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ORIGINAL ARTICLE

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Platelet-rich concentrate in serum-free medium enhances cartilage-specific extracellular matrix synthesis and reduces chondrocyte hypertrophy of human mesenchymal stromal cells encapsulated in alginate

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Abstract

Platelet-rich concentrate (PRC), used in conjunction with other chondroinductive growth factors, have been shown to induce chondrogenesis of human mesenchymal stromal cells (hMSC) in pellet culture. However, pellet culture systems promote cell hypertrophy and the presence of other chondroinductive growth factors in the culture media used in previous studies obscures accurate determination of the effect of platelet itself in inducing chondrogenic differentiation. Hence, this study aimed to investigate the effect of PRC alone in enhancing the chondrogenic differentiation potential of human mesenchymal stromal cells (hMSC) encapsulated in three-dimensional alginate constructs. Cells encapsulated in alginate were cultured in serum-free medium supplemented with only 15% PRC. Scanning electron microscopy was used to determine the cell morphology. Chondrogenic molecular signature of hMSCs was determined by quantitative real-time PCR and verified at protein levels via immunohistochemistry and enzyme-linked immunosorbent assay. Results showed that the cells cultured in the presence of PRC for 24 days maintained a chondrocytic phenotype and demonstrated minimal upregulation of cartilaginous extracellular matrix (ECM) marker genes (*SOX9, TNC, COL2, ACAN, COMP*) and reduced expression of chondrocyte hypertrophy genes (*Col X, Runx2*) compared to the standard chondrogenic medium ($p < 0.05$). PRC group had correspondingly higher levels of glycosaminoglycan and increased concentration of chondrogenic specific proteins (COL2, ACAN, COMP) in the ECM. In conclusion, PRC alone appears to be very potent in inducing chondrogenic differentiation of hMSCs and offers additional benefit of suppressing chondrocyte hypertrophy, rendering it a promising approach for providing abundant pool of chondrogenic MSCs for application in cartilage tissue engineering.

Keywords

Blood, cartilage repair, chondrogenesis, extra-cellular matrix, regenerative medicine, stem cells, tissue engineering

History

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Introduction

Articular cartilage is an avascular connective tissue comprised of sparsely distributed highly specialized cells of mesenchymal origin. These cells, called the chondrocytes, are responsible for secreting, maintaining and repairing the extracellular matrix (ECM), which in turn regulates the shape and stability of mature cartilage [1–3]. The ECM also influences various aspects of the cell behavior, including proliferation, survival and migration [4]. Injuries to the cartilage tissue result in the loss of ECM proteins and this increase the risk of joint degeneration leading to post-traumatic osteoarthritis. Despite its high durability, the articular cartilage has limited ability to repair itself, mainly due to its avascular nature and inability of mature chondrocytes present in the cartilage to migrate and significantly proliferate [5,6]. In recent years, many cell-based therapies that use cells capable of producing cartilage-specific ECM to repair damaged tissues have been developed. Among these, autologous chondrocyte implantation (ACI) or matrix-induced ACI have been widely used in the clinical setting to overcome the intrinsic constraint of cartilage repair. However, these approaches have limitation in producing repaired tissue with similar mechanical and functional characteristics as the native articular cartilage [7–9]. More recently, further work to explore an alternative source of chondrocytes have been carried out in an attempt to minimize the morbidity associated with autogenic cartilage harvest and the two-stage surgical procedures of ACI [7]. Such effort includes exploring the role of mesenchymal stromal cells in cartilage repair. Mesenchymal stromal cells (MSCs) have emerged as a promising cell source owing to their ability to proliferate and differentiate to multiple cell types such as osteocyte, chondrocyte and adipocyte [5]. Various experimental approaches have been developed to optimize the isolation, expansion and differentiation of these cells *in vitro*. These include using either carrier scaffolds or delivering cells to

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the defect site in conjunction with specific active biomolecules such as growth factors [10]. Currently, there is a resurgence of interest in exploring the potential benefit of natural growth factors such as those in platelet-rich plasma (PRP) to avoid the risk of viral or prion transmission and immunological reaction to non-human proteins present in animal serum usually used for MSC expansion.

PRP contains various growth factors that aid in cell proliferation, differentiation and tissue angiogenesis. Different forms of PRP have been defined based on the method of preparation, one of which is the platelet-rich concentrate (PRC). This preparation constitutes of platelet pellets that are resuspended in sterile phosphate buffered saline (PBS) [11]. Platelet rich preparations have been previously shown to stimulate chondrocyte proliferation and ECM biosynthesis [12] and also enhance the chondrogenic differentiation capacity of MSCs in pellet culture system [13,14]. However, MSCs and chondrocytes cultured in this culture model were found to express higher concentration of chondrocyte hypertrophy marker i.e. collagen type X [15] and proteolytic enzyme i.e. matrix metalloproteinase13 (MMP13) [16]. The presence of these molecules, which is also implicated in the pathogenesis of osteoarthritis, renders this culture model impractical for cartilage repair. Hence, efforts have been directed to explore the use of alternative 3D constructs made of natural polysaccharide, alginate. Whilst culturing cells encapsulated in alginate in PRP-supplemented medium has been shown to successfully induce differentiation of MSCs to chondrocytes, it is unclear to what extent is the contribution of the platelet rich preparation itself to the desired outcome, since the medium was also supplemented with differentiation inducing factors such as glucose, ascorbate and dexamethasone. These external factors could have mainly contributed to the observed enhancement of cellular differentiation [17]. Moreover, it is not known whether the use of PRC alone in serum-free media is more efficacious in inducing chondrogenic differentiation compared to the commercially available chondrogenic medium (CM), since the effects of both have not been directly compared in a single experimental setting. Hence, this study aimed to investigate the effect of PRC alone in inducing chondrogenesis of hMSCs encapsulated in alginate beads, and compare the chondrogenic molecular signature of cells cultured in the presence of PRC alone to those cultured in the commercially available CM.

