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Effect of cell density on formation of three-dimensional cartilaginous constructs using fibrin & human osteoarthritic chondrocytes

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***Background & objectives*: Seeding density is one of the major parameters affecting the quality of tissue-engineered cartilage. The objective of this study was to evaluate different seeding densities of osteoarthritis chondrocytes (OACs) to obtain the highest quality cartilage.**

***Methods*: The OACs were expanded from passage 0 (P0) to P3, and cells in each passage were analyzed for gross morphology, growth rate, RNA expression and immunochemistry (IHC). The harvested OACs were assigned into two groups: low (1×10^7 cells/ml) and high (3×10^7 cells/ml) cell density. Three-dimensional (3D) constructs for each group were created using polymerised fibrin and cultured for 7, 14 and 21 days *in vitro* using chondrocyte growth medium. OAC constructs were analyzed with gross assessments and microscopic evaluation using standard histology, IHC and immunofluorescence staining, in addition to gene expression and biochemical analyses to evaluate tissue development.**

***Results*: Constructs with a high seeding density of 3×10^7 cells/ml were associated with better quality cartilage-like tissue than those seeded with 1×10^7 cells/ml based on overall tissue formation, cell association and extracellular matrix distribution. The chondrogenic properties of the constructs were further confirmed by the expression of genes encoding aggrecan core protein and collagen type II.**

***Interpretation & conclusions*: Our results confirmed that cell density was a significant factor affecting cell behaviour and aggregate production, and this was important for establishing good quality cartilage.**

Key words Cartilage - chondrocytes - collagen-fibrin - osteoarthritis - seeding density

Chondrocytes in articular cartilage are embedded within an extracellular matrix (ECM) containing collagen, proteoglycan and non-collagenous proteins. Normally, articular cartilage undergoes continuous remodelling to replace degraded matrix. However, ageing and cartilage injury cause overexpression of matrix metalloproteinase which shifts the balance towards matrix degradation[1] and ultimately leads to osteoarthritis (OA)[2]. The first human chondrocyte transplantation was performed in 1994 to induce regeneration[3,4]. However, expansion of chondrocytes in two-dimensional (2D) environment leads to dedifferentiation evident by morphological change, increased expression of collagen type I and reduced expression of collagen type II and aggrecan[5]. Interestingly, dedifferentiated chondrocytes were able

to re-differentiate in three-dimensional (3D) culture environment[6].

The success of 3D constructs for cartilage tissue regeneration depends on the composition of the culture medium, surrounding environment during expansion and tissue construction[7]. It is well known that chondrogenic properties of chondrocytes can be maintained in 3D cell spheroid[4]. Collagen and alginate scaffolds have been extensively used in tissue engineering of cartilage. However, collagen was reported to induce chondrocyte dedifferentiation, while the formation of alginate scaffolds is difficult to control and inconsistently stimulates the ECM production[2]. In contrast, fibrin is biocompatible and non-toxic and can be autologous, thus suitable for tissue reconstruction[8,9]. Fibrin was known to promote the migration and proliferation of cells[10]. Thus, this study was aimed to evaluate the effect of the seeding density of osteoarthritis chondrocytes (OACs) on fibrin construct.

Material & Methods

Isolation and culture of chondrocytes: Following approval by the Universiti Kebangsaan Malaysia Research and Ethics Committee (UKM 1.5.3.5/244/FF-2014-215), and obtaining written informed consent six human OA cartilage samples were collected from Universiti Kebangsaan Malaysia Medical Centre, Kuala Lumpur, Malaysia, from consenting patients (54-69 yr) undergoing total knee arthroplasty surgery. Cartilages were collected from the lateral and medial parts of femoral condyle of OA patients' varus knee (Fig. 1). Cartilage samples were diced and digested using collagenase type II (Gibco, USA). The isolated cells were resuspended in chondrocyte growth medium (CGM) with an initial seeding density of 5000 cells/ml. The CGM was prepared according to the previous method[11,12]. CGM was replaced every 2-3 days. The total cell number and viability were recorded until passage 3 to determine the growth rate (cells/day/cm^2).

