Gene Expression Profiling of Chondrogenic Markers in Cultured Ovine Chondrocytes

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ABSTRACT

This study investigated the ability of ovine chondrocytes to maintain their chondrogenic potential at different passage levels under *in vitro* culture conditions using real-time PCR. Articular cartilage tissue was collected from apparently healthy sheep from a local slaughterhouse. Chondrocytes were isolated from the articular tissue using enzymatic digestion. The isolated chondrocytes were cultured using suitable media and conditions till passage 3. The cultured chondrocytes were characterized by real-time PCR using chondrogenic markers, namely Collagen type – II (COLII), Aggrecan (AGC), and Collagen type – I (COLI). There was a significant ($p < 0.01$) decrease in the mRNA expression of COLII in passaged cells when compared to the primary culture. The mRNA expression of COLI increased with increasing passage number. Thus, from this study, it was evident that ovine chondrocytes get dedifferentiated into a fibroblast phenotype with increasing passage number. This simple and reliable assessment can thus be used to screen cultured chondrocytes for deviation from their chondrogenic phenotype to be used for tissue engineering applications.

Keywords: Ovine chondrocytes, Collagen II, Aggrecan, Real-time PCR

INTRODUCTION

Chondrocytes are the sole cellular constituent of the articular cartilage. Chondrocytes make up 5 – 10 % of the total cartilage volume (Hunziker *et al*., 2002). They are the only source of Extracellular Matrix (ECM) macromolecules such as Collagen type- II and Aggrecan, which are responsible for cartilage homeostasis. After skeletal maturity is attained, the chondrocytes in the articular cartilage stop dividing and only persist. Adding up, cartilage tissue is devoid of a vascular and neural network. Thus, the treatment of cartilage injuries is challenging because of their low self-healing capacity. A multitude of cartilage injuries often progress to permanent degeneration and dysfunction of the joint (Richter *et al*., 2016). Many techniques have been developed for the treatment of cartilage damage. Autologous Chondrocyte Implantation (ACI) is one of the promising treatment strategies that involves culture and expansion of autologous chondrocytes and their transplantation into injured tissue using tissue engineering techniques. However, a major drawback of the approach is that chondrocytes tend to dedifferentiate during *in vitro* expansion and gradually lose their chondrogenic phenotype (Su *et al*., 2012). Thus, a simple yet reliable preliminary method of phenotype confirmation for the evaluation of chondrogenic potential is highly essential. This study, therefore, evaluated the chondrogenic potential of ovine chondrocytes under culture conditions up to the level of P -3 by cartilage-specific markers, namely COLII, AGC and COLI, using real-time PCR.

MATERIALS AND METHODS

The study was carried out in the Department of Veterinary Physiology and Centre for Stem Cell Research and Regenerative Medicine (CSCR&RM), Madras Veterinary College, Chennai. Ovine joint tissue was collected soon after slaughter in Phosphate Buffered Saline (PBS) containing gentamicin (80 µg/ml) and transported to the laboratory, keeping the tissue at 4˚ C with the use of ice packs. The tissue was then washed twice with 1x PBS and once with 1% antibiotic-antimycotic solution. The articular cartilage was gently scraped from either end of the joint with the help of a

sterile scalpel. The cartilage tissue was washed thoroughly with PBS in a Petri dish and then cut into small pieces of approximately 2 x 2 cm. Chondrocytes were isolated from the cartilage tissue by enzymatic digestion using 0.15 % collagenase type II for 18 hrs at 37˚ C and 5% CO_2 (Jesslyn *et al.,* 2022). The isolated chondrocytes were cultured in growth media containing Dulbecco's Modified Eagle Medium (DMEM) and 10% Fetal Bovine Serum (FBS). After the cells reached 80% confluency, they were trypsinized and subcultured with a split ratio of 1:3. The cells were maintained up to passage 3. RNA was isolated from the primary chondrocytes and passaged cells using the Trizol method as described by Jakob e*t al.,* 2003. Subsequently, c-DNA was synthesized from the isolated RNA using iScript™ cDNA Synthesis Kit (Bio-Rad). Quantitative real-time PCR was performed using SYBR Green (Bio-Rad) reagent on a Bio-Rad CFX connect Real-time PCR system. Data was analysed using Bio-Rad CFX Manager Software Version 2.1. mRNA expression was normalized according to the expression of the housekeeping gene Ovine Glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Ovine COL II, Ovine AGC and Ovine COL I were targeted to analyse the expression in primary chondrocytes and passaged cells. The details of primers used in the study are given in Table I. The results of the study were statistically analysed using One-way ANOVA followed by Duncan's *post hoc* test.

