Protection of the Differentiated Chondrocyte Phenotype during Monolayer Expansion Cultures Using Heat Inactivated Fetal Calf Serum

Tat Fong Ng*[1], Mourad Matmati[1], Thomas M. Quinn[1]

[1]Department of Chemical Engineering, McGill University, 3610 University Street Montreal, Quebec, Canada H3A 2B2

ABSTRACT

Introduction: Autologous Chondrocyte Implantation (ACI) is one successful treatment for osteoarthritic defects *(1-4)*. However, chondrocytes undergo a conversion to a fibroblastic phenotype during their expansion *in vitro*. This process can lead to degradation and failure of the repaired tissue, and is termed "dedifferentiation" *(5-9)*. We hypothesized that culturing chondrocytes in Heat Inactivated Fetal Calf Serum (HIFCS) would lead to a slowing of the process of dedifferentiation. This study specifically examines how HIFCS affects gene expression and proliferation of bovine chondrocytes in short-term monolayer culture. **Methods**: We cultured primary chondrocytes extracted from bovine joints in FCS or HIFCS for four passages (P0-P4). We then analysed the relative gene expression of aggrecan (*agg*), type II-alpha1 collagen (*col2a1*), cartilage oligomeric matrix protein (*comp*), SRY (sex determining region Y)-box 9 (*sox9*), type I-alpha2 collagen (col1a2) and Bcl-2-associated X protein (*bax*) using quantitative real-time Polymerase Chain Reaction (qPCR). **Results**: Our results show significant down-regulation of col1a2 and up-regulations of *agg*, *col2a1*, *comp* and *sox9* in HIFCS cultures when compared to those in FCS cultures. **Discussion**: *Agg*, *col2a1* and *comp* are associated with the synthesis of cartilage matrix molecules while *sox9* is associated with chondrogenesis *(5-11)*. *Col1a2* is associated with the presence of fibrous tissue which lacks the desired mechanical properties *(5-9)*. Therefore, our results suggest that HIFCS can partially protect the chondrocytic phenotype from dedifferentiating to fibroblastic after one passage. To improve upon our results, more experiments are required to reveal the mechanism underlying the protection of chondrocytes offered by the heat inactivation of FCS.

KEYWORDS

Autologous Chondrocyte Implantation, articular cartilage, chondrocyte expansion, heat inactivated fetal calf serum

*Corresponding author:
tat.ng@mail.mcgill.ca

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INTRODUCTION

Articular cartilage is a dense connective tissue which ensures smooth motion and proper weight transfer in synovial joints. In adult articular cartilage, which is avascular and aneural, nutrients are mainly transported via diffusion instead of direct delivery by blood vessels *(12)*. After an injury, this system can make the *in vivo* natural repair process very difficult to heal itself because of the inefficient delivery of required nutrients and removal of undesired waste by diffusion. Physical or biochemical damage can alter the chondrocytes' phenotype and lead to the formation of

fibrocartilage, which is a mechanically inferior tissue and can be broken down after prolonged use *(13)*.

To recover the native functionality of the injured articular cartilage, one of the tissue engineering techniques is used to assist the healing process – Autologous Chondrocyte Implantation (ACI) *(14)*. It is the most common surgical treatment for cartilage damage since it is economical and technically simple. In ACI, healthy articular chondrocytes are first extracted from patients and then expanded in monolayer cultures, in which all chondrocytes are growing side by side on the same growth surface and none growing on top of another. This *in vitro* chondrocyte proliferation is repeated for multiple passages to reach a minimum density of 15 to 20 million cells before implantation into cartilage defects *(1, 3, 15)*. There are two advantages to this technique. Firstly, the healthy chondrocytes transferred from other patients might be recognized as foreign cells by the immune system of the host patient, which would then trigger immune defense in the recipient. Therefore, the use of autologous cells prevents such negative immune responses and can provide an active repair process to the joint *(16)*. Secondly, only a small quantity of chondrocytes is needed, and they can be extracted from a non-load-bearing site in the patient *(17)*. As a clinical practice, ACI achieves functional recovery for a majority of patients *(2-4)*. However, a critical drawback of this technique is that chondrocytes dedifferentiate during proliferation, which affects the ability of the implanted chondrocytes to regenerate and serve as a long-term functional replacement for the original tissue *(5-9)*.