Cell cycle analysis of monolayer cultured chondrocytes: Flow cytometric analysis was performed using FACScan (Becton Dickinson, San Jose, CA, USA), and CELLQuest software (Becton Dickinson) with doublet discrimination was used to analyze the DNA content of freshly isolated and cultured OAC. Cell suspensions (n=6) were centrifuged at 400×*g* for five minutes, and pellet was washed and incubated with propidium iodide according to a previous method[11]. For each sample, 12,000 gated events were collected, and cell cycle analysis was performed using Modfit LT software (Verity Software House, Topsham, ME, USA).

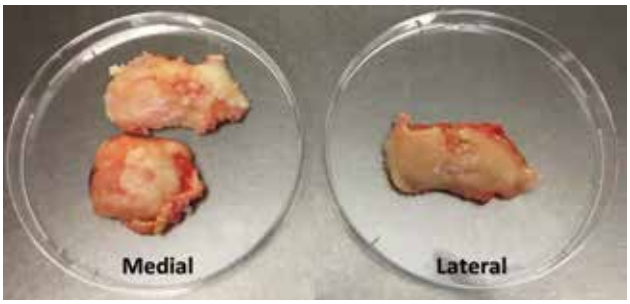

Fig. 1. Gross view of the collected samples.

Preparation of plasma-derived fibrin (PDF): Blood (5 ml) was collected from human volunteers (N=6) into vacutainer tubes containing sodium citrate, and the blood and anticoagulant were carefully mixed followed by centrifugation at 2500 × *g* for 10 min. The plasma layer was aspirated and centrifuged to remove the remaining insoluble matter. Plasma-derived fibrin (PDF) was stored at −80ºC for future use.

Formation of the osteoarthritis chondrocyte-fibrin (OACF) constructs: The OACs at 1×10^7 and 3×10^7 cells/ml were added to 1 ml PDF, and the mixture was polymerized with 250 mM calcium chloride ($CaCl_2$). The resulting gel-like osteoarthritis chondrocyte-fibrin (OACF) constructs were cultured for 7, 14 or 21 days. The constructs were used for histological and biochemical analyses.

RNA extraction and real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR): Total RNA was extracted from OAC monolayer and OACF construct using TRI Reagent (Molecular Research Centre, Cincinnati, USA) according to the previous method[12]. The yield and purity of the extracted RNA were determined by a NanoDrop™ 2000 spectrophotometer (Thermo Scientific, Wilmington, DE). Two-step qRT-PCR for chondrocytes specific genes; aggrecan, type I and type II collagen were performed using iScript™ cDNA Synthesis Kit followed by amplification using iQ™ SYBR Green® Supermix (BioRad, Hercules, CA, USA) on MyiQ™ iCycler Real-Time PCR Detection System (BioRad) as per kit instructions. Relative quantification of the gene expression was performed using the ΔCt method[13]. Housekeeping gene glyceraldehyde phosphate dehydrogenase (GAPDH) was used for internal

Table . List of primers for real-time polymerase chain reaction

Gene	Sequence
GAPDH	F: TCCCTGAGCTGAACGGGAAG
	R: GGAGGAGTGGGTGTCGCTGT
Type I collagen	F: AGGGCTCCAACGAGATCGAGA
	R: TACAGGAAGCAGACAGGGCCA
Type II collagen	F: CTATCTGGACGAAGCAGCTGGCA
	R: ATGGGTGCAATGTCAATGATGG
Aggrecan	F: CACTGTTACCGCCACTTCCC
	R: ACCAGCGGAAGTCCCCTTCG

GAPDH, glyceraldehyde phosphate dehydrogenase; F, forward; R, reverse

normalization. The primer sequences (Biobasic, Markham, Ontario, Canada) are shown in the Table.

Histological and immunochemistry (IHC) analyses: Histological and immunochemistry (IHC) analyses of the OACF construct were performed at 7, 14 and 21 days. Gross observation was conducted prior to fixation. The constructs were fixed with 10 per cent formalin, embedded in paraffin and cut into 5 µm slices. For histological analysis, samples were stained with haematoxylin (Sigma-Aldrich, St. Louis, Mo., USA), fast green (Sigma-Aldrich) and Safranin O (Sigma-Aldrich) to visualize collagen deposition. For IHC, the sections were pre-treated with proteinase K (Dako Cytomation, Ely, UK) and peroxidase blocker (Dako Cytomation) prior to incubation with the primary antibodies (mouse anti-human collagen type I and II; Abcam, Cambridge, MA, USA), horseradish peroxidase-labelled polymer conjugated to goat anti-mouse Ig (DAKO Cytomation) and peroxidase substrate 3,3'-diaminobenzidine (DAB; DAKO Cytomation) sequentially. The sections were counterstained with haematoxylin and mounted in a glycerol gel (DAKO Cytomation).

