk membentuk rawan hialin. Kajian ini telah dijalankan untuk mengkaji potensi BMSCs untuk membentuk sel rawan secara in vitro dan in vivo. BMSCs dipencilkan daripada kambing dan dibahagikan kepada dua kumpulan; iaitu kumpulan kajian dan kumpulan kawalan. Sel di dalam kumpulan kawalan disemai di dalam medium yang mengandungi 10% serum anak lembu (FBS). Manakala, sel di dalam kumpulan kajian telah dikultur di dalam medium yang diperkaya dengan 1% FBS, 1% ITS, 5 ng/mL TGF-ß3, 50 ng/mL IGF-1, 40 ng/mL L-proline, 100 nM dexamethasone dan 50 µg / fosfat. Analisis pembentukan sel rawan telah dijalankan secara in vivo pada model mencit gondol atimus. Sel didapati beragregasi di dalam kumpulan kajian dan menunjukkan permulaannya kondrogenesis telah berlaku dalam pengkulturan sel. Kerangka fibrin yang telah disemai bersama sel yang telah atau tidak diaruh disediakan dan ditanamkan di dalam lapisan subkutaneus mencik gondol atimus selama 5 minggu. Eksplan tersebut kemudiannya dikeluarkan daripada lokasi penempelan untuk pemeriksaan histologi. Berdasarkan pewarnaan Safranin O dan pengekspresan GAG, eksplan daripada kumpulan kajian didapati menghasilkan matriks rawan pada kadar yang tinggi berbanding eksplan di dalam kumpulan kawalan. Hasil kajian ini menunjukkan keberkesanan medium rendah serum yang

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mengandungi TGF-ß3 merangsang pembezaan sel stem mesenkima daripada sum-sum tulang untuk menjadi sel rawan.

Kata kunci: sum-sum tulang, rawan, kondrogenesis, faktor tumbesaran, medium rendah serum, sel stem

ABSTRACT

Tissue engineering has emerged as a new promising field that allow *in vitro* construction of whole transplantable tissue. Recently, bone marrow stem cells (BMSCs) fulfil the requirements as appropriate cell source that is renewable for cartilage tissue engineering since they were able to form hyaline-like cartilage *in vitro* and *in vivo* when cultured in media supplemented with specific growth factors. This study aimed to examine the potential of BMSCs chondrogenesis *in vitro* and *in vivo*. BMSCs were isolated from sheep and divided into a test group and control group. Control group was cultured in medium supplemented with 10% fetal bovine serum (FBS). Test group was cultured in medium supplemented with 1% FBS, 1% ITS, 5ng/mL TGF-ß3, 50ng/mL IGF-1, 40 ng/mL L-proline, 100 nM dexamethasone and 50 μg/mL ascorbic acid-2 phosphate. The experiment was carried out on athymic nude mice. Cell aggregates were formed in test group and indicated the early chondrogenesis in cell culture. Later, cells-fibrin constructs were made and implanted subcutaneously into nude mice for 5 wks, then explanted for histological examination and glycosaminoglycans (GAG) quantification. Test group constructs showed higher cartilage matrix synthesis as confirmed by Safranin O staining and GAG production. These results demonstrated the effectiveness of low serum media supplemented with TGF-ß3 in promoting chondrogenesis in BMSCs.

Keywords: bone marrow, cartilage, chondrogenesis, growth factors, low serum media, stem cells

INTRODUCTION

Tissue engineering has emerged as a new promising field that allow in vitro construction of a transplantable tissue. The three main parts required to construct an engineered tissue are: a scaffold, a suitable cells type and the addition of substances like growth factors (Kou et al. 2006). Recently researches focusing on cartilage repair used mesenchymal stem cells (MSCs) to achieve the therapeutic purpose (Miljkovic et al. 2008). MSCs are a good cell source to engineered cartilage tissue due to their ability to undergo chondrogenic differentiation after culture-expansion with specific growth factors mix (Baksh et al. 2004). MSCs fulfil the requirements as appropriate cell source that is renewable for cartilage