Since chondrocytes dedifferentiate during their proliferation in monolayer culture, extensive research has revealed relationships between the degree of chondrocytes' dedifferentiation and their corresponding morphogy, gene expression profile and proliferation rates. *Col2a1* is the major collagen produced by chondrocytes, and it makes up 50% of all protein in cartilage *(18)*. *Col1a2* is the major collagen produced by fibroblasts, and it is presented in scar tissue, dentin, dermis and tendon *(18)*. When chondrocytes attach to the flat surface of a cell culture dish, their cell structure gradually changes from a spherical to a spindle-shaped morphology after multiple monolayer passages *(19, 20)*. In addition, protein production shifts from *col2a1* to *col1a2* and the proliferation rate increases with higher number of passages *(5, 7-9, 10, 21, 22)*. Although the underlying mechanism of this shift is still poorly understood, previous research has eliminated as a possible cause for the relationship between the cell shape and types of collagen synthesized in passaged monolayer cultures of chondrocytes *(23)*. Researchers believe that the shift in types of collagen initiates in the early stages of *in vitro* expansion *(24)*.

In this project, we hypothesized that chondrocytes expanded in HIFCS would maintain their differentiated phenotype, but would dedifferentiate in FCS. Although morphology, genotype, cell growth and protein analysis are all essential to understand the underlying mechanism of decelerated chondrocytes dedifferention, we focused primarily on genotype and cell growth analysis in this study; morphology and protein analysis were performed by other collaborators in the same laboratory. For genotype analysis, gene expression of *col1a2* indicated the degree of dedifferentiation, and the expression of *agg*, *col2a1*, *comp* and *sox9* reflected the extent of differentiation. *Agg* is a major structural component of articular cartilage that facilitates chondrocyte-chondrocyte and chondrocyte-matrix interactions *(25)*. *Comp* is a noncollagenous ECM protein responsible for collagen fibril formation *(26, 27)*. *Sox9* acts as the first high-mobility-group domain transcription factor that is required for chondrocyte differentiation and cartilage formation *(11, 28)*. *Bax* gene expression was used to see whether a decline in cell proliferation and an increase in cell death were due to the extracellular environment, the heat inactivation of FCS, or intracellular activities *(29, 30)*. Hence, the objectives of this study were to compare the relative gene expression profiles and proliferation rates of articular chondrocytes expanded in monolayer FCS or HIFCS cultures during passaging and determine the minimum number of passages required for the clinical application of ACI.

EXPERIMENTAL METHODS

TISSUE HARVEST

Approximately 3-5 g of articular cartilage was first aseptically dissected from each bovine joint provided by a local abattoir. Mixed population of chondrocytes from the superficial and middle zones (top 10-20%) could then be isolated as most of the proliferating chondrocytes were presented in these zones. Chondrocytes were then enzymatically digested from cartilage tissue segments using previously published techniques *(31)*. For each sample, the tissue was minced using a scalpel and digested for 24 h in 20 mL of Dulbecco's Modified Eagle Medium (DMEM) High Glucose 1X (Invitrogen, Grand Island, NY), supplemented with 2 mg/mL type II collagenase (Sigma Aldrich, St. Louis, MO), in an incubator with 5% CO2 at 37°C. The resulting cell suspensions were strained from undigested tissue debris and cell clumps using a 70 μm pore size polyethersulfone mesh, and the cell density was obtained using a Zeiss Axiovert 40°C microscope with a hemacytometer. Chondrocytes obtained at this stage were considered as a control for comparison with passaged chondrocytes.

