Isolation and characterization of human articular chondrocytes from surgical waste after total knee arthroplasty (TKA)

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Cartilage tissue engineering is a fast evolving field of biomedical engineering, in which the chondrocytes represent very commonly used cell type in related studies. Since research in tissue engineering always consumes a lot of cells, an efficient and cheap isolation method could form a powerful basis to boost such studies and enable their faster progress to the clinics. Isolated chondrocytes can be used for autologous chondrocyte implantation in cartilage repair, and are the base for valuable models to investigate cartilage phenotype preservation, as well as enable studies of molecular features, nature and scales of cellular responses to alterations in the cartilage tissue. In this study, we developed a protocol for isolation and consequent cultivation of primary human adult articular chondrocytes with the desired phenotype from the surgical waste obtained during total knee arthroplasty (TKA). TKA is a common and very frequently performed orthopaedic surgery during which often both femoral condyles are removed. The latter present the ideal source for an efficient and relatively cheap isolation of chondrocytes. Cartilage specific gene expression of collagen type 2 and aggrecan have shown that the proposed protocol enables the chondrocyte specific phenotype preservation up to the second passage. Collagen type 1 expression was performed to evaluate the dedifferentiation of the isolated cells into a fibroblast-like phenotype.

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Short title: Alternative human chondrocyte isolation

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AUTHOR CONTRIBUTIONS

J.N., L.G., M.V. and U.M. designed the study. J.N., L.G. and M.G. performed the experiments. J.N., L.G. and U.M. wrote the manuscript and prepared the figures. All authors reviewed the manuscript.

ABSTRACT

Cartilage tissue engineering is a fast evolving field of biomedical engineering, in which the chondrocytes represent very commonly used cell type in related studies. Since research in tissue engineering always consumes a lot of cells, an efficient and cheap isolation method could form a powerful basis to boost such studies and enable their faster progress to the clinics. Isolated chondrocytes can be used for autologous chondrocyte implantation in cartilage repair, and are the base for valuable models to investigate cartilage phenotype preservation, as well as enable studies of molecular features, nature and scales of cellular responses to alterations in the cartilage tissue.

In this study, we developed a protocol for isolation and consequent cultivation of primary human adult articular chondrocytes with the desired phenotype from the surgical waste obtained during total knee arthroplasty (TKA). TKA is a common and very frequently performed orthopaedic surgery during which often both femoral condyles are removed. The latter present the ideal source for an efficient and relatively cheap isolation of chondrocytes. Cartilage specific gene expression of collagen type 2 and aggrecan have shown that the proposed protocol enables the chondrocyte specific phenotype preservation up to the second passage. Collagen type 1 expression was performed to evaluate the dedifferentiation of the isolated cells into a fibroblast-like phenotype.

KEYWORDS: human articular chondrocytes, isolation protocol, total knee arthroplasty, TKA, phenotype preservation, gene expression, collagen 2, aggrecan

1 INTRODUCTION

Damage to articular cartilage has important clinical implications since the cartilage tissue possesses a limited intrinsic healing potential and tends to an incomplete regeneration by local chondrocytes, accompanied with an inferior fibrocartilage formation (Camp et al. 2014; McNickle et al. 2008; Richter et al. 2016). Surgical intervention is often the only option, although the repair of damaged cartilage is often less than satisfactory, and rarely restores full function or returns the tissue to its native state (Kerker et al. 2008; Kock et al. 2012; Tuli et al. 2003). Over the past decade a number of viable options of cartilage regeneration have been introduced into clinical practice (Camarero-Espinosa et al. 2016; Hettrich et al. 2008; Schrobback et al. 2011). Among these, autologous chondrocyte implantation (ACI) seems the most promising since it relies on the use of biodegradable materials that serve as temporary cell-carriers, enabling *in vitro* cell growth and subsequent implantation into the defective cartilage (Bomer et al. 2016; Niemeyer et al. 2016; Robb et al. 2012).