Sulphated glycosaminoglycan (sGAG) production assay: All OACF constructs were digested with a papain digestion solution (125 µg/ml papain, 5 mM L-cystein, 100 mM Na_2HPO_4 and 5 mM EDTA; *p*H 6.8) at 60°C for 16 h. Sulphated glycosaminoglycan content was analyzed using a 1,9-dimethylmethylene blue (DMMB) assay (Biocolor, Belfast, UK). A 20 µl aliquot of each sample was pipetted into the microplate reader and added with 200 µl DMMB. Samples were analyzed immediately by measuring the absorbance at 525 nm.

Statistical analysis: The data were analyzed using two-way ANOVA and Tukey's multiple comparison tests.

Results

The characteristics of OACs were evaluated by their morphology, growth rate and expression of matrix proteins. OAC cultured in CGM preserved the small polygonal morphology throughout the passage (Fig. 2A). The OAC growth rate was significantly (P<0.05) increased from P0 (7144±786.6 cells/day/cm^2) to P1 (14363±1279 cells/day/cm^2), and then subsequently decreased until P3. The growth rate of OAC at P3 (8548±968.2 cells/day/cm^2) was significantly (P<0.05) lower than that of P1. Comparison of growth rate between patients in each passage showed no significant difference (Fig. 2B). Results from the qRT-PCR showed that OAC expressed aggrecan and type I and II collagen during the subculture (Fig. 2C). Type II collagen was highly expressed by OAC in all passages relative to type I collagen and aggrecan. The expression of type II collagen was significantly higher at P0 (1.82±0.10) compared to that of subsequent passages. A similar trend was observed for type I collagen expression; however, type I collagen expression at P0 showed no significant differences with any other passages except P3 (0.70±0.02). There were no significant differences for aggrecan expression between the passages. Gene expression for patients in each passage also showed no significant differences. The changes in the expression of type I and type II collagen were further confirmed by results from the immunostaining analysis (Fig. 2D). Prominent staining for type II collagen was observed at P0 but showed weaker staining in subsequent passages until P3. In contrast, type I collagen staining was high for all the passages.

Cell cycle analysis: The results from the cell cycle analysis suggested that cultured OAC maintained 100 per cent diploidy with no evidence of aneuploidy, haploidy or tetraploidy (Fig. 3A). The proportion of cells in G1 at P0 was 96.53±0.67 per cent, which significantly decreased to 88.1±0.80 per cent at P3 (P<0.05). The G2 phase index significantly increased from 1.53 per cent at P0 to 5.2 per cent at P3 (P<0.05). The index for the S phase was slightly increased at P3 compared to other passages; however, this was not significant (Fig. 3B).

Effect of cell density on in vitro OACF constructs: The OACF constructs were prepared by culturing