CELL CULTURE

For each donor, a low density of 1×10^5 chondrocytes were plated in a 100mm×20mm non-pyrogenic sterile polystyrene cell culture dish (Corning Incorporated, Corning, NY) filled with 6mL of FCS or 6mL of HIFCS. The FCS was composed of DMEM

enriched with 10% Fetal Bovine Serum (FBS) and 1% Prostate Specific Antigen (PSA). HIFCS was prepared with the same protocol, except that the FBS used was first heat inactivated at 56°C for 30mins before mixing with DMEM and PSA *(32)*. In addition to the original 6mL of FCS or HIFCS added in the first day of initial seeding, an extra of 3mL FCS or HIFCS were added after 5 days of initial seeding to provide fresh nutrients. After 10 days, near confluence (~50,000cell/cm^2) was achieved *(33)* and cells in both cultures (P0) were isolated by trypsinization using previously published techniques *(34)*. Their cell densities were then obtained by counting the total number of cells in the plastic plate and dividing it by the total volume of FCS or HIFCS added. Then, 1×10^5 P0 chondrocytes were passaged using the same protocol and to the same level of confluency—but for only 5 days due to accelerated proliferation—to obtain P1 chondrocytes. This procedure was repeated a total of four times to obtain P1 to P4 chondrocytes.

RNA EXTRACTION

Samples of ribonucleic acid (RNA) were extracted from control and P0 to P4 chondrocytes expanded in FCS and HIFCS cultures using Invitrogen RNA isolation protocol as recommended by the manufacturer. The RNA samples were then diluted with diethylpyrocarbonate (DEPC)-treated water (Invitrogen, Carlsbad, CA) and stored at -80°C. Diluted RNA solutions were measured at optical density (OD) 260 for total RNA and at OD 280 for total protein readings using the Eppendorf BioPhotometer Plus. The measured concentrations of diluted RNA solutions can be accepted with minimal accuracy as long as they had RNA-to-protein ratios of 1.8 to 2.2, indicating adequate relative purities *(35)*.

cDNA SYNTHESIS

RNA was reversed transcribed into complementary deoxyribonucleic acid (cDNA) using qScript™ cDNA Synthesis Kit (Quanta BioSciences, Gaithersburg, MD) as recommended by the manufacturer. In this procedure, 15 µL RNA and nuclease-free water, 1 µL reverse transcriptase and 4 µL Reaction Mix (5x) were mixed together, reversed transcribed at 42°C for 1h, and stored at -20°C. The synthesized cDNA was amplified using PCR and this process can be verified by obtaining proper fragment size in gel electrophoresis. Glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) was used as an endogenous control *(31, 36, 38)*.

QUANTIFICATION OF GENE EXPRESSION

qPCR is a technique used to simultaneously amplify and quantify the amount of selected DNA sequence presented in a sample. It was performed on a 96-well/plate ABI Prism 7900HT Sequence Detection machine (Applied Biosystems, Foster City, CA) using Standard Cycle SYBR Green qPCR protocol (Quanta Biosciences, Gaithersburg, MD). The total volume (20 µL) of each PCR reaction consisted of 10 µL PerfeCTa™ SYBR™ Green FastMix™ ROX, 7 µL de-mineralized water (ddH2O), 2 µL cDNA, 0.5 µL for each of 20% forward and reverse primers. Table 1 lists the forward and reverse sequences for each primer used (Invitrogen, Carlsbad, CA). Primer sequences for all genes were designed using bovine messenger RNA (mRNA) data published on the National Center for Biotechnology Information (NCBI) website. The qPCR reaction was carried out at 95°C for 10 min (activation), 40 cycles of 95°C for 15 s, 60°C for 20 s and 72°C for 20s (amplification), and 72°C for 1 min (final extension) *(37)*.

RELATIVE GENE EXPRESSION

Gene expression for *agg*, *col2a1*, *comp*, *sox9*, *col1a2* and *bax* were normalized using the endogenous control gene, *gapdh*, so that the variations in the number of cells per sample became insignificant. The2$-T$ method of relative quantification of gene expression was chosen because it relates the PCR signal of the target transcript in a treatment group to that in an untreated control *(38)*. To obtain the value of T from raw T data, we used this equation:T=$(CT,Target-CT,gapdh)Time\ x$-$(CT,Target-CT,gapdh)Time\ 0$, where our targets were the genes *agg*, *col2a1*, *comp*, *sox9*, *col1a2* and *bax*. To fully comprehend why the amount of target can be calculated by finding $2-T$, please refer to derivation and explanation written by Livak and Schmittgen *(38)*. Relative gene expression values greater than 1 indicated that the gene of interest was expressed to a greater level than the endogenous gene. Since the expression value of *gapdh* was assumed to be constant among all the chondrocytes analyzed, the relative expression of the gene of interest could be quantified independently of the amount of RNA extracted from chondrocytes in each sample *(30, 38)*.