tissue engineering since they can be manipulated in vitro to differentiate into chondrocytes and able to form hyaline-like cartilage in vitro and in vivo when they were cultured in media supplemented with specific growth factors such as transforming growth factor (TGF-ßs) and insulin like growth factor (IGF-1) (Magne et al. 2005). Chondrogenic differentiation happens when MSCs were grown in three-dimensional culture system, serum-free medium and with the induction from members of the TGF-ß super-family. In these culture environment, MSCs rapidly lose their fibroblastic characteristic and start to express a group of cartilage-specific extracellular matrix genes (Barry et al. 2001). There are protocols to differential MSCs into chondrogenic cells successfully by inducing the expression of cartilage-specific molecules such as collagen type II, SOX9 and aggrecan that known to promote cartilage formation (Pelttari et al. 2008). In the presence of TGF-ß3 for differentiation protocol, MSCs synthesize cartilage oligomeric matrix protein, aggrecan, fibromodulin, collagen type II and link protein as the main matrix components of the normal articular cartilage (Rodeo 2001). MSCs can be cultured and differentiated along multiple phenotypic pathways with specific media containing different types of growth factors and substances such as transforming growth factor beta family (TGF-ßs), insulin-like growth factor, dexamethasone and vitamin C (Jones et al. 2002). Moreover, their expansion in vitro is easier than primary autologous chondrocytes (Miljkovic et al. 2008). This is due to MSCs having

much longer life span compared to chondrocytes (Hui et al. 2005). Bone marrow-derived stem cells (BMSCs) can differentiate into chondrogenic cells in various culture conditions and TGF-ßs were added to enhance chondrogenesis process in many culture methods (Coleman et al. 2007). The chondrogenic differentiation of MSCs can be induced by growth factors such as IGF-1 and TGF-ß3. Both factors can maintain the phenotype of chondrocyte; promote the secretion of extracellular matrix and promote cartilage matrix macromolecules synthesis during chondrogenesis (Worster et al. 2001; Indrawattana et al. 2004; Gelse et al. 2003). In vitro study had demonstrated that MSCs can form hyaline-like cartilage when cultured in medium containing TGF-ß3 (Magne et al. 2005). It was reported that IGF-1 regulated the synthesis of collagen type II and aggrecan at the transcription level during chondrogenic differentiation in rat articular chondrocytes while TGF-ß3 is the important factor in the initial stages of MSCs chondrogenesis (Messei et al. 2000; Mastrogiacomo et al. 2001) While addition of another factor; basic fibroblast growth factor (bFGF) was demonstrated to increase the proliferation rate and life span of dog, rabbit and human MSCs (Mastrogiacomo et al. 2001; Tsutsumi et al. 2001). Previous studies had showed the mitogenic effect of bFGF in human chondrocytes and chondrogenic effect when combined with TGF-ß2, IGF-1 and insulin-transferrin-selenium in cartilage regeneration (Chua et al. 2005; Chua et al. 2007). However, there were few studies if

any conducted on combination of medium with different growth factors on BMSCs chondrogenesis either in vitro or in vivo. Thus this study aimed to investigate the effect of combination medium on marrow stem cells (BMSCs) in vitro and in vivo chondrogenesis.

MATERIALS AND METHODS

The study was conducted at the Institute of Medical Research and Tissue Engineering Centre, Universiti Kebangsaan Medical Centre Malaysia (UKMMC). All experiments in this study were performed in accordance with Animal Ethical Committee of the Faculty of Medicine, Universiti Kebangsaan Malaysia (UKMAEC) with approval Number FISIO/2006/RUSZYMAH-AUGUST/175-JANUARY-2009.

About 10 mL of bone marrow was aspirated from iliac crest of healthy male sheep (n=5). Sterile bone marrow aspiration needle (Jamshidi-Dominica Republic) connected to heparinized 20 mL sterile syringe was inserted into hip bone (iliac crest) of sheep. The syringe was pulled out slowly to collect the bone marrow.

Mesenchymal stem cells (MSCs) were isolated from the specimen via gradient centrifugation (Universal 32 R, Hettich, Germany) over a Ficoll-Paque PLUS (GE Healthcare-Sweden) layer at 3000 rotations per min (r.p.m.) for 30 mins and subsequently rinsed twice with phosphate buffered saline (PBS; Invitrogen, USA). Cells were resuspended in the culture medium (Ham's F12: high glucose DMEM-FD 1:1; GIBCO, Rockville,

MD) supplemented with 10% fetal bovine serum (GIBCO), 1X antibiotic-antimycotic (GIBCO), 1X GlutaMAX-1 (GIBCO), 1% antibiotic/antimycotic, 50 µg/ml L-ascorbic acid (SIGMA, St. Louise, MO), 0.02 M HEPES buffer (GIBCO) and plated into T-75 cm^2 flasks. The cells were incubated at 37°C in an incubator containing 5% CO_2. The culture medium was changed every 2 to 3 days.