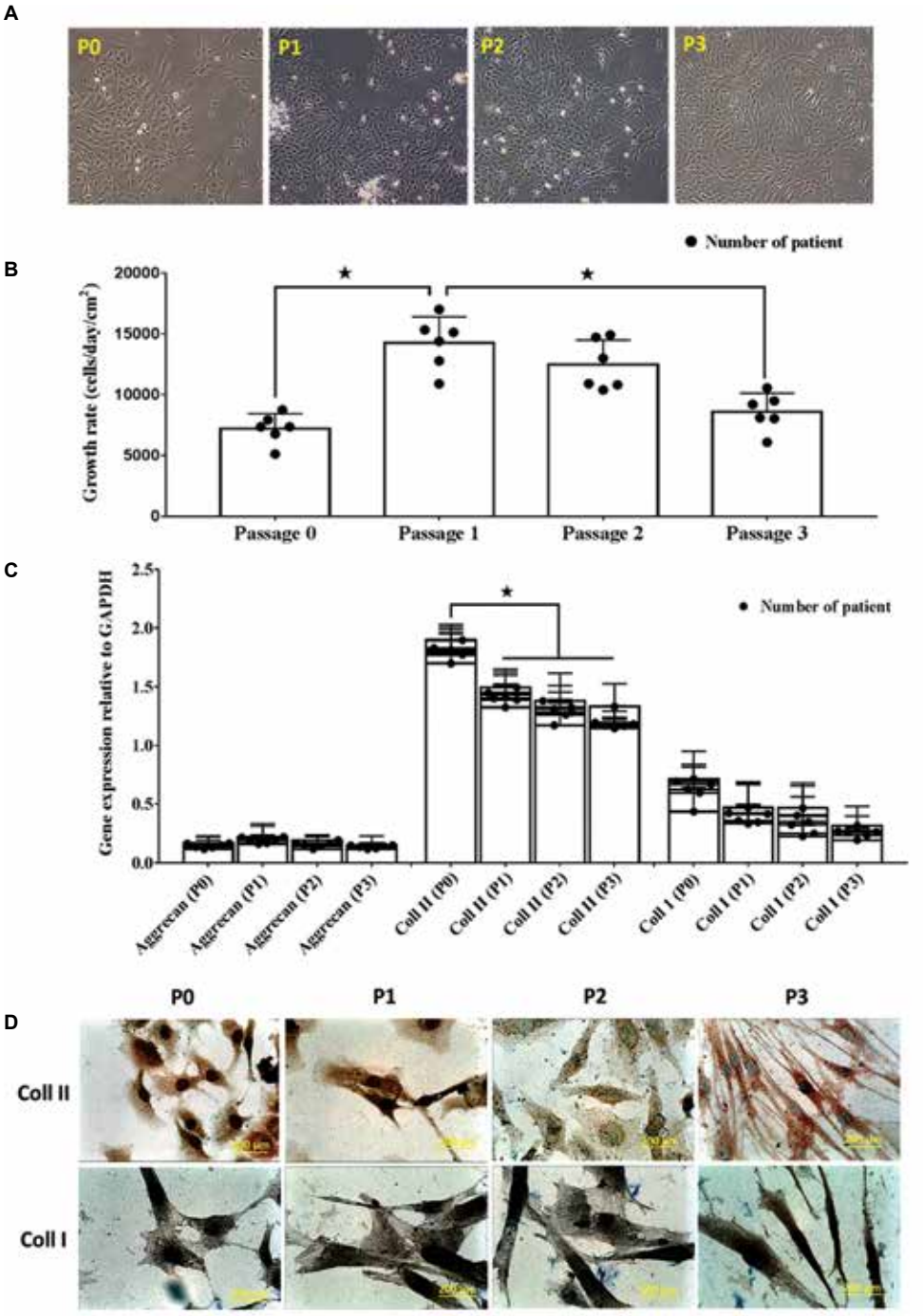

Fig. 2. (A). Photomicrographs (×40) of human osteoarthritis chondrocytes at day 7 from P0, P1, P2 and P3. After seven days of *in vitro* culture, all chondrocytes demonstrated polygonal morphology. (**B**) The growth rate of cultured chondrocytes decreased over successive passages **P*<0.05. (**C**) Gene expression for collagen types I and II reduced after several passages, whereas aggrecan gene expression was consistently expressed. (**D**) Prominent staining of collagen type II in P0 detected by immunocytochemistry, which became weaker by P3. Collagen type I staining was observed for all passages.

1×10^7 or 3×10^7 cells/ml with PDF. Gross observation revealed that all constructs formed stable 3D cartilage-like structures, with a smooth, white and glossy appearance (Fig. 4A and 4B). However, the texture, size and shape of the constructs changed as culture progressed. As shown in Fig. 4C, the diameter of the OACF constructs decreased with time for both cell densities, but the value was comparatively higher for