Tissue engineering of articular cartilage remains challenging due to the specific structure of cartilage tissue, i.e. its multiphasic cellular architecture together with a remarkable weight-bearing characteristics (e.g. resistance to mechanical stress and wear) (Kim et al. 2012; Su et al. 2012). Good understanding of the cartilage structure, physiology, and the molecular basis of chondrogenesis is key to *in vitro* cartilage production, either for use in tissue engineering or clinics (Bhat et al. 2011; Lee et al. 2013; Li et al. 2012). The state-of-the-art concept of *in vitro* cartilage tissue development combines the use of biocompatible and biodegradable carrier materials, the application of growth factors, the use of different cell types (stem or already differentiated) and different approaches to simulate the native mechanical stimulation (Gardner et al. 2013; Hildner et al. 2011; Khan et al. 2013; Naranda et al. 2016).

More specific challenges of articular cartilage tissue engineering remain the high consumption of cells and related costs, as well as the preparation of an ideal host scaffold. Although solutions to both mentioned challenges have been introduced in recent years (Bassleer et al. 1998; Stellavato et al. 2016), is the cell part gaining far less research momentum. Therefore, it comes to no surprise that novel approaches for chondrocyte isolation are highly desired, especially considering the high prices of ordered cells. Optimisation of isolation yields, abundant cell sources and efficient culturing procedures that lead to preparation of desired, reproducible and relatively affordable cell cultures or/and material-cell constructs with good durability, are therefore highly rated novelties in recent research (Dehne et al. 2009; Naranda et al. 2016; Otero et al. 2012).

The most demanding part in the process of *in vitro* culturing still presents the preservation of the desired phenotype to a high enough passage to yield sufficient cells to perform planned experiments (Pei & He 2012; Rosenzweig et al. 2012; Schnabel et al. 2002a; Wu et al. 2014). Since the latter depends on numerous factors and can therefore be confirmed only by a combination of (often) expensive techniques (different microscopies, molecular analysis, immunohistochemistry etc.), it is important to prepare protocols for an easier and cheaper preliminary phenotype confirmation by means of methods, available in most cell laboratories around the world. Since the desired phenotype can be identified by chondrocyte specific production (Han et al. 2010), we believe that the easiest and safest preliminary method to prove phenotype preservation could be

the analysis of gene expression. More specifically, this analysis should include the evaluation expression of genes related to cartilage specific markers (e.g. collagen type 2 and aggrecan). To follow-up possible dedifferentiation towards the fibroblastic phenotype (Goldring et al. 2006; Makris et al. 2015; Otero et al. 2012), we propose simultaneous measurement of up-regulation of collagen type 1.

Based on all mentioned it is clear that chondrocyte isolation from an abundant source with a high yield, together with an effective and cheap preliminary phenotype confirmation method, would be greatly beneficial to boost the cartilage tissue engineering (Cetinkaya et al. 2011; Goepfert et al. 2010; Schrobback et al. 2011). This study was therefore designed to provide a relatively simple, yet effective procedure for isolation and culturing of human tissue derived primary chondrocytes up to the second passage. As the preliminary method of phenotype confirmation we chose the evaluation of chondrocyte specific gene expression, together with morphological evaluation of cells. Such an approach provides a cheap and effective protocol to be considered an alternative to other available methods (Hu et al. 2002; Li et al. 2015; Strzelczyk et al. 2001; Xu & Zhang 2014), especially suitable for other laboratories to boost their respective entry level cartilage tissue engineering studies.

2 MATERIALS AND METHODS

2.1 Materials

All used materials and chemicals were of laboratory grade and purchased from Sigma-Aldrich, Germany, if not stated otherwise. For specific parts of the isolation process and cultivation, all used labware and chemicals were additionally sterilized using the standard autoclavation procedure (Avtoklav A-21, Kambič, Slovenia).

2.2 Isolation of primary chondrocytes

Full-thickness cartilage was surgically removed from the femoral condyle of arthritic knee of a 50 years old patient who underwent total knee arthroplasty (TKA) performed at the University Medical Centre Maribor, Slovenia (application reference: 123/05/14). Prior to surgery, no systemic disease or any treatment was reported for the donor patient. The study was conducted in accordance with the *Declaration of Helsinki* and its subsequent amendments and was approved by the Republic of Slovenia National Medical Ethics Committee (Ljubljana, Slovenia). The patients' informed consent was obtained.