Methods

Isolation of human mesenchymal stromal cells (hMSC)

This study was approved by the University of Malaya Medical Centre Medical Ethics Committee (UMMC, reference number 967.10). Written informed consent was obtained from each subject prior to the study. Bone marrow was aspirated from six patients undergoing total knee/hip arthroplasty in the hospital. The aspirated bone marrow was added to equal volume of phosphate-buffered saline (PBS; pH 7.2) and layered onto Ficoll-Paque Premium of density 1.073 g/mL (GE Healthcare, Sweden) and centrifuged at 960×g for 25 minutes. The mononuclear cells were then isolated and resuspended in 10 mL of low glucose Dulbecco's modified eagle medium (L-DMEM) and centrifuged again at 645×g for 5 minutes. The supernatant was discarded and the cell pellet obtained was cultured in growth medium (L-DMEM supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin (100 U/mL, Invitrogen-Gibco) and 1% Glutamax (Invitrogen-Gibco, USA) in T-25 tissue culture flasks. Medium was changed every 3 days until the cultures were 80% confluent. The cells were then serially passaged and cells from passage 2 to 3 were used for further experiments. The isolated cells were confirmed to be MSCs based on their potential to differentiate to adipogenic, osteogenic and chondrogenic lineages (Supplementary Figure 1).

Preparation of PRC

Twenty-five milliliters of blood from each six healthy volunteers was collected in ACD tubes. Platelets were prepared using double centrifugation method, as mentioned previously with slight modifications [18–20]. In brief, the whole blood was first centrifuged at 450×g for ten minutes to separate the red blood cells, buffy coat containing the platelets and the white blood cells, and the plasma. The plasma along with buffy coat were pooled in a 50 mL falcon tube. Prostacyclin (PGI_2) was added to prevent transient activation of platelets during the centrifugation and resuspension steps. The pooled plasma was then centrifuged at 1740×g for another ten minutes. The supernatant portion of the plasma was discarded and only the platelet pellet was resuspended in sterile phosphate buffered saline (PBS, pH 7.2), at 1/10th the initial blood volume. This preparation was referred to as platelet rich concentrate (PRC). The amount of platelets in PRC and whole blood were determined using Sysmex XE 5000 hematology analyzer.

Preparation of hMSC- alginate constructs

hMSC in alginate beads were prepared by resuspending the cells in 1.2% low-viscous alginate (Sigma-Aldrich, St. Louis, MO) in 0.15 M sodium chloride as previously described by Kamarul et al. Briefly, hMSCs were first detached from the T75 flask surface using TypLE^{TM} express (Invitrogen) and resuspended in sterile alginate at cells densities of 1×10^6 cells/mL to form about 22 alginate beads, each containing approximately 4.5×10^4 cells. The cell suspension was dropped into a 102 mM calcium chloride solution using a micropipette tip. The resulting beads were washed with 0.15 M sodium chloride after 10 min of polymerization [21]. Two beads were transferred into each well of a low attachment 24-well plate (Corning® Costar®, USA) and cultured in DMEM low glucose medium containing 1% Glutamax, 1% penicillin/streptomycin and supplemented with either 10% FBS or 15% PRC. Medium was changed every 4 days.

Morphological analysis of hMSC- alginate constructs

Scanning electron microscopy was used to verify the porosity of the alginate bead, which would allow the transfer of growth factors from media into the 3-dimensional construct, as well as facilitate encapsulation of hMSCs in the alginate scaffold. Both the alginate beads alone and hMSCs encapsulated in alginate beads were viewed using a Scanning Electron Microscope (SEM) (model JEOL JSM-6360, Japan). After 21 days of culture in 10% FBS or 15% PRC, beads were first fixed in 4% glutaraldehyde and then dehydrated in an ascending series of ethanol followed by a mixture of ethanol/acetone in the following ratios: 3:1, 2:2 and 1:3 each for 15 min. The samples were transferred to the critical point drier and mounted onto aluminum stubs with adhesive carbon tapes and sputter coated with gold and subsequently examined by the scanning electron microscope.

In vitro cell proliferation assay

Cell proliferation assay was performed to verify that hMSCs encapsulated in alginate were able to proliferate in a medium containing PRC alone prior to undergoing the differentiation process. Two hMSC-alginate beads were transferred to each well of an ultra-low attachment 24-well culture plates (Corning® Costar®, USA) and cultured in DMEM low glucose

medium containing 1% Glutamax, 1% penicillin/streptomycin and supplemented with 10% FBS. After 48 hours of culture in growth medium, the cells were subjected to a serum reduction of 1% FBS to arrest cell cycle progression for 24 hours. Cells were then cultured in medium supplemented with either 10% FBS (control) or 15% PRC. The medium was changed every 4 days and fresh PRC was added once in four days to the experimental groups. Cell viability was determined at 0, 8, 16 and 24 days using alamarBlue® assay (Life Technologies, CA) according to the manufacturer's protocol. A standard curve of total number of hMSCs versus absorbance was plotted (data not shown) and used to extrapolate the cell numbers in the PRC treated and control samples. All experiments were performed in triplicate and repeated six times.