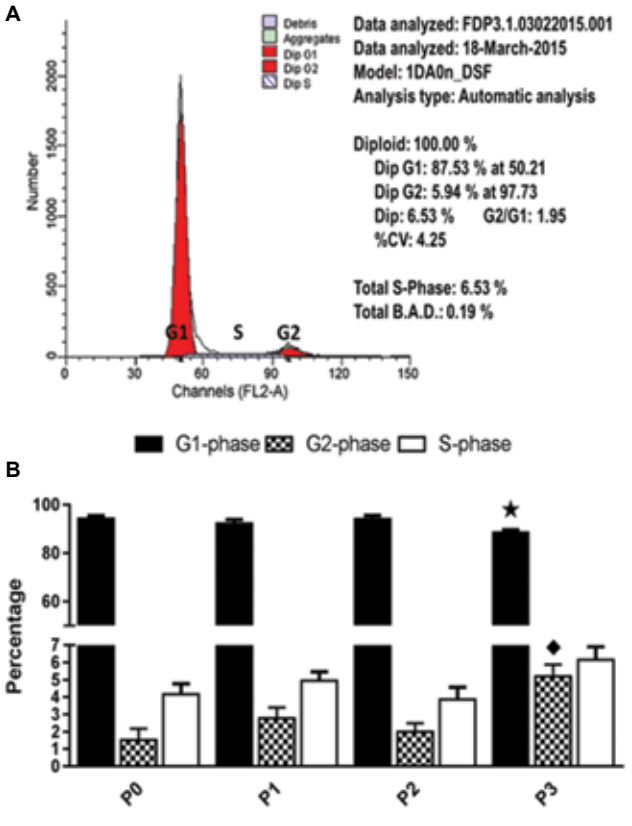

Fig. 3. (A) Cytofluorometry with propidium iodide staining showing diploid DNA content in cultured chondrocytes, suggesting that cultured chondrocytes maintained 100 per cent diploidy in the defined chondrocyte growth medium, with no evidence of aneuploidy, haploidy or tetraploidy. The X-axis represents the relative fluorescence intensity proportional to the DNA content. (**B**) Percentage of cells in the G1, G2 and S phases over successive passages. S phase index indicated that chondrocytes were actively proliferating during monolayer cultivation $P<0.05$ compared to P0.

high-density constructs than low-density constructs. The diameter of high-density OACF constructs was significantly ($P<0.05$) higher at days 14 (8.85±0.09 mm) and 21 (6.98±0.06 mm) compared to that of low-density constructs. There were no significant differences of diameter from patient to patient in each evaluated time point. *In vitro* Safranin O staining of both OACF constructs at day 7 demonstrated a homogeneous distribution of cells, with no sign of lacunae and minor accumulation of proteoglycan (Fig. 4A and B). At days 14 and 21, however, good distribution of cells embedded within the basophilic ground substance was detected, with matrix protein accumulation observed in both groups. To confirm the spatial distribution of cells in the constructs, fluorescence-labelled chondrocytes were used to create low- and high-density OACF constructs. In both OACF constructs, the number of cells increased with time, also demonstrating homogeneous distribution (Fig. 4D and E). In low-density OACF constructs, cells were found to form loose aggregates. In contrast, chondrocytes in high-density OACF constructs exhibited dense aggregates at day 21, consisting of both rounded and spindle-shaped cells. In addition, the chondrocytes formed a thick layer at the periphery of the OACF constructs.

Production of ECM by OACF constructs: The production of type I and II collagen and aggrecan was evaluated to determine the quality of the *in vitro* OACF constructs. Low- and high-density OACF constructs demonstrated the expression of aggrecan and type I and II collagen (Fig. 5A). However, the expression of type II collagen was comparatively higher in both low- and high-density constructs compared to that of type I collagen and aggrecan. In addition, the high-density OACF constructs showed significantly higher expression of type II collagen compared to low-density OACF constructs at each time point ($P<0.05$). The expression of type II collagen in high-density OACF constructs showed a significant increase with increasing culture time, with type II collagen expression at day 21 being 1.71- and 1.44-times higher than that at days 7 and 14, respectively. In the low-density seeded OACF constructs, type II collagen was expressed almost equally at each time point. In contrast, the expression for aggrecan and collagen type I showed no significant differences between the low- and high-density OACF constructs. No significant difference was found between patients for gene expression either within time point or seeding density. Immunostaining observation revealed that, at day 7, both groups (Fig. 5B and C) demonstrated low expression of type II collagen. However, by days 14 and 21, higher type II collagen expression was observed in both OACF constructs. Unlike type II collagen, the expression of type I collagen did not differ throughout the culture period. In addition, as shown in Fig. 5B proteoglycan synthesis gradually increased with increasing cultured periods for both OACF constructs. High-density OACF constructs produced more proteoglycan compared to that of low-density constructs; however, no significant differences were observed.