Cell morphology was examined using an inverted phase contrast microscope (CK40; Olympus, Japan). Upon 95% confluence, cells were trypsinized. Viability and total cell number were determined by manual counting using a bright field microscope with the aid of the trypan blue exclusion dye and a haemocytometer.

Adult bone marrow mesenchymal stem cells were divided into 2 groups; test and control groups (n=6): Five million of BMSCs were cultured in medium supplemented with 10 % FBS alone for 3 wks and constructed and implanted subcutaneously in nude mice. Test group (n=6): Five million of BMSCs were cultured in medium supplemented with 1% antibiotic/antimycotic, 1% FBS, 1% ITS, 1% ascorbic acid, 1% GlutaMAX-1 (GIBCO), 50ng/mL IGF-1, 5ng/mL transforming growth factor beta 3 (TGF-ß3), 50 µg/mL ascorbic acid-2 phosphate, 40ng/mL L-proline and 100nM/mL dexamethasone. Each group of the cells were plated into five T-75 culture flasks and incubated at 37°C in an incubator containing 5% CO_2 with media changed every 2 to 3 days for 3 wks.

After 3 wks, the cells from test group

and control groups were trypsinized and suspended in 0.5 mL of sheep plasma. A three dimensional (3D) cells-fibrin construct was formed by adding 30 μL calcium chloride (CaCl$_2$) solution into the mixture. The resulted three dimensional constructs were maintained in 5mL of the same culture medium and incubated overnight at 37°C in an incubator containing 5% CO$_2$.

Eighteen healthy athymic nude mice were divided into two groups (test and control); Mice were anesthetized by 0.1 ml of mixed xylazine-ketamin1:1/25g of body weight. Small incision on the dorsum part of the mice was made, BMSCs construct was implanted subcutaneously. After 5 wks, mice were euthanized by overdose of anaesthetic drugs. Dorsal skin of nude mouse was dissected and BMSCs construct was carefully removed from surrounding tissues, fixed in 5% formaldehyde overnight. Each implant was divided into two parts for histology examination and glycosaminoglycans (GAG) quantification.

For histological examination, constructs were imbedded in paraffin, sectioned into 4 μm thickness and stained with Safranin O staining. GAG quantification assay, constructs were dried and weighed before treated with papain digestive solution (125 μg/mL of papain, 5 mM L-cystein, 100 mM Na$_2$HPO$_4$, 100 mM EDTA, pH 6.8) at 60°C for 16 hours. The GAG contents were measured using a 1, 9 dimethylmethylene blue (DMMB) assay. The sample was mixed with DMMB solution and the absorbance at 590 nm wavelength was measured.

Total GAG of was determined using a standard plot of shark chondroitin sulfate (Sigma) and normalized with dried-weight of each sample (n=4) as the relative GAG content (%).

STATISTICAL ANALYSIS

Statistical analysis, data was expressed as mean   standard error of the mean (SEM). Result was analyzed using SPSS, Mann-Whitney test and the difference were significance when $p < 0.05$.

RESULTS

The control group of BMSCs cultured in medium supplemented with 10% FBS demonstrated spindle to polygonal cells adhered to the flasks at day 7 in culture in passage zero (Figure 1A). The cells density increased fast and formed pack monolayer cells in the culture at day 21 (Figure 1B). However, no cell aggregation was detected in this group. Interestingly in the test group, BMSCs cultured in medium supplemented with TGFß-3, IGF-1, ITS and 1% FBS exhibited plenty of cell aggregates as early as the first week in culture (Figure 1C), and the aggregates were increased in number and formed big cells aggregates at day 14 in culture (Figure 1D).