Quantitative real-time PCR

Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) analysis was performed to compare the relative expression of chondrogenic genes. The hMSC-alginate constructs were cultured in medium supplemented with either 10% FBS, chondrogenic medium (positive control) or 15% PRC. The constructs were washed once in PBS and incubated with 1mL of dissolving buffer (55mM sodium citrate) for 10 min. Cells extracted were centrifuged for 2 min at 20 000×g, washed with PBS, and further centrifuged for 2 min at 40 000×g. Cell pellets were lysed with buffer RLT (Qiagen, Valencia, CA) and total RNA was isolated from the cells at days 0, 8, 16 and 24 using RNeasy Mini Kit (Qiagen) following the manufacturer's recommendations and quantified with a nanophotometer (Implen GmbH, Germany). The measured 260/280 ratio was consistently 2.0 ± 0.1 in all samples. Total RNA was reverse transcribed and gene expression were determined using quantitative real-time PCR kit (Qiagen). One μg of RNA was used to generate cDNA using QuantiTect Reverse Transcription kit (Qiagen) following the manufacturer's instructions. Real-time PCR analysis (CFX96 Real-time system, Bio-Rad) was performed to assess the mRNA levels using QuantiTect SYBR® Green PCR Kits (Qiagen). Each RT-PCR was performed in triplicate for PCR yield validation. After verifying the stable expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), it was used as the housekeeping gene. Data were analysed by the $2^{-\Delta\Delta Ct}$ method, with normalization to the Ct value of GAPDH. Gene expression levels at each time point was expressed relative to the expression of hMSCs cultured in FBS. The forward and reverse primers used for the experiment are shown in Table I.

Table I. List of primers used for Q-PCR.

Gene	Sequence	Length (base pair)
COL II F	AGCCCTGCCGGATCTGTGTCTGT	23
COL II R	TGGCGAGGTCAGTTGGGCAGA	21
COL X F	CCACCAGGCATTCCAGGATTCC	22
COL X R	CCGGTGGGTCCATTGAGGCC	20
TNC F	CAGCCAAACCCACCTCCACCAT	22
TNC R	GCCGGATGACTTTCTTGAGGACC	23
ACAN F	ATCCCGCTACGACGCCATCTG	21
ACAN R	GCTCCATGTCAGGCCAGGTCACT	23
RUNX2 F	CCGCCATGCACCACCACCT	19
RUNX2 R	CTGGGCCACTGCTGAGGAATTT	22
COMP F	GCGCCAGTGCCGTAAGGACA	20
COMP R	CGTCCGTGTTGCGCTGGTCT	20
SOX9 F	AGGCGGAGGCAGAGGAGGC	19
SOX9 R	GGAGGAGGAGTGTGGCGAGTCG	22
GAPDH F	GCCCCCTCTGCTGATGCCC	19
GAPDH R	GGGTGGCAGTGATGGCATGGA	21

Immunocytochemical staining for chondrogenic ECM markers

Immunocytochemical staining for chondrogenic markers collagen type II, aggrecan and COMP were performed at day 8, 16 and 24. hMSC-alginate beads cultured in medium supplemented with 10% FBS or 15% PRC or standard chondrogenic medium (CM) were washed once with PBS and fixed with 4% paraformaldehyde for 30 min. It was then washed again with PBS, and stored in PBS containing 10mM $CaCl_2$ to prevent gel dissolution. Hydrogel beads that were frozen on dry ice were embedded in Optimum Cutting Temperature Compound (OCT) (ThermoScientific, Denmark). The samples were then sectioned into (5μm) thickness using a cryotome FSE Cryostats (Thermo Scientific, Denmark) and mounted on polysine coated microscope slide (Thermo Scientific, Denmark). The samples were kept in −20°C until further processing. The slides were fixed in ethanol and then washed with 10mM $CaCl_2$ in PBS for immunostaining. The samples were incubated with 1% bovine serum albumin (BSA) and 2% goat serum in PBS for 20 minutes at room temperature to block nonspecific binding. Samples were then incubated with primary antibodies specific against collagen type II (Abcam, England), aggrecan (ACAN) (Abcam) and cartilage oligo matrix protein (COMP) (Abcam) at 1:100 dilutions in 1% BSA in PBS overnight at 4°C. Samples were washed thrice in PBS and incubated with 1:400 Alexa-Fluoro 488 goat anti-mouse secondary antibody (Abcam) for 1h at room temperature. The nucleus was counterstained with DapiNucblue® Fixed Cell Ready Probes® (Invitrogen, USA). Controls were prepared following the same procedure but without the primary antibody. Images of the samples were captured using fluorescence microscope (NikonEclipse TE2000-S, Japan).

Quantitative ELISA for quantitative determination of chondrogenic ECM markers

After 8, 16 and 24 days, two hMSC-alginate constructs cultured in medium supplemented with 10% FBS or 15% PRC or standard CM, from each time point, were washed with PBS and the cells were retrieved by incubating the constructs in 55 mM sodium citrate. The cells were then lysed in papain (Worthington) for 3 hours at 60°C and the concentrations of collagen type II (USCN, Cloud-Clone Corp, USA), aggrecan (DIAsource Immuno Assays, Belgium) and COMP (R&D system, USA) in the supernatants were measured with human enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions.