Table 1. Primer sequences used for qPCR analysis

Primers	Forward Sequence (5' to 3') Reverse Sequence (5' to 3')
gapdh	ACCCTCAAGATTGTCAGCAA ACGATGCCAAAGTGGTCA
agg	GCTACCCTGACCTTCATC AAGCTTTCTGGGATGTCCAC
col2a1	AACGGTGGCTTCCACTTC GCAGGAAGGTCATCTGGA
comp	TTCGGAACGCACTGTGG TGCAGGACAGCGGTA
sox9	ACGCCGAGCTCAGCAAGA CACGAACCGCTTCT
col1a2	CATTAGGGGTCACAATGGTC TGGAGTTCCATTTTCACCAG
bax	AACATGGAGCTGCAGAGGAT CAGTTGAGTTGCCGTCAGA

STATISTICAL ANALYSIS

A total of 6 groups of experiments were performed, each with a sample size of n=3. Among the 6 groups of experiments, 3 groups were obtained only from chondrocytes without passaging (P0) to determine the baseline effect of HIFCS. The other 3 groups of experiments analyzed all of the passaged chondrocytes (P0 to P4). As a result, a total sample size of n=18 were analyzed for P0 chondrocytes but n=8 were analyzed for P1 to P4 chondrocytes due to a loss of one sample during the first passage. The means and standard error of the mean were calculated using Excel spreadsheet software. Finally, z-test, F-test and t-test were applied to test the significance of the results. P-values less than 0.05 were deemed to be significant.

RESULTS

EFFECT OF HEAT INACTIVATION

In order to test the hypothesis that the heat inactivation of FCS would decelerate chondrocyte dedifferentiation, three analyses were required. The first analysis compared the gene expression of P0 chondrocytes cultured in HIFCS relative to those cultured in FCS. As seen in Fig. 1, the expression of genes *agg*, *col2a1*, *comp* and *sox9* were significantly augmented in HIFCS cultures when compared to that in FCS cultures, while the expression of gene *col1a2* was significantly reduced in HIFCS cultures when compared to that in FCS cultures. In order to assess the effect of heat inactivation of FCS, P0 chondrocytes cultured in HIFCS or FCS was compared to freshly isolated chondrocytes. As shown in Fig. 2, the gene expression of *agg*, *col2a1*, *comp* and *sox9* in HIFCS cultures were significantly decreased when compared to that in FCS cultures; and the expression of gene *col1a2* is significantly increased in HIFCS cultures when compared to that in FCS cultures (all P=0.00). *Agg* gene expression in HIFCS was about 54% higher than that in FCS cultures (P=0.012) while *col1a2* gene expression in HIFCS cultures was about 42% less than that in FCS cultures (P=0.045). As the total yield of P0 chondrocytes was much lower than the minimal requirement for clinical applications, which is about 15 to 20 million cells, multiple passages were essential. The third analysis compared the gene expression of passaged chondrocytes cultured in HIFCS to those cultured in FCS. From Fig. 3, all the gene expression of *agg*, *col2a1*, *comp* and *sox9* were significantly amplified for P0 to P4 chondrocytes in HIFCS cultures when compared to that in FCS cultures, except the *agg* gene expression in P3 chondrocytes. However, when the gene expression of *col1a2* was examined for passaged chondrocytes, only P0 to P1 chondrocytes were significantly declined in HIFCS cultures when compared to that in FCS cultures. Finally, the expression of the bax gene was consistently insignificant.