The cartilage tissue was surgically removed under sterile conditions, transferred into a previously sterilized 250 ml glass bottle filled with phosphate buffered saline (PBS, Sigma-Aldrich, Germany) and immediately transferred to the cell isolation laboratory. The cartilage tissue was transferred to a petri dish filled with PBS to prevent drying of the tissue and subsequently cut into pieces of approximately 2 x 2 mm. PBS was carefully removed by a pipette and the petri dish was immediately filled with 10 mL solution of 0.25 wt.% Trypsin/EDTA (Sigma, France). The as-prepared cartilage pieces were incubated for 3 hours at 37°C and 5 wt.% CO_2 (CO_2 Incubator MCO-19AICUVH-PE, Panasonic, Japan), followed by addition of 20 mL of Advanced Dulbecco's modified Eagle's medium (Advanced DMEM, Gibco, Grand Island, NY, USA) to the cell

suspension. The suspension was transferred to a 50 mL falcon tube and centrifuged at 300 x g for 10 minutes (Centrifuge 5804 R, Eppendorf, USA). The supernatant was carefully discarded and the cell pellet was re-suspended in 20 mL of Advanced DMEM and centrifuged at 200 x g for 5 minutes (Centrifuge 5804 R, Eppendorf, USA). The supernatant was again carefully discarded and the cell pellet re-suspended in 10 mL Advanced DMEM supplemented with 100 IU/ml Penicillin, 1 mg/ml Streptomycin, 2mM L-glutamine and 5 wt.% foetal bovine serum (FBS, Gibco, Grand Island, NY, USA) and plated on 25 cm^2 flasks (in triplicates). The primary chondrocytes were then left until confluence was reached. Growing cells were regularly observed with an Axiovert 40 inverted optical microscope (Zeiss, Germany) at several magnifications. The culturing medium was changed every three days. The general steps of the procedure are schematically depicted in **Fig. 1**.

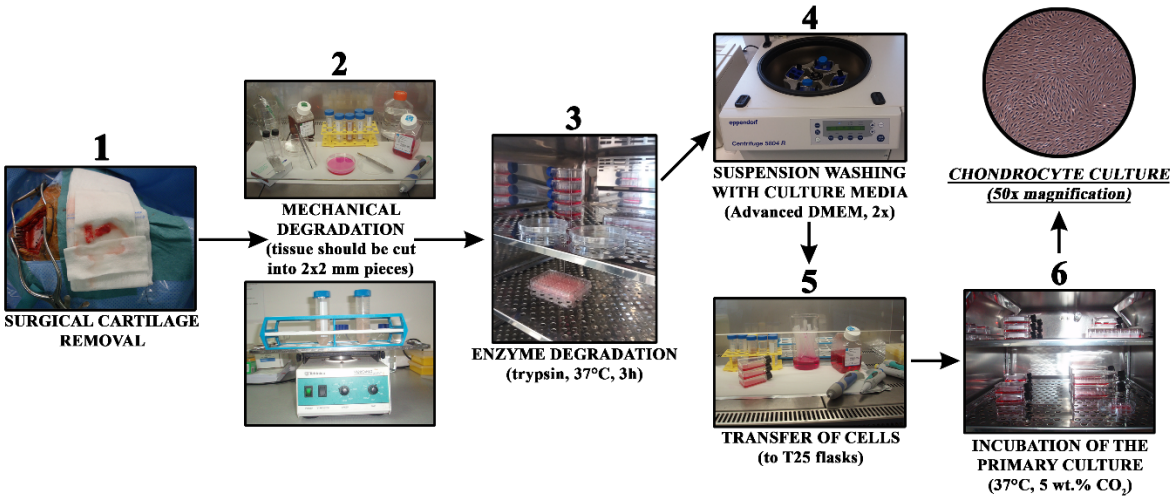

Fig. 1: Chondrocyte isolation from cartilage in a short overview of the most important preparation steps.

2.3 Isolated primary chondrocyte cell culture characterization

Gene expression analysis of cartilage specific markers collagen type 2 (COL2) and aggrecan (ACAN) was performed in order to determine the primary chondrogenic phenotype. Possible dedifferentiation to a more fibroblast like cell type was evaluated by monitoring the expression of collagen type 1 (COL1). After confluence was reached in all respective samples (triplicates) (see above section 2.2. for details), the cell suspension was transferred to micro-centrifuge tubes, and 1.4 mL of TRI reagent (Sigma-Aldrich, Germany) was added. The tubes were vortexed for 30 min at room temperature. Afterwards, 280 µL of chloroform (Sigma-Aldrich, Germany) was added and the tubes were further vortexed for 15 min and centrifuged at 12.000 rpm and 4°C. RNA extraction was carried out according to the manufacturer's instructions (Chomczynski 1993; Louveau et al. 1991). Concentration and purity of the extracted cellular RNA was determined using NanoDrop 2000c (Thermo Scientific, Delaware, USA) through optical density readings at 260 nm and a 260/280 nm ratio. cDNA was obtained by using a cDNA reverse transcription kit (Applied Biosystems, California, USA). Primer sequences for cartilage target genes ACAN and COL2 were obtained from Caterson et al. (Caterson et al. 2001), while the corresponding mRNA sequences were retrieved from PubMed Nucleotide database (www.ncbi.nlm.nih.gov/nuccore/) and the