Glycosaminoglycan (GAG) quantification

After 24 days of chondrogenic induction, the cell pellets were collected and papain-digested at 60°C. Total sulfated glycosaminoglycan (GAG) was then quantified by the 1,9-dimethylmethylene blue-based Blyscan GAG Assay Kit (Biocolor, Carrickfergus, United Kingdom). The result was normalized with the total amount of DNA, determined separately by the Quanti-iT™ PicoGreen®dsDNA assay kit (Life Technologies, USA) according to the manufacturer's protocol.

Statistical analysis

Values are expressed as mean ± standard deviation. The differences between groups were analysed using a non-parametric test

(Kruskal-Wallis). If values were significant, Mann Whitney U tests were performed to evaluate the level of significance between the groups. Differences were considered to be significant at $p < 0.05$. Data were analysed with SPSS software version 17.0 (IBM Corp., Armonk, NY, USA).

Results

Microstructures of hMSC encapsulated in alginate beads

The microstructures of alginate beads with or without encapsulated hMSCs are shown in Figure 1. Alginate hydrogel without cells (Figure 1A) had an intrinsic porous structure with submicron size pores, which would facilitate the diffusion of nutrients. Cells encapsulated in alginate and cultured in PRC (Figure 1C1) and those cultured in FBS medium (Figure 1B1) were both spherical in shape similar to that of chondrocytes. Cross-sectional view showed that the cells were distributed within the pores of the alginate beads cultured in both FBS medium (Figure 1B2) and PRC supplemented medium (Figure 1C2).

Platelet yield and hMSC proliferation in PRC-supplemented 3D-culture

The PRC yielded an average of approximately four fold higher platelet count ($1157.00 \pm 92.37 \times 10^3$ platelets/μL) than that in the whole blood ($263.71 \pm 22.83 \times 10^3$ platelets/μL) ($p = 0.021$). There was a significant increase in cell proliferation rate in the PRC group ($p < 0.05$) compared to the control at days 8 and 16. Proliferation of hMSCs in the PRC groups reached a peak at day 16, and declined thereafter (Figure 2).

Chondrogenic molecular signatures of hMSCs cultured in PRC vs. chondrogenic medium

Changes in the chondrogenic gene expression in the PRC-treated cells and standard chondrogenic medium (CM) are shown in Figure 3. The cells in both PRC and CM groups expressed transcription factor *Sox 9* and Tenascin C (*TNC*) as early as day 8, followed by the expression of other markers, collagen type II (*COL II*), aggrecan (*ACAN*) and cartilage oligomeric matrix protein (*COMP*). At the later time point, the expression of *Sox9* in the PRC-treated cells was significantly higher compared to CM-treated cells. The gene associated with early stages of chondrogenesis, *TNC*, was expressed about1.3-fold higher in the PRC group compared to CM group at day 8 ($p < 0.05$). The gene expression of *COL II, COMP* and *ACAN* increased throughout the experimental duration, and by day 24, it was significantly higher in cells cultured in PRC compared to CM ($p < 0.05$). The expression of the hypertrophic markers, collagen type X (*COLX*) and runt-related transcription factor 2 (*Runx2*), was significantly lower in the PRC group compared to the CM group day 24 ($p < 0.05$). In general, the increase in the expression of chondrogenic markers in the PRC group compared to the standard chondrogenic medium was minimal.

Qualitative and quantitative evaluation of ECM proteins by immunocytochemical staining and ELISA

Cells treated with PRC exhibited a more intense staining for cartilage specific ECM macromolecules, collagen type II, COMP and ACAN compared to cells cultured in CM, indicating a greater ECM protein deposition in this group (Figure 4). The cells cultured in FBS supplemented medium (control) stained negative for all the three markers with only slight staining for collagen type II at day 24.

The concentration of the ECM proteins Col II, ACAN and COMP determined by ELISA on the cell lysate were significantly higher ($p < 0.05$) in the PRC group compared to the CM group (Figure 5). By day 24, the concentration of ACAN in the PRC group increased to 72.19 ± 4.11 ng/mL whilst that of the control was 24.28 ± 2.64 ng/mL and the CM group was 57.90 ± 3.11 ng/mL ($p < 0.05$). The results show that PRC induced significantly