Table I: Primers used in Real-time PCR (Ude *et al*., 2014)

Name of the Primer	Primer Sequence
Ovine GAPDH	(F) 5'- CTGGTGCTGAGTACGTGGTG -3'
	(R) 5'- CGTCAGCAGAAGGTGCAGAG -3'
Ovine COLII	(F) 5'- CCTCAAGAAGGCTCTGCTCA -3'
	(R) 5' - ATGTCAATGATGGGGAGACG -3'
Ovine COLI	(F) 5' - CGGCTCCTGCTCCTCTTAGCG -3'
	(R) 5'- CTGTACGCAGGTGACTGGTG -3'
Ovine AGC	(F) 5' - TAGGTGGCGAGGAAGACATC -3'
	(R) 3'– AAACGTGAAAGGCTCCTCAG -3'

RESULTS AND DISCUSSION

This study investigated the ability of cultured chondrocytes to maintain their chondrogenic phenotype at different passages. Real-time PCR analysis was performed to assess the mRNA expression of Collagen type II (COLII) and Aggrecan (ACG) to determine the chondrogenic phenotype and the expression of Collagen type 1 (COLI) to monitor the possibility of chondrocyte dedifferentiation into fibroblast-like cells. The relative mRNA expression of COLII, AGC and COLI is given in Figures 1, 2 and 3, respectively.

Collagen – II

The mRNA expression of COL II showed 1.97-fold, 2.89-fold and 3.25-fold decrease in P1, P2 and P3 when compared to the primary culture. Collagen type II is the predominant type of collagen in cartilage tissue and accounts for 90 per cent of total collagen in hyaline cartilage. Chondrocytes lose their ability to produce collagen II on serial passages in monolayer culture. In the present study, a significant decrease was observed in the expression of collagen II with increasing passage level. In a similar study by Muhammad *et al.* (2019) in human chondrocytes, there was a significant decrease in the level of collagen 2 production between primary chondrocytes and P1 cells. Ma *et al.* (2003) observed that ovine chondrocytes in monolayer culture showed stable expression of collagen II until seventeen days of culture, after which there was downregulation in collagen II production. The downregulation of collagen II is linked to the down-streaming of BMP – 2 when

chondrocytes are cultured in a monolayer. Perturbation of the BMP–2 signalling pathway can aid in the prevention of chondrocyte dedifferentiation and preservation of chondrocyte phenotype (Enomoto-Iwamoto *et al.,* 1998).