Discussion

Several studies have demonstrated that OACs have similar characteristics as normal cartilage cells and proven to have more mesenchymal progenitor cell (MPC) properties[2,14,15]. One study has shown a

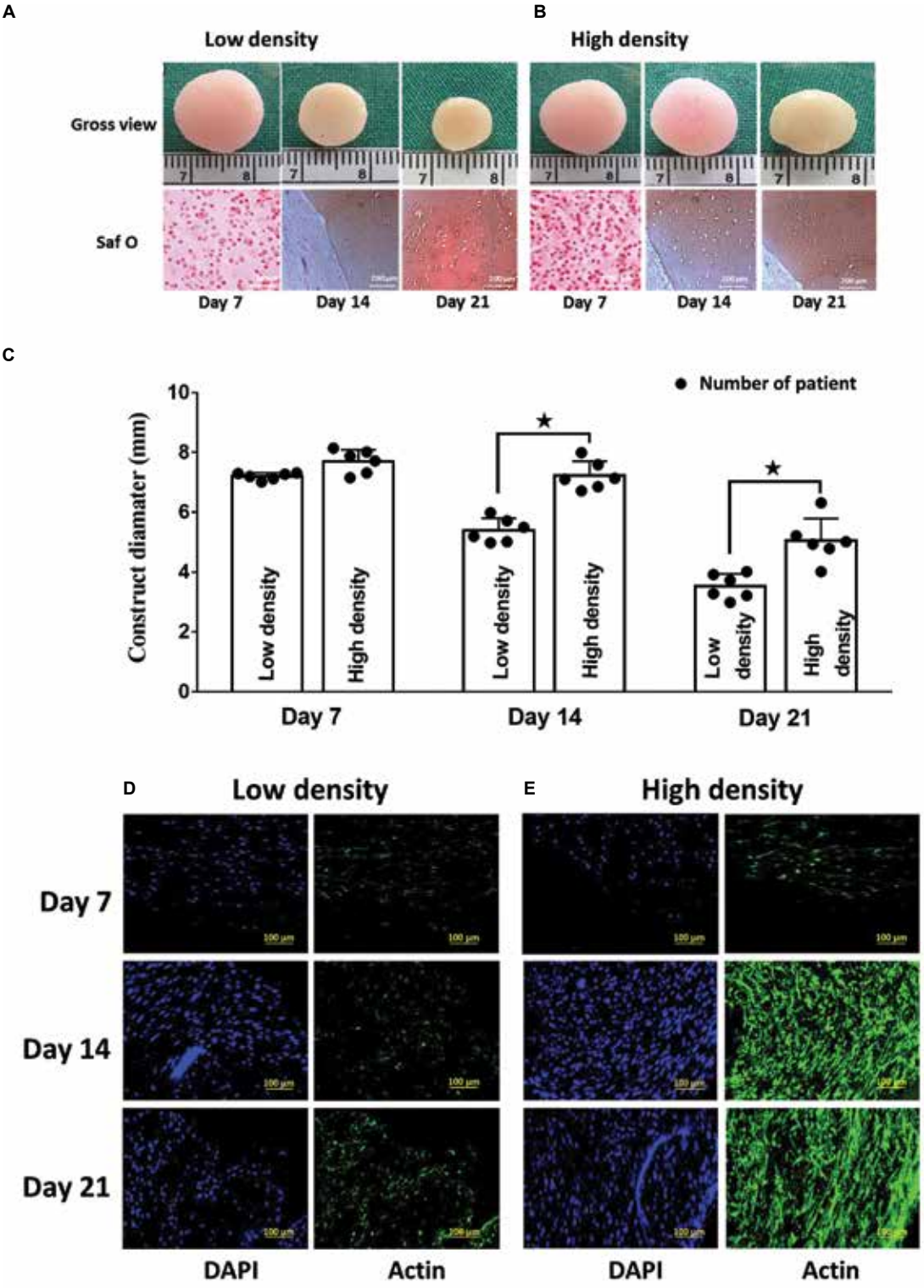

Fig. 4. (**A&B**) *In vitro* osteoarthritis chondrocyte-fibrin constructs derived from cells seeded at low and high densities at days 7, 14 and 21, showing cells of various shapes and sizes. Positive Safranin O (magnification: 200µm) staining confirmed the presence of cartilage-specific proteoglycan (**C**). The diameter of the high-density seeding construct was significantly bigger compared to that of the low-density construct at days 14 and 21 ($^{*}P<0.05$), with a difference of 2.1 and 1.6 mm, respectively (**D&E**). Image showing the cell distribution and aggregates in the fibrin construct seeded with chondrocytes at low and high densities at different time points (day 7, 14 and 21).