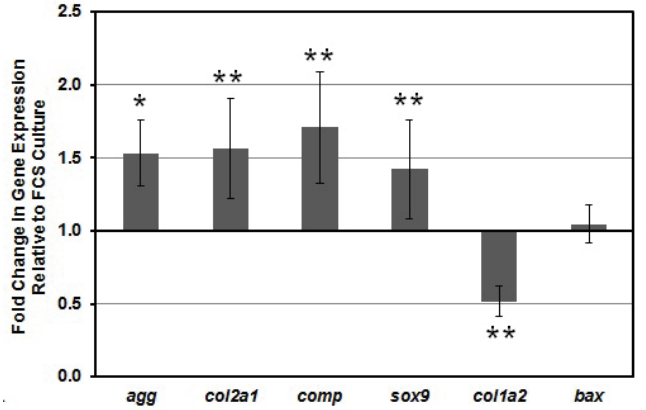

Fig. 1. Gene expression of P0 chondrocytes in HIFCS cultures relative to that of FCS cultures. Values represent mean ± standard error of the mean for n=18. Statistically significant differences are indicated by *=P<0.05 and **=P<0.01.

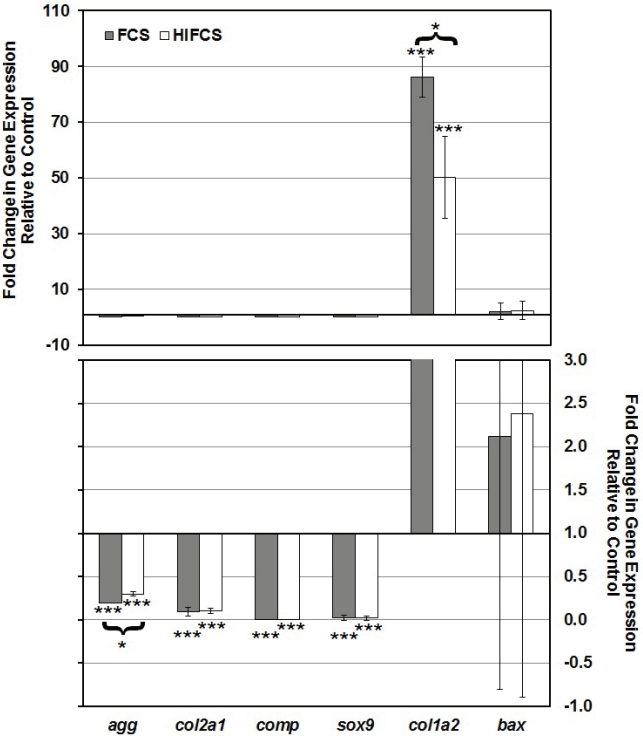

Fig. 2. Gene expression of P0 chondrocytes in FCS and HIFCS cultures relative to that of freshly isolated chondrocytes. Values represent mean ± standard error of the mean for n=9. Statistically significant difference is indicated by *=P<0.05 and ***=P<0.001.

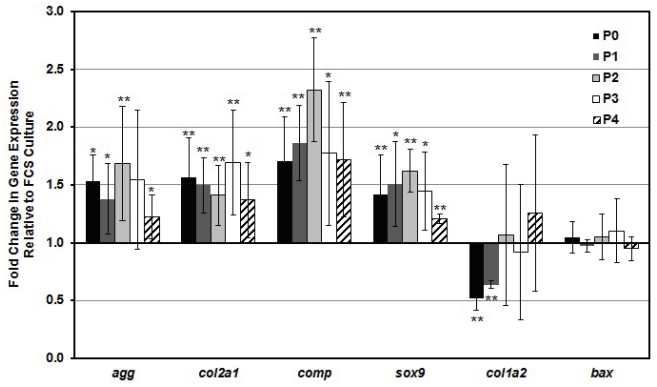

Fig. 3. Gene expression of P0 to P4 chondrocytes in HIFCS cultures relative to that of FCS cultures. Values represent mean ± standard error of the mean for n=18 for P0 chondrocytes and n=8 for P1 to P4 chondrocytes. Statistically significant differences are indicated by *=P<0.05 and **=P<0.01.

