Growth medium with low serum and transforming growth factor beta 3 promotes better chondrogenesis of bone marrow-derived stem cells *in vitro* and *in vivo*

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Adult mesenchymal stem cells are candidate cells for cartilage tissue engineering due to their ability to undergo chondrogenic differentiation after extensive expansion, and induced with growth factors.[1] Sustained delivery of transforming growth factor beta (TGF-ß)3 induces differentiation of human bone marrow-derived stem cells (BMSCs) into chondrocytes.[2] Bone marrow-derived stem cells undergo chondrogenesis in a variety of culture conditions, and the addition of TGF-ßs has generally enhanced chondrogenesis regardless of culture method.[3] The chondrogenic differentiation of BMSCs induced by growth factors, such as TGF-ß3, and insulin like growth factor (IGF)-1 can inhibit the instability of the chondrocyte phenotype, promote extracellular matrix secretion, and maintain cartilage matrix macromolecules during chondrogenesis.[4] The shift from TGF-ß3 to IGF-I at week 3 resulted in a significant increase of cartilage-like extracellular matrix.[5] This study aimed to examine the effect of TGF-ß3 and TGF-ß2 on BMSCs chondrogenesis *in vitro* and *in vivo*.

All experiments in this study were performed from August 2008 to January 2009, with approval from the Ethical Committee of Universiti Kebangsaan Malaysia (UKMAEC), and in accordance with the Policy for Humane Care and Use of Laboratory Animals. Five ml bone marrow aspirate was drawn from the iliac crest of healthy male sheeps. The BMSCs were suspended in standard culture medium Ham's F-12: high glucose Dulbecco's Modified Eagle Medium (DMEM) (1:1) supplemented with 10% fetal bovine serum (FBS). The cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2. Cell morphology was assessed using an inverted phase contrast microscope. Eighteen Athymic mice (nude mice) were used in this study. Food and water was given ad libitum. The mice were exposed to normal day and night cycle. In the control group (n=6), $5x10^6$ of BMSCs were cultured in FD 1:1 supplemented with 10% FBS (Gibco, Grand Island, NY, USA). In group A (n=6), $5x10^6$ of BMSCs were cultured in a medium supplemented with a 1% FBS, 1% antibiotic/antimycotic, 1% ascorbic acid, 1% GlutaMAX-1 (Gibco, Grand Island, NY, USA), 1% insulin transferrin selenium (ITS [Gibco, Grand Island, NY, USA]), 5 ng/ml TGF-ß3 [Peprotech Inc, Rocky Hill, NJ, USA], 50 ng/ml IGF-1 [Peprotech Inc, Rocky Hill, NJ, USA], 50 µg/ml ascorbic acid-2 phosphate, 40 ng/ml L-proline, and 100 nM/ml dexamethasone. In group B (n=6), $5x10^6$ of BMSCs were cultured in a medium supplemented with 1% FBS, 1% antibiotic/antimycotic, 1% ascorbic acid, 1% GlutaMAX-1, 1% ITS, 5 ng/ml IGF-1, 3 ng/ml basic-fibroblast growth factor (b-FGF [Peprotech Inc, Rocky Hill, NJ, USA]), and one ng/ml TGF-ß2. After a 3-week culture, the BMSCs from the control group and the test groups were harvested and suspended in 0.5 ml sheep plasma. A 3-dimensional (3-D) cells-fibrin construct was formed by adding 30 µl calcium chloride solution into the mixture. The cells-fibrin constructs were then implanted onto the dorsum of nude mice from all groups. After 5 weeks, all the nude mice were euthanized, and the cells-fibrin constructs were removed, and fixed in 5% formaldehyde overnight. The cells-fibrin constructs were embedded in paraffin, sectioned into 4 µm thickness, and stained with Safranin O (Sigma-Aldrich Corp, St. Louis, MO, USA). Sulphate glycosaminoglycan (sGAG [Sigma-Aldrich Corp, St. Louis, MO, USA]) quantification were measured using a 1, 9 dimethyl methylene blue (Sigma-Aldrich Corp, St. Louis, MO, USA) assay, and the absorbance was measured at 590 nm wavelength. The total sGAG of each sample was determined using a standard plot of shark chondroitin sulfate, and normalized with dried-weight of each sample as the relative sGAG content (%). Data was expressed as mean ± standard error of mean. The results were analyzed using Mann-Whitney test. $P<0.05$ was considered significant.