AceView database (Thierry-Mieg & Thierry-Mieg 2006). Primers for the target gene COL1 were designed using IDT oligo analyser (eu.idtdna.com/calc/analyzer). The primer sequences with the corresponding mRNA sequences and the corresponding NCBI accession numbers, are given in **Table 1**. 2 µL of each cDNA sample with concentration of 15 ng/µL was used for quantitative real time PCR (qPCR) analysis performed using LightCycler 480 thermocycler (Roche, Switzerland) and with 2× Maxima SYBR Green qPCR master mix (Life Technologies, California, USA) according to the manufacturer's instructions. The quality and specificity of PCR amplicons were checked using melting curve analyses and agarose gel electrophoresis (see below **Fig. 4**). All shown results are presented as average values with the standard errors.

Table 1: Primer sequences with the corresponding mRNA sequence and the corresponding NCBI accession numbers.

Gene	Gene name	Accession number	Primer sequence 5'→3'
ACAN	*Aggrecan*	NM_013227.3 NM_001135.3	TGAGGAGGGCTGGAACAAGTACC GGAGGTGGTAATTGCAGGGAACA
COL1	*Collagen type 1, alpha 1*	NM_000088.3	CGGCTCCTGCTCCTCTTAG CACACGTCTCGGTCATGGTA
COL2	*Collagen type 2, alpha 1*	NM_001844.4 NM_033150.2	TTTCCCAGGTCAAGATGGTC CTGCAGCACCTGTCTCACCA
GAPDH	*Glyceraldehyde-3-phosphate dehydrogenase*	NM_001289745.1 NM_002046.5 NM_001289746.1 NM_001256799.2	GGGCTGCTTTTAACTCTGGT TGGCAGGTTTTTCTAGACGG

3 RESULTS

3.1 Isolation of primary chondrocytes

As mentioned in the Materials and methods section, the full-thickness cartilage was obtained from the femoral condyle of an arthritic knee during knee arthroplasty (TKA) performed at the University Medical Centre Maribor, Slovenia. TKA is a common procedure at the mentioned hospital, considering that approximately 700 such surgeries are performed each year (Univerzitetni klinicni center 2014). Since the removed cartilage tissue is considered surgical waste, this presents a reliable and continuous source for isolation of primary chondrocytes.

The primary chondrocytes were isolated as described in the Materials and methods section. During their cultivation, their morphology and proliferation were regularly observed using inverted optical microscopy (**Fig. 2**). **Fig. 2A** shows the thin slice of cartilage that was used for their cultivation, while **Fig. 2B-D** present the primary human chondrocytes in a monolayer culture at different cultivation times. This initial examination was performed to follow possible morphological changes in the cell shapes, which would indicate possible dedifferentiation.