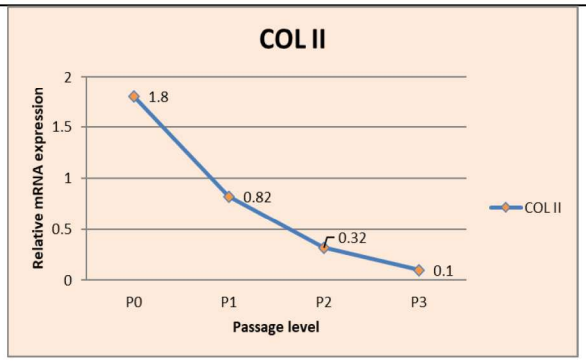

Fig.1. Relative mRNA expression of COLII from P0 to P3

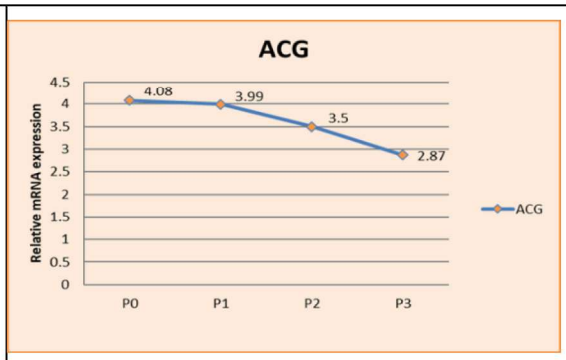

Fig.2. Relative mRNA expression of ACG from P0 to P3

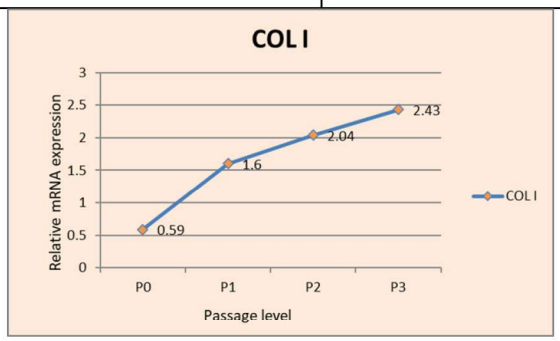

Fig.3. Relative mRNA expression of COLI from P0 to P3

Aggrecan

The mRNA expression of ACG showed 1.06-fold, 1.49-fold and 2.31-fold decrease in P1, P2 and P3 when compared to the primary culture. Aggrecan is a large proteoglycan that bears numerous chondroitin sulphate and keratin sulphate chains and provides articular cartilage with its ability to withstand comprehensive loads. Chondrocytes, when cultured for a longer duration, gradually shift from synthesis of aggrecan to low molecular weight proteoglycans such as versican and a decrease in alkaline phosphatase activity (Roughley and Mort, 2014).

In the study, there was a downregulation of aggrecan expression with increasing passage level, which was parallel to the finding by Muhammed *et al.* (2019) in human chondrocytes. There was a fluctuating aggrecan expression across the three passages, with primary chondrocytes showing the highest expression. However, there was no significant difference in aggrecan expression among various passages. The variation in aggrecan expression is attributed to the lack of three dimensional matix micro-environment in the monolayer culture system (Lin *et al.,* 2008).

Collagen – I

There was upregulation of COL I at different passage levels. The passaged cells exhibited 2.14-fold, 2.73-fold and 3.58-fold increase in the level of mRNA expression at different passage levels when compared to the primary culture. The expression pattern of collagen type I was studied to assess the dedifferentiation of chondrocytes to a fibroblast-like phenotype. Cultured

chondrocytes switch from collagen II to collagen I production with increasing passage levels. In the present study, there was a significant increase in collagen I production across P1 to P3. According to Stewart *et al.* (2000), expression of collagen type I peaked on day 14 of monolayer culture of human chondrocytes. Muhammed *et al*. (2019) reported a significant increase in collagen I expression in P1 when compared to the primary culture. The increased expression of collagen type I may be linked to down regulation of TGF – ß signalling. TGF – ß signalling plays an important role in chondrogenesis by initiating mesenchymal condensation, cell proliferation and preventing terminal differentiation towards hypertrophy. TGF – ß levels were found to be drastically decreased at both gene and protein levels along chondrocyte passages.

SUMMARY

Ovine articular cartilage was cultured up to 3 passage levels in monolayer culture. From this study, it was evident that ovine chondrocytes tended to shift towards a fibroblast phenotype during subsequent passages. When implanted into cartilage defects, the dedifferentiated cells may proliferate to form fibrous tissue that does not possess the mechanical properties of cartilage. Hence, chondrocytes from primary culture and passage 1 are more suitable for tissue engineering approaches as they possess greater expression of cartilage-specific COLII.

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