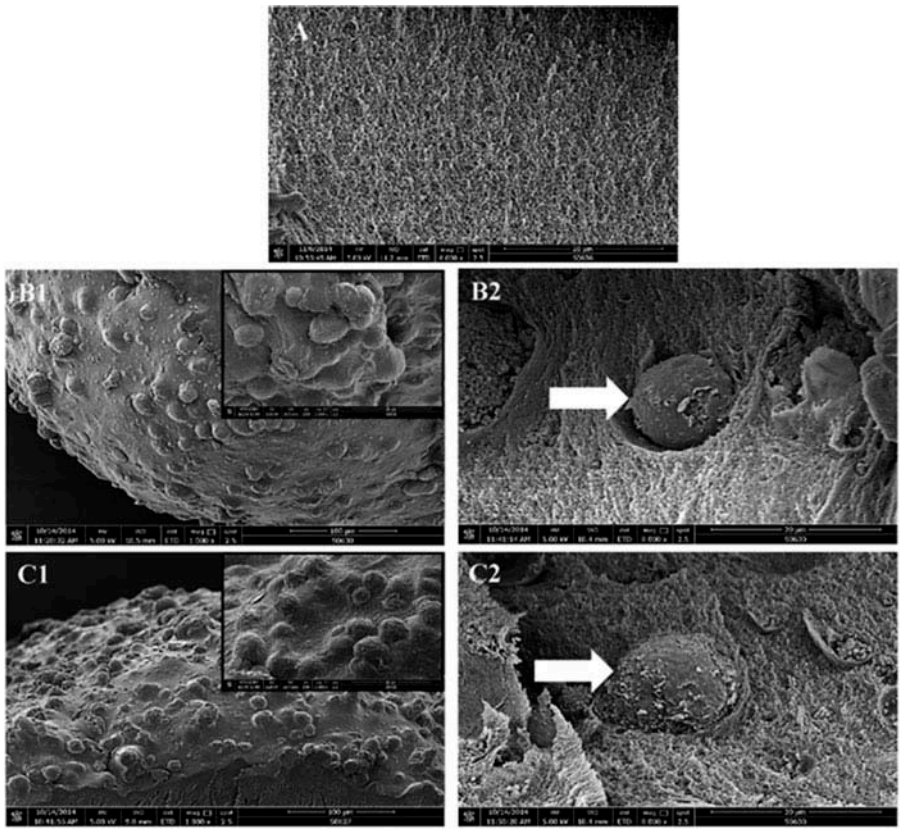

Figure 1. Scanning Electron Microscope Images. Alginate bead without cells had a porous structure (A). Cells encapsulated in alginate cultured in FBS medium (B1), (B2) and in PRC supplemented medium(C1), (C2) at 24 days post-seeding show a spherical morphology.The arrows point to the cells in the alginate bead. Scale bar of images B1 and C1 is 100 μm and B2 and C2 is 20 μm.

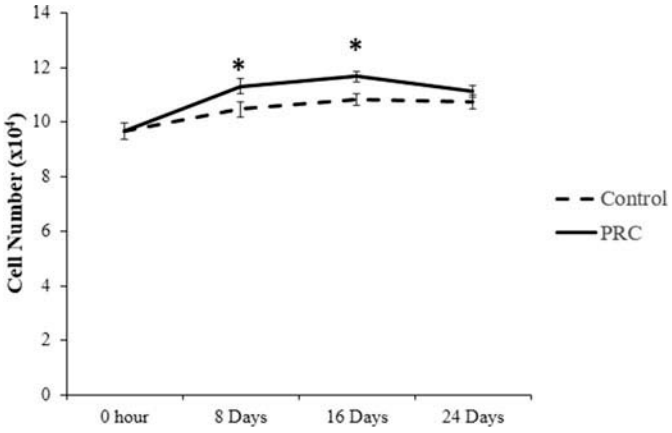

Figure 2. Proliferation of hMSCs encapsulated in alginate. Proliferation of hMSCs in medium supplemented with 15% PRC was higher compared to cells cultured in 10% FBS (control). Data are represented as the means ± SD. *p < 0.05 denotes statistical significance between the two groups.

higher synthesis of ECM proteins compared to the chondrogenic medium. However, the differences were minimal.

Glycosaminoglycan (GAG) concentration

The GAG/DNA ratio, which showed GAG production by individual cell, gradually increased in both the PRC-supplemented medium and CM, peaking at day 16 (Figure 6). GAG/DNA concentration in the PRC group was significantly higher at day 16 and day 24 compared to the CM group ($p < 0.05$). By day 24, the ratio of GAG/DNA was 308.71 ± 41.40 ng/ng in the PRC-treated cells whilst the cells cultured in the CM had a GAG/DNA level of only 237.70 ± 38.42 ng/ng ($p < 0.05$).

Discussion

This study showed that PRC alone in serum-free medium is effective in inducing chondrogenesis of human mesenchymal stromal cells without the need for addition of external chondrogenic differentiation factors in the media. This study highlights 2 major benefits of PRC as a chondroinductive medium for providing abundant pool of chondrogenic MSCs for application in cartilage tissue engineering. Firstly,