Fibrin-cells construct appeared soft, shiny and white in colour on the day of construction for both groups (test and control) before implanted into nude mice (Figure 2A). The constructs appeared firm and hard on week three in the dorsum part of the nude mouse (Figure 2B). There was no inflammatory response observed around constructs
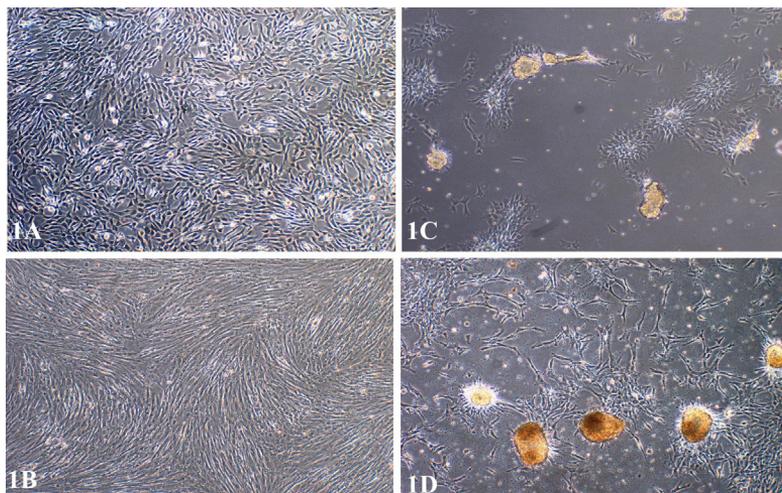
Figure 1: A) Phase-contrast microscopic evaluation on BMSCs culture in media in vitro. Control group; BMSCs adhered fast and formed monolayer within 7 days of culture; B) formed dense monolayer cells after 3 weeks in culture. C) Test group BMSCs formed early cells aggregates at the first week of culture and; D) big cells aggregates can be seen at the end of third week. Magnification x 40.
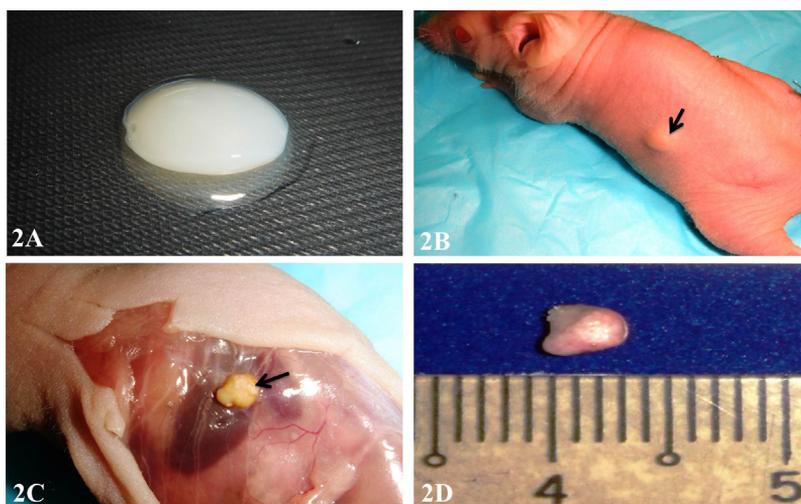

Figure 2: A) Morphology of cells-fibrin constructs. In vitro construct before implantation; B) Construct in the dorsum part of nude mice (arrows); C) Construct at the end week five of implantation, note the implant site showed no inflammation response (arrow); D) Explanted construct appeared white and hard in consistency when harvest.