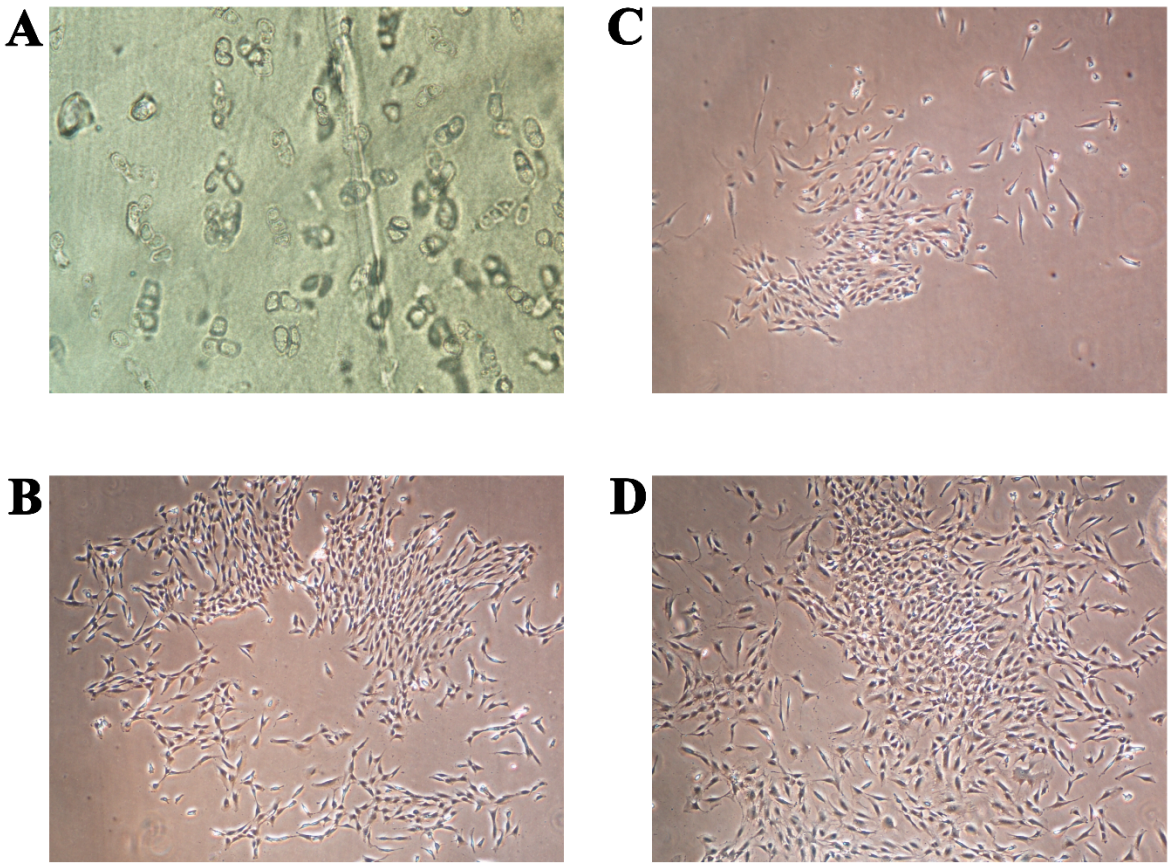

Fig. 2: A) Thin slice of cartilage for primary chondrocyte isolation; B-D) the primary human chondrocyte culture in a monolayer after 3, 6 and 9 days, respectively. The magnification of all shown images is 50x.

The full confluence of the isolated cells for the first and second passage was reached after two (14 days) and after one (7 days) week, respectively. Cell growth stopped presumably due to contact inhibition (Lackie 2013). A comparison between the primary chondrocyte culture and the obtained chondrocyte cultures after the first and second passages, is shown in **Fig. 3**. The cells formed confluent monolayers (after the above mentioned cultivation times) and appeared polygonal in shape (**Fig. 3A-C)**. It can be observed that the chondrocyte morphology became more spindle-like in the second passage (**Fig. 3C)**, showing their tendency for dedifferentiation, most likely towards fibroblast like cells (Hong & Reddi 2013). Observing the mentioned changes was an indication that the third passage will not yield a high percentage of chondrocytes only, using the proposed cultivation conditions.