CELL GROWTH PROFILE

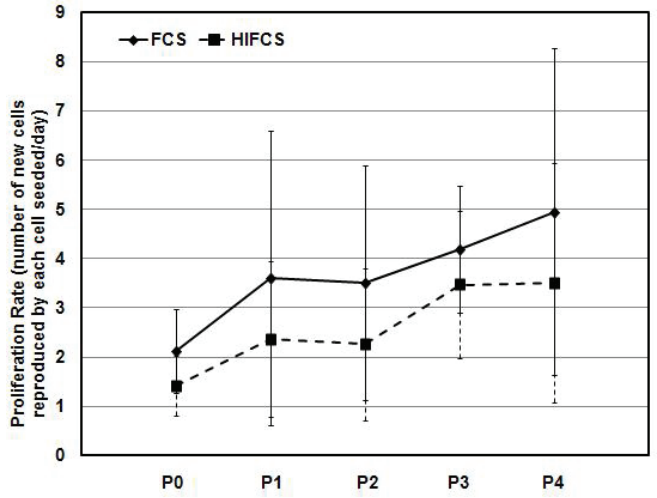

Fig. 4. Proliferation rates (number of new cells reproduced by each cell seeded per day) of P0 to P4 chondrocytes. Values represent mean ± standard error of the mean for n=9 for P0 to P2 chondrocytes and n=8 for P3 and P4 chondrocytes.

After investigating the effect of HIFCS on the genotypes of passaged chondrocytes, the proliferation rate and cumulative cell numbers of chondrocytes expanded in all passages were analyzed to give a cell growth profile. Proliferation rate, which was defined as the number of new cells reproduced by each cell seeded initially per unit of time, was plotted in Fig. 4 for all passaged chondrocytes expanded in FCS and HIFCS cultures. Noticeably, chondrocyte proliferation rate increased with the number of passages in both FCS and HIFCS cultures. Additionally, the proliferation rates of FCS cultures were generally higher than the rate in HIFCS cultures for each passage. Finally, Fig.5 depicts the cumulative cell numbers of P0 to P4 chondrocytes cultured in FCS and HIFCS. It can be observed that the cumulative cell numbers in FCS cultures are always higher than that in HIFCS cultures and this difference is increasing as we passaged the chondrocytes from P0 to P4.

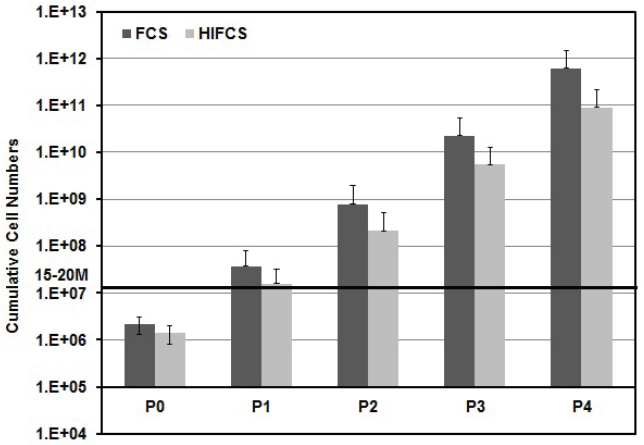

Fig. 5. Cumulative Cell Numbers of P0 to P4 chondrocytes. Values represent mean ± standard error of the mean for n=9 for P0 to P2 chondrocytes and n=8 for P3 and P4 chondrocytes. The horizontal line at 15-20M indicates the minimum number of cells required for clinical treatments of cartilage defects.

DISCUSSIONS

EFFECT OF HEAT INACTIVATION

Fig. 1 reveals the effect of heat inactivation of FCS on chondrocytes by comparing the gene expression of P0 chondrocytes cultured in HIFCS relative to those grown in FCS. The significant up-regulation of chondrocytic genes (*agg*, *col2a1*, *comp*, *sox9*) and significant down-regulation of fibroblastic gene (*col1a2*) in HIFCS cultures when compared to FCS cultures prove that HIFCS was indeed more effective than FCS in protecting the chondrocytes from dedifferentiation. In addition, the insignificant difference between *bax* gene expression levels in FCS and HIFCS cultures might indicate that the heat inactivation of FCS did not cause fatal damage in cultured chondrocytes' organelles, but the confirmation of this statement required repeated experimentation.