at the implantation site (Figure 2C). The removed constructs were white in colour and firm in consistency which resembled hyaline cartilage when harvested at the end of week five (Figure 2D). Histological evaluation of constructs from control group showed no response to Safranin O staining (Figure 3A). Interestingly the test group was stained positive with Safranin O, which denoted abundant cartilage matrix production (Figure
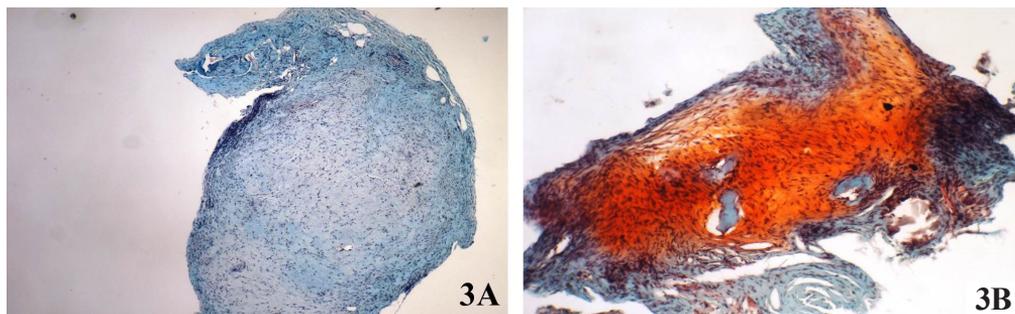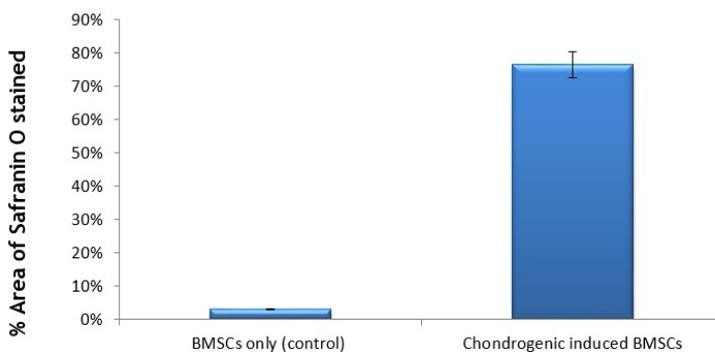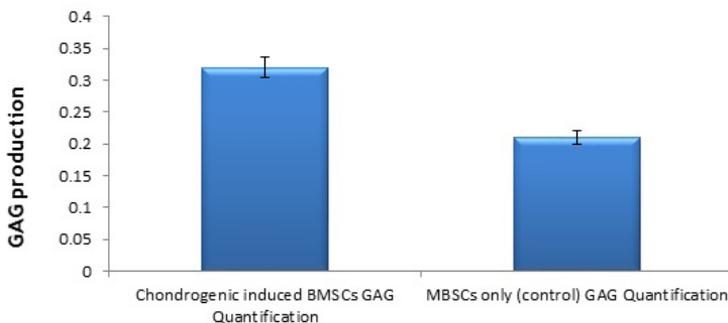
Figure 3: A) Histological evaluation of in vivo BMSCs constructs. Control group showed negative staining with safranin O; B) Test group demonstrated positive orange-red staining with Safranin O. Magnification x40.

Safranin O Staining of implanted BMSCs construct

Figure 4: Percentage of area stained positive with Safranin O in the various constructs: Control group: medium supplemented with 10% FBS and the test group: medium supplemented with 1% FBS and 10ng/ml TGFβ3. #Total area stained with Safranin O in the test group was significantly higher than control group. Data were presented as mean ± standard error of mean and analyzed using Mann-Whitney test.

GAG Quantification of implanted MSCs construct

Figure 5: GAG quantification after 5 wks of implantation. *GAG production level in the test group was significantly higher ($P < 0.05$) compared to GAG production level in control group. Data was analyzed using Mann-Whitney test. Data were presented as mean ± standard error of mean of GAG production.

3B). Data analysis showed that positive area stained with Safranin O was significantly higher in test group (scored 75.73 ± 7.68 % of the total area stained positive with Safranin O; p< 0.05) (Figure 4), when compared to control which were weakly stained and only scored 2.89 ± 2.84 % of total area with Safranin O (Figure 4). Total GAGs production was normalized in each group by dried weight and showed as the relative sGAG content in percentage (%) after 5 wks of in vivo implantation of cells-fibrin constructs in the nude mice. Constructs from the test group BMSCs cultured in medium supplemented with 1% FBS and 10ng/mL TGF-ß3 exhibited the highest sGAG production and was significant higher than control group (P < 0.05; 0.32 ± 0.014; Figure 5). Control group BMSCs cultured in medium supplemented with 10% FBS showed the lowest GAG production (0.211 ± 0.006; Figure 5).

DISCUSSION

Previous study showed that human bone marrow MSCs can expand to large number and encapsulate as osteochondral constructs to form synovial joint condyles for *in vivo* implantation. MSCs has the ability to differentiate into different cell lineages such as chondrocytes, myoblasts, osteoblasts, adipocytes and fibroblasts. Some differentiated cells can express the key markers of neuron-like cells, endothelial cells and cardiomyocytes (Marion & Mao 2006). A study was conducted by Al Faqeh et al. (2012), which showed that BMSCs grew in low serum medium containing IGF-1, TGF-ß3 and dexamethasone for 3 wks were able to form colonies and aggregates. This was in contrast compared to cells culture in basic medium that only can formed multilayer cells. In this study we demonstrated that bone marrow mesenchymal stem cells (BMSCs) cultured in medium supplemented with 1% FBS and TGF-ß3 showed great potential in chondrogenesis in vitro. Transforming growth factor beta 3 (TGF-ß3) is needed to stimulate MSCs chondrogenesis in the early stages and this cause the cells aggregation (Roark & Greer 1994). In the monolayer culture-expansion, BMSCs changed from polygonal shape to spinal-like appearance as early as first passage. The same result was reported previously in human chondrocytes that proliferate fast (Ruszymah et al. 2007). Subsequently, cultured BMSCs regardless of culture media used were mixed well with sheep plasma. Addition of $CaCl_2$ initialled the polymerization of fibrin and formation of physically stable construct for transplantation.