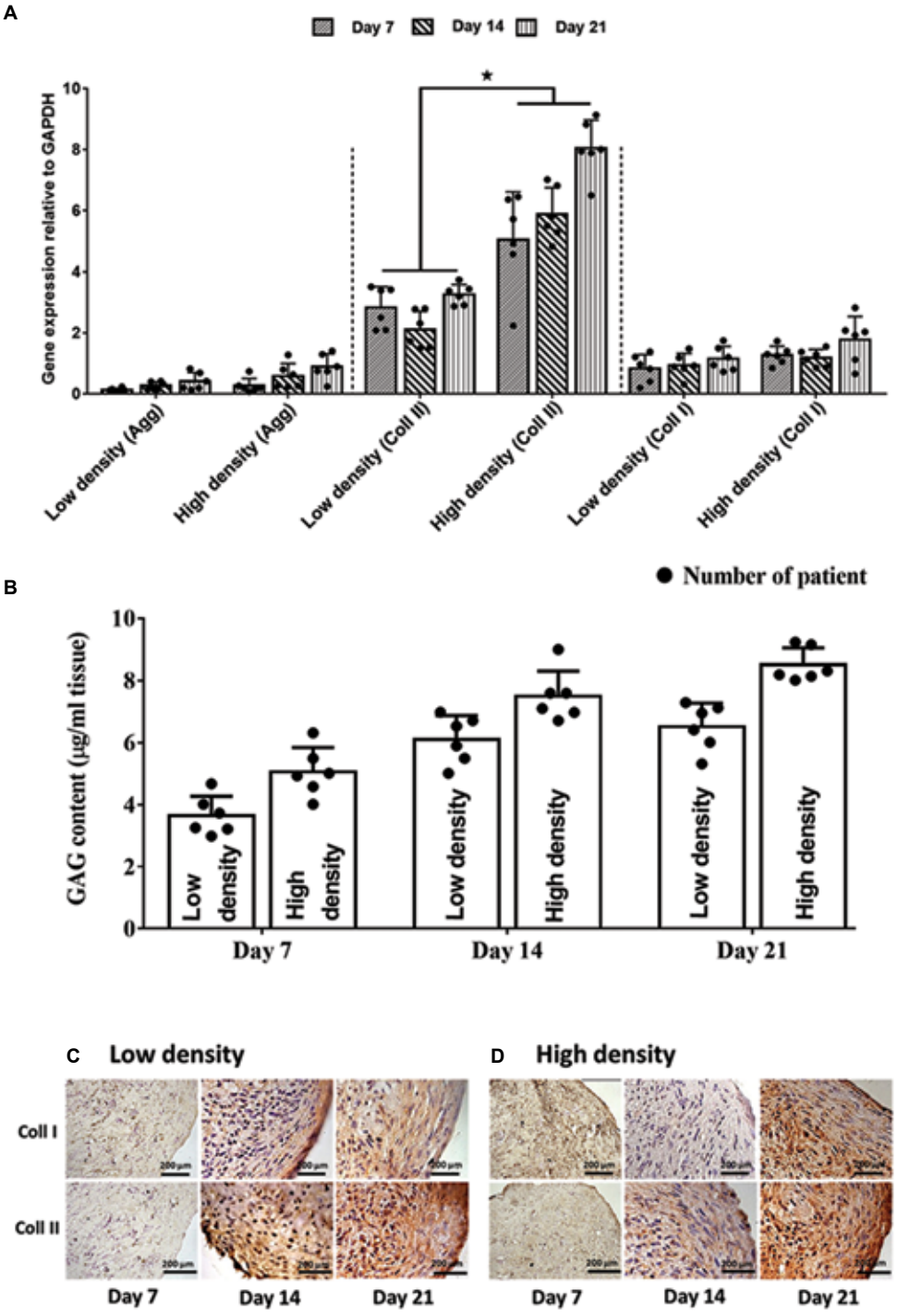

Fig. 5. (A) Significant ($^{*}P<0.05$) expression of collagen type II (coll II), particularly in high-density seeded cells. All evaluated genes showed progressive expression throughout the culture period. Constructs showed strong expression of collagen type II in the pericellular matrix area and throughout the extracellular matrix, confirming an immature cartilage phenotype at days 14 and 21 for both groups glyceraldehyde phosphate dehydrogenase (GAPDH) and glycosaminoglycan (GAG) (**B**). Proteoglycan deposition showed no significant differences between constructs (**C&D**). Immunohistochemistry staining. Collagen type I and II (coll I & II) deposition was also detected in both constructs at day 7, 14 and 21 in low and high seeding chondrocytes fibrin construct.