The comparison of gene expression of P0 chondrocytes cultured in FCS or HIFCS relative to that of freshly isolated chondrocytes was necessary to determine the degree of dedifferentiation in *in vitro* chondrocyte cultures and provide an indirect assessment of the effect of heat inactivation of FCS. From Fig.2, the significant down-regulation of all chondrocytic genes (*agg*, *col2a1*, *comp* and *sox9*) and strong up-regulation of fibroblastic gene (*col1a2*) shows that the dedifferentiation of chondrocytes was initiated

in the beginning of *in vitro* expansion in both FCS or HIFCS cultures. Again, the relative insignificance of *bax* gene expression might imply that the cell death that occurred in both FCS and HIFCS was mainly caused by the extracellular environment instead of intracellular activities, but more experiments would be needed in order to prove this statement.

The comparison between gene expression of passaged chondrocytes cultured in HIFCS and those grown in FCS cultures demonstrated how multiple passages impacted the effectiveness of HIFCS. In Fig. 3, the consistency of significantly up-regulated chondrocytic genes (*agg*, *col2a1*, *comp* and *sox9*) for P0 to P4 chondrocytes in HIFCS cultures when compared to that in FCS cultures implied that the advantageous protection offered by HIFCS was persistent for four passages. This protection by HIFCS was unaffected by passaging as no increasing nor decreasing trend could be observed in these chondrocytic gene expression. Nevertheless, when the fibroblastic gene expression (*col1a2*) was examined for passaged chondrocytes, the down-regulation of the gene *col1a2* was significant for only one passage. This infers that P2 to P4 chondrocytes are not practical for clinical treatments as the implantation of them will not be functional in patients' joints. Finally, the expression of the *bax* gene was consistently insignificant which might provide additional suggestion that HIFCS did not kill the intracellular organelles of cultured chondrocytes. However, more experiments are needed to reduce the error rate and acquire accurate results in order to draw affirmative conclusion about the effect of passaging on the efficiency of HIFCS for chondrocytes.

CELL GROWTH PROFILE

After investigating the effect of HIFCS on the genotypes of passaged chondrocytes, the proliferation rate and cumulative cell numbers of chondrocytes expanded in all passages were analyzed to give a cell growth profile. Proliferation rate was plotted in Fig. 4 for all passaged chondrocytes expanded in FCS and HIFCS cultures. Noticeably, chondrocyte proliferation rate increased with the number of passages in both FCS and HIFCS cultures. Additionally, the proliferation rates of FCS cultures were generally higher than the rate in HIFCS cultures for each passage. In spite of the fact that higher proliferation rates in FCS cultures were achieved when compared with that in HIFCS cultures in Fig. 4, this difference is statistically insignificant, which might suggest that the slower dedifferentiation rate observed in HIFCS chondrocyte cultures of was not due to lower proliferation rates. This result might be crucial because decelerated dedifferentiation of chondrocytes originating from a lower proliferation rate is scientifically reasonable; any findings that could not rule this out should not be accepted as a new discovery. Lastly, Fig. 5 shows that the chondrocytes needed to be expanded in HIFCS cultures for at least one passage to reach the clinically required minimum of 15 to 20 million cells for ACI therapy.

CONCLUSIONS

This project aims to investigate the effect of heat inactivation of Fetal Calf Serum (FCS) on the process of chondrocytes' dedifferentiation. Bovine articular chondrocytes were isolated and expanded through monolayer cell culture supplemented with either FCS or Heat Inactivated Fetal Calf Serum (HIFCS) for four passages. The study indicates that the addition of HIFCS to the culture media limits the dedifferentiation from chondrocytes to fibroblasts for one passage. Furthermore, we show that one passage creates sufficient amounts of chondrocytes for clinical applications. Nevertheless, our results, while potentially significant, are preliminary. More experiments will be required to determine whether the limited dedifferentiation in HIFCS cultures is originated from lower proliferation of chondrocytes. Moreover, further experiments are necessary to disclose the mechanism underlying the protection of chondrocytes by the heat inactivation of FCS in order to advance successful clinical applications of articular cartilage regeneration.

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