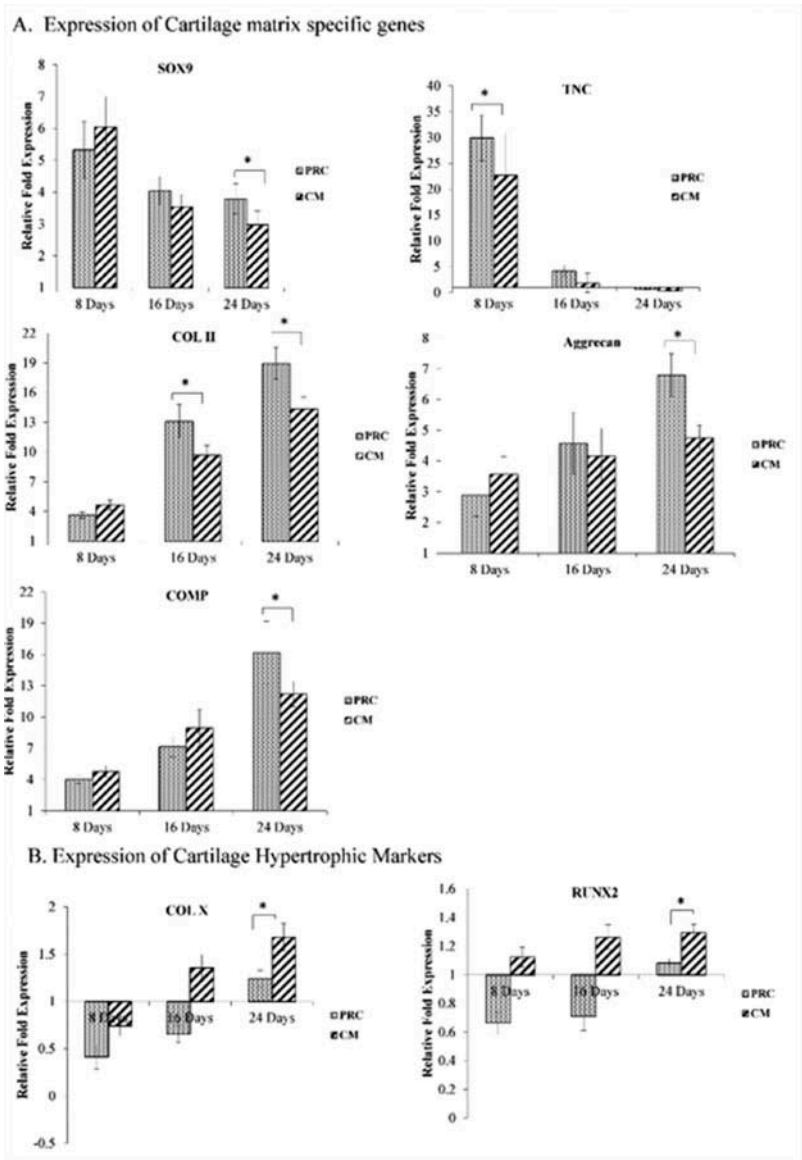

Figure 3. Quantitative polymerase chain reaction analysis of genes expressed in hMSC cultured in PRC and chondrogenic medium (CM). (A) Expression of cartilage extracellular matrix markers show significantly higher TNC level, an early chondrogenic marker, in the PRC group at day 8, while expression of other chondrogenic markers (i.e. SOX9, COL2A1, ACAN, and COMP) in hMSCs cultured in PRC-supplemented medium were significantly higher compared to the standard CM by day 24. (B) Gene expression of hypertrophic markers COL10A1 and RUNX2 were expressed at significantly lower levels in the PRC group compared to CM by day 24. Results are expressed as fold change relative to the control (FBS medium). Data are represented as the mean ± SD. *p < 0.05 denotes statistical significance between the indicated pairs.

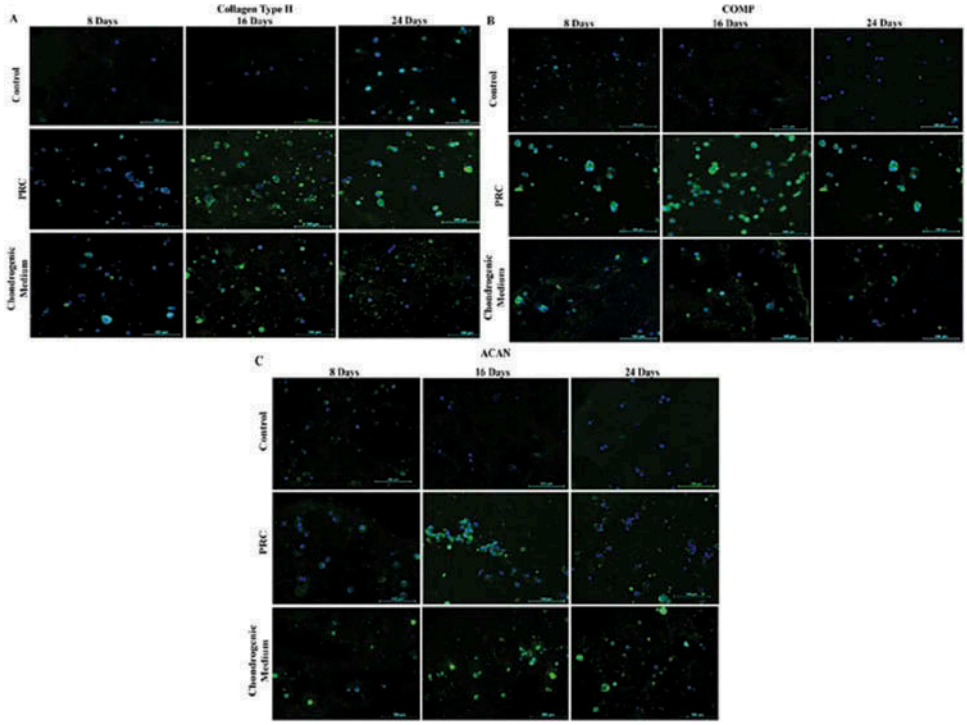

Figure 4. Immunocytochemical staining of cells encapsulated in alginate. Qualitative evaluation of chondrogenic differentiation by immunocytochemical staining of cryostat sections of alginate beads for COL II (A), COMP (B) and ACAN (C) at 8, 16 and 24 days, shows stronger staining of cells cultured in PRC-supplemented medium. Protein depositions are stained with FITC (green) and nuclei are counterstained with DAPI (blue). Results are representative of three independent experiments. Scale bar: 100 μm.