In the present study the chondrogenesis of BMSCs in vivo was induced successfully after BMSCs cultured in medium supplemented with 1% FBS and 10ng/mL TGF-ß3. This result was supported by histological evaluation (Safranin O staining) and glycosaminoglycan's (GAG) assay. TGF-ß3 is more chondrogenic efficient when added to BMSCs culture to induce chondrogenesis.

The result of this study showed no cell aggregation in the control group cultured in medium supplemented with 10% FBS. In this study the

chondrogenesis was induced in high-density of cells 5 x10^6 cells/20mL in the culture. Numbers of cell aggregates was formed as early as day two and day three of the culture and the size of cell aggregates increased by time in test group of BMSCs cultured in medium supplemented with 1% FBS and 10ng/mL TGF-ß3. This phenomenon was not observed in the control. These results showed that TGF-ß3 possesses great potential to induce early chondrogenesis in BMSCs in vitro and in vivo. The implanted constructs of BMSCs from the test and control groups were appeared white in color and hard in consistency when harvested after 5 wks of implantation. Although the constructs from control group appeared larger than the test group construct, it did not show sign of chondrogenesis. The Safranin O staining was negative and the level of GAG production was the lowest compared to the test group. This result could contribute by higher concentration of FBS in the medium promote BMSCs proliferation but inhibit GAG production.

The test group BMSCs constructs showed significant higher GAG production when compared with the control group (BMSCs only). Interestingly the group of BMSCs constructs that was supplemented with TGF-ß3 showed the highest cell aggregates in vitro, highest Safranin O stained area with high GAG production in vivo. These findings agree with the previous study that BMSCs differentiation using TGF-ß3 was 10 times more active than other TGF-ß family. The combination of TGF-ß3 and IGF-1 was more effective to induce chondrogenesis (Indrawattana et al. 2004). The result of the present study suggests that TGF-ß3 should be considered as specific growth factor to induce BMSCs chondrogenesis. The histological evaluation and GAGs production of BMSCs construct of the test group (BMSCs cultured in medium supplemented with 1% FBS and TGF-ß3) were clear evidence of BMSCs chondrogenesis enhancement. Our result suggested that growth factor TGF-ß3 and BMSCs in chondrogenesis are relevant for developing cartilage in future. This has provided a new therapeutic alternative for the treatment of osteoarthritis (OA) in the joint. The lowest quality of chondrogenic feature of BMSCs cultured in medium supplemented with 10% FBS (control) suggested medium with high concentration of serum is not desirable for chondrogenic induction.

Our result was supported by another study that showed the chondrogenic differentiation of MSCs was stained positive for collagen type II antibody and the expression of COL2A1 was upregulated significantly (Ullah et al. 2013). Another study showed GAG production and collagen type II synthesis in MSCs were inhibited in high percentage of serum (Bilgen et al. 2007). The formation of cartilage matrix in vivo could contribute by the cell aggregation in the test groups. Our results demonstrated that BMSCs cultured in medium supplemented with TGF-ß3 and 1% FBS induced higher chondrogenesis activity in BMSCs. In conclusion, bone marrow mesenchymal stem cells (BMSCs)

cultured in medium supplemented with 1% FBS and 10 ng/mL TGF-ß3 showed chondrogenic capability in vitro and in vivo compared to BMSCs cultured in medium supplemented with 10% FBS. This study suggests that TGF-ß3 could enhance BMSCs chondrogenesis phenotype in vivo and promotes the chondrocyte phenotype by enhancing sulfated glycosaminoglycans accumulation.

LIST OF ABBREVIATIONS

BMSC: bone marrow mesenchymal stem cells; TGF: transforming growth factor; DMEM: Dulbecco's Modified Eagle Medium; MSCs: mesenchymal stem cells, FBS: Fetal bovine serum; PBS: phosphate buffered saline; FD: Ham's F12: DMEM (1:1); GAG: Glycosaminoglycan; DMMB: 1, 9-dimethylmethylene blue; IGF: insulin like growth factor; ITS: Insulin transferrin selenium; b-FGF: basic-fibroblast growth factor.

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