The cell numbers in the control group increased dramatically, and formed dense monolayer cells at day 21 in the culture. However, there was no cell aggregation detected in the control group. Interestingly in Group A, there were plenty of cell aggregates that coalesced to form big cell aggregates at day 14. In group B, there was cell aggregation activities, but it was less than group A. The cells-fibrin constructs were white in color, and firm in consistency resembling hyaline cartilage when harvested after 5 weeks. There was no inflammation response detected around constructs

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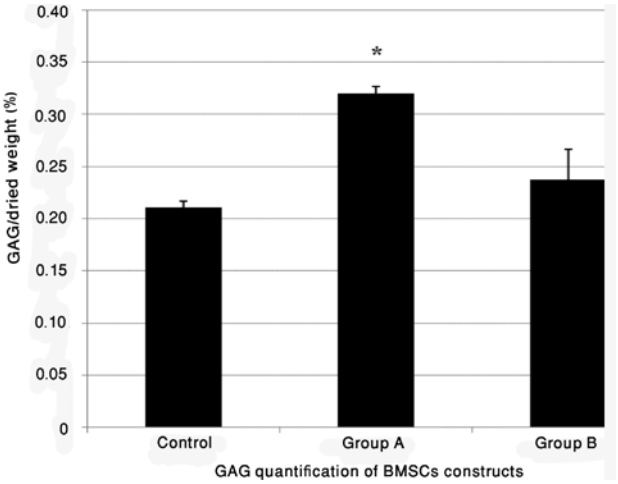

Figure 1 - The chart exhibits the effect of transforming growth factor beta (TGF-ß)3 and TGF-ß2 supplements in combination medium on *in vivo* chondrogenesis. Sulphate glycosaminoglycan (GAG) production level in group A was significantly higher with **p*=0.03 compared to the control group, but not significant to group B. Both combination medium promotes *in vivo* chondrogenesis but better with the addition of TGF-ß3. BMSCs - bone marrow-derived stem cells

at the implantation site of the cells-fibrin constructs. Constructs from groups A and B stained positive with Safranin O denoting abundant cartilage matrix production. Data analysis demonstrated that positive area stained with Safranin O was significantly higher in group A (75.73 ± 7.68%, *p*=0.03) compared to group B that scored 56.33 ± 17.42%. In contrast, cells-fibrin constructs in the control group were poorly stained, and only scored 2.89 ± 2.84% of the total area stained with Safranin O. Total sGAG production in the cells-fibrin constructs from group A exhibited the highest sGAG production (0.32 ± 0.01 µg/ml), and is significantly higher than group B (0.24 ± 0.03 µg/ml) and control group (0.21 ± 0.01 µg/ml) (**Figure 1**). The size of cell aggregates increased with time in Group A more than in Group B. No cell aggregates was observed in the control group. The cell aggregates formation in the test groups may contribute to the later *in vivo* formation of cartilage matrix. The combination medium with TGF-ß3 possesses greater potential to induce chondrogenesis in BMSCs *in vitro* and *in vivo*. Although cells-fibrin constructs from the control group appeared as the largest constructs compared to the other 2 test groups, it did not exhibit any significant chondrogenesis of BMSCs. This was confirmed with negative staining with Safranin O, and showed the lowest level of sGAG production compared to the test groups. The higher concentration of FBS in the medium could inhibit sGAG production, but support the proliferation of BMSCs. In this study, BMSCs cells-fibrin constructs from both test groups showed no significant differences in sGAG production level, which reveals that the combination medium with TGF-ß2 also has chondrogenic potential effect on BMSCs. In the histological evaluation of Group A, the cells-fibrin constructs showed the highest positive staining area with Safranin O and sGAG production *in vivo*. In conclusion, BMSCs cultured in medium supplemented with the combination medium with TGF-ß3 showed higher chondrogenic capability *in vitro* and *in vivo* compared to BMSCs cultured in the combination medium supplemented with TGF-ß2. This study suggests that both combination medium, but better with the addition of TGF-ß3 could enhance BMSCs chondrogenesis *in vivo*, and promotes the maintenance of the chondrocyte phenotype by enhancing sGAG. This will provide a new therapeutic approach and useful tool for the treatment of human knee joint with osteoarthritis.

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