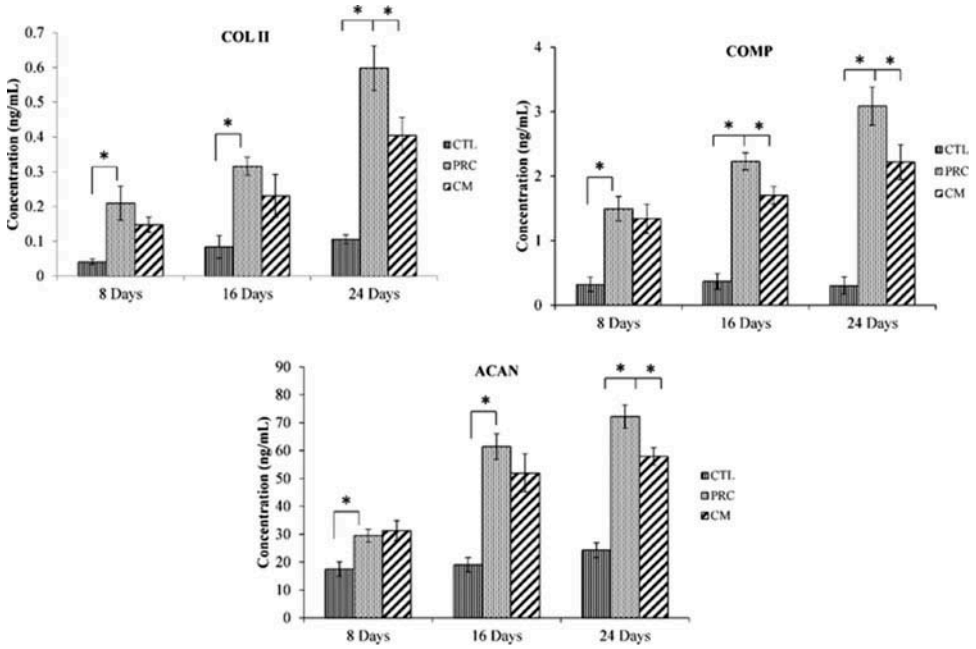

Figure 5. Quantification of ECM proteins by ELISA. Concentrations of proteins were significantly higher in cells cultured in PRC-supplemented medium. Data are represented as mean ± SD. *$p < 0.05$ denotes statistical significance between the indicated pairs.

PRC appears to be equally effective as the standard commercially available chondrogenic medium in inducing proliferation and differentiation of hMSC to chondrogenic lineage. This is reflected by a slightly higher expression of cartilage-specific markers, both at the gene and proteins levels, in cells treated with PRC compared to CM. Secondly, the use of PRC seem to be associated with less likelihood for development of bone ossification in the engineered cartilage tissue, as reflected by significantly lower expression of the hypertrophic markers in the PRC group compared to the CM group.

PRC increased cell proliferation, which peaked at day 16. However, there was a decline in cell proliferation beyond that time, which could be attributed to various factors. This includes replicative senescence, whereby cells lose their potential to proliferate after a certain number of cell divisions, a phenomenon

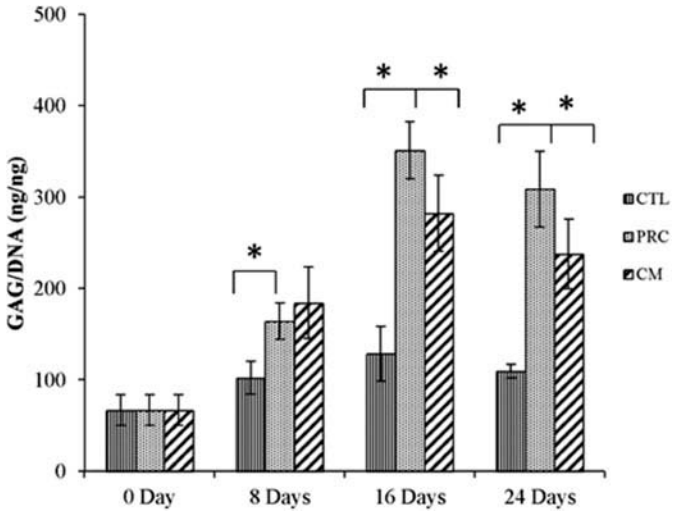

Figure 6. Concentration of GAG in different groups. Synthesis of GAG was significantly higher in cells cultured in the PRC-supplemented medium compared to FBS-supplemented medium and chondrogenic media (CM) from day 16 onwards. Data are represented as the mean ± SD. *p < 0.05 denotes statistical significance between the indicated pairs.

referred to as the hayflick limit [22,23]. Increased cell numbers could also result in contact dependent inhibition of cell proliferation [24,25]. The decrease in cell proliferation after day 16 in the PRC group as seen in our study corroborates with a previous study that showed a gradual decline in cell numbers in the PRP treated group after day 6. The cells were observed to have undergone morphological transformation and showed signs of cellular differentiation beyond day 6 [26], which could have contributed to the decrease in cell numbers. Thus, the transition to active cellular differentiation, which would arrest further proliferation, could also explain the observation in the present study. Another factor that may account for the decline in cell proliferation is poor diffusion of nutritive substances, particularly to the cells at center of the alginate beads. A previous study has shown an increase in the number of dead cells population in the center of 3D pellets, which has been speculated to be due to reduced access to nutrients and oxygen [27].