significant increase of CD105+/CD166+ MPC markers in OAC[15]. However, no concrete evidence was reported on the effect of cell density of abnormal chondrocytes on cartilage formation. Thus, the present study was

aimed to evaluate the effect of OAC seeding densities in the fibrin scaffold for cartilage regeneration.

In cartilage tissue engineering, the major challenge is to produce enough chondrocytes with limited starting materials and dedifferentiation of cultured chondrocytes. A previous study showed that combination of transforming growth factor-β2 (TGF-β2), insulin-like growth factor-1 (IGF-1) and basic fibroblast growth factor (bFGF) effectively improved the proliferation of chondrocytes and cartilage matrix production[16]. The use of IGF-1 and bFGF induced cell proliferation but was less effective without TGF-β. The use of a defined CGM containing ITS, bFGF, IGF-1 and TGF-2 supports the growth and chondrogenic properties of OAC. Based on the cell cycle analysis, it was found that aneuploidy or tetraploidy of cultured OAC was consistent with that of previous cytofluorometry studies that used normal auricular chondrocytes[17]. Although rapid cell expansion was observed, the results of the cell cycle analysis suggested that there were no changes in the cell cycle properties.

In this study, fibrin derived from human plasma was used as a scaffold to form stable constructs. It fulfils the criteria of being controllable degradation rate, non-toxic, biocompatible and stimulates cell proliferation and ECM production[9]. As shown in histology and immunofluorescent staining, fibrin held the embedded cells together, in addition to promoting cell attachment, proliferation and ECM secretion. It was similarly observed during the earlier evaluation of fibrin as a natural 3D scaffold that promotes cartilage regeneration[18-20]. It was hypothesized that the cell density would facilitate cell-cell and cell-matrix interactions, in addition to promoting chondrogenesis within the transplanted cells. In this study, two different densities, 1×10^7 and 3×10^7 cells/ml, of OAC were encapsulated within a fibrin gel. The *in vitro* OACF constructs for both high- and low-seeding densities demonstrated a similar gene expression pattern to that of chondrocytes following serial passage. Type II collagen was highly expressed in the high-density OACF constructs when compared to the low-density constructs, suggesting that the high-density seeding constructs favoured the formation of a cartilage-specific phenotype. Although both low- and high-density constructs were of the same size, histological analysis showed that the high-density OACF constructs were superior, displaying histologic architectural features consistent with normal cartilage, and the results were consistent with a previous study[21].

The expression of aggrecan and collagen type I in low- and high-density seeding constructs may be due to the accumulation of protein during the development of articular cartilage. In this study, the engineered cartilage was newly formed, and therefore showed characteristics of immature tissue. Chondrocytes adopt a fibroblast-like morphology in monolayer culture, accompanied by increased proliferation and an altered phenotype[22]. This might have occurred to a certain degree but was not sufficient to affect the properties of chondrocytes used as the cell source. This could explain why the constructs were stained positive for Safranin O. After 21 days of *in vitro* culture, there was no difference in GAG quality between constructs with a cell seeding density of 3×10^7 and 1×10^7 cells/ml. The diameter was slightly larger for the high-density constructs compared to the low-density constructs.

Overall, our results support the hypothesis that chondrogenic process starts in the areas of cell condensation. Cell-scaffold construct showed homogeneous cell distribution, and chondrogenic activity was found to be higher with a seeding density of 3×10^7 cells/ml. *In vitro* experiments with OAC seeded to human fibrin showed a linear relationship between biological activity and the number of seeding cells, and it was similarly claimed by other researchers[23,24]. In conclusion, higher seeding density (3×10^7 cells/ml) significantly enhanced ECM production and biochemical properties of human OAC on fibrin scaffolds compared to that of lower seeding density.

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

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