Under the influence of PRC, the cells followed the normal temporal pattern of chondrogenesis, with early expression of *Sox9* and *TNC*, followed by increasing levels of other cartilage matrix-specific markers such as *COL II, COMP* and *ACAN*. The observation that PRC group had significantly higher expression of these proteins as well as GAG synthesis suggests the presence of potent chondroinductive factors in PRC. Platelets are known to contain a cocktail of growth factors in their alpha-granules including but not limited to the transforming growth factor β (TGFβ), insulin like growth factor (IGF) and basic fibroblast growth factor (bFGF), which are known to promote chondrogenesis of MSCs. Previous studies have shown that MSC pellet culture treated with TGFβ1 decreased collagen type I and increased collagen type II expressions [28]. IGF-1 has been shown to induce chondrogenesis in adipose-derived stem cells [29], and bFGF was also found to enhance the chondrogenic differentiation of MSCs [30]. We speculate that the presence of these growth factors in PRC may be the main contributing factor towards the enhancement of chondrogenic differentiation by PRC seen in our study. Our results also showed that PRC not only induces chondrogenesis of hMSCs but also delays their transition to the hypertrophic state, as reflected by reduced expression of hypertrophic genes, *Col X* and *Runx2*, in the PRC compared to CM group. The presence of hypertrophic phenotype seen in MSC-derived chondrocytes would be of a great concern for MSC applications in cartilage repair therapies, since the ideal repaired tissues should be hyaline cartilage that does not normally undergo hypertrophy. Hypertrophy in the neocartilage could ultimately lead to apoptosis, vascular invasion and ossification as sometimes seen in osteoarthritis [31]. In a similar study by Mardani et al., the effect of PRP on adipose derived stem cells was compared with TGFβ1 supplemented medium. While their findings showed a significant increase in the expression of *Col X* (about 45 fold) by day 14, our results showed only a slight upregulation of the chondrocyte hypertrophic marker (about 1.8 fold) by day 24. This variation in the results could be due to the difference in the cell culture technique and the composition of the culture medium. In the study by Mardani et al., the cells were cultured in a transwell and the medium was supplemented with differentiation inducing supplements like dexamethasone [32], and both of these factors are known to contribute to the induction of cell hypertrophy [33,34]. In contrast, we used alginate as a scaffold, which could have contributed to the considerably lower magnitude of upregulation of *Col X* gene expression in our study compared to that reported previously. Whilst cells in both PRC and CM groups were encapsulated in alginate, the presence of a cocktail of chondroinductive growth factors in PRC could have contributed to the further reduction in the expression of the hypertrophic genes. Studies have shown that PRP reduced myocyte hypertrophy *in vivo* [35] and synovial hypertrophy in primary OA patients [36]. However, the specific growth factor in platelets involved in reducing hypertrophy is not known and remains to be defined. Collectively, our results suggest that the use of PRC as a chondroinductive adjunct might be beneficial in cartilage tissue engineering applications, as it increases the synthesis of cartilage-specific ECM and also decreases the expression of hypertrophic genes.

Despite our attempt to produce a robust experimental design, this study has a limitation worth noting. There was a technical limitation related to the immunocytochemical staining, which precludes accurate sampling at the same site at each time point of assessment. This could have resulted in a lower number of cells from a particular section of the alginate bead being included at a specific time point of assessment. This may have explained the discrepancy in results between day 16 and 24, and also between the immunocytochemical and ELISA results. Immunocytochemical images provide qualitative data that are only representative of the whole sample. Nevertheless, it can be used to complement the ELISA, which on the other hand, provides a more accurate and reproducible quantitative measure since it involves digestion of the entire cells for protein determination.

To date, only one study has investigated the effect of PRP in inducing chondrogenesis of canine marrow stromal cells encapsulated in alginate. However, the cells were encapsulated in beads made of a mixture of alginate and PRP. Apart from the need for additional external supplementation of PRP to the culture medium, the encapsulated cells were also exposed to other differentiation inducing supplements such as dexamethasone, proline and ascorbate [17]. In this instance, the extent of contribution of PRP alone to the chondrogenic differentiation cannot be deduced due to the presence of other confounding factors. In this study, we cultured hMSCs encapsulated in alginate in medium supplemented with only PRC, which is advantageous as it reveals the magnitude of effect of PRC alone on hMSC differentiation without the confounding effect of other substances in the standard chondrogenic medium.

We believe that the alginate hydrogel in our model helps in the controlled delivery of the platelet growth factors supplemented in the medium to the encapsulated cells. Since alginate has been shown to be porous, the encapsulated cells in alginate would still be able to respond to the growth factors and cytokines known to affect chondrocyte metabolism available in the culture medium [37]. Alginate hydrogels are also lacking in ligands recognizable by the cell surface receptors, thus limiting their interaction with the cell matrix, helping the cells to maintain a spherical chondrocytic cell phenotype mimicking the microenvironment of chondrocytes in cartilage tissue [38,39].

The mechanism of action of PRC in enhancing chondrogenesis was not delineated in the present study, as this was not the primary objective of the study. Further studies on the effect of individual growth factors in PRC on hMSC chondrogenesis would be of great interest in obtaining a greater insight on the mechanism of action of PRC. Nevertheless, this study is valuable in determining the merit of using PRC alone to differentiate hMSCs for enhancement of cartilage repair.

In conclusion, PRC appears to be equally as effective as the commercially available chondrogenic medium in enhancing chondrogenesis of the hMSC. PRC also renders benefit in delaying chondrocyte hypertrophy in the differentiated cells. Altogether, the results of this study suggest that the use of PRC alone could be a valuable adjunct for obtaining sufficient pool of pre-differentiated cells to accelerate cartilage repair process.

Declaration of interest

The authors report no declarations of interest.

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