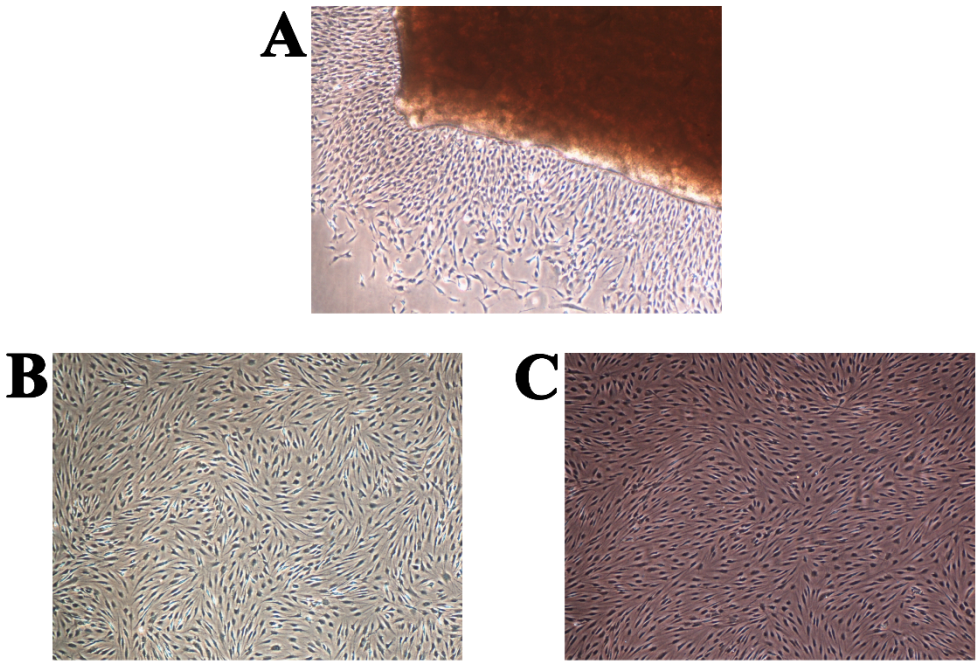

Fig. 3: Human chondrocyte culture: A) the explant culture of chondrocytes ("passage zero"), B) monolayer of chondrocytes after first passage, and C) monolayer of chondrocytes after the second passage. The magnification of all shown images is 50x (the inlay images were taken with a magnification of 100x).

3.2 Characterization of isolated chondrocytes

Now that we determined the suitable number of passages presumably yielding a high percentage of chondrocyte cells, we performed additional characterization to confirm the chondrocytes' desired phenotype. Analysis of gene expression was chosen due to its affordability and availability. The isolated cells from the human articular cartilage were characterized in regard of the genes related to specific chondrogenic production, namely collagen type 2 (COL2) and aggrecan (ACAN). To detect possible dedifferentiation towards fibroblast like cells, expression of collagen type 1 (COL1) was also determined. Expression of all three mentioned genes was performed after the confluence was reached for the first (after 14 days – not shown) and second passage (after 7 days). As shown in **Fig. 4 and Fig. 5**, both cartilage specific genes (COL2 and ACAN) and also the marker of fibrocartilage (COL1) were expressed in the isolated chondrocytes in both passages. qPCR results are presented as absolute Ct values. Reference gene GAPDH was used as an internal control (Chen et al. 2016).

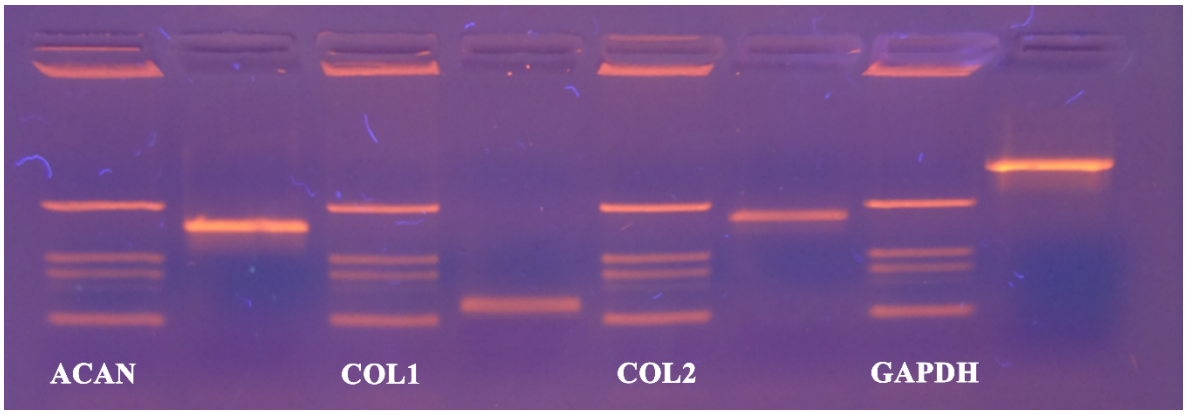

Fig. 4: cDNA products of analysed genes (GAPDH, collagen type 1, collagen type 2 and aggrecan) at the end-point of qPCR on agarose gel electrophoresis.
Analyzed genes: GAPDH (702 bp), COL2 (377 bp), COL1 (137 bp), ACAN (350 bp) and DNA markers (433 bp, 245 bp, 203 bp, 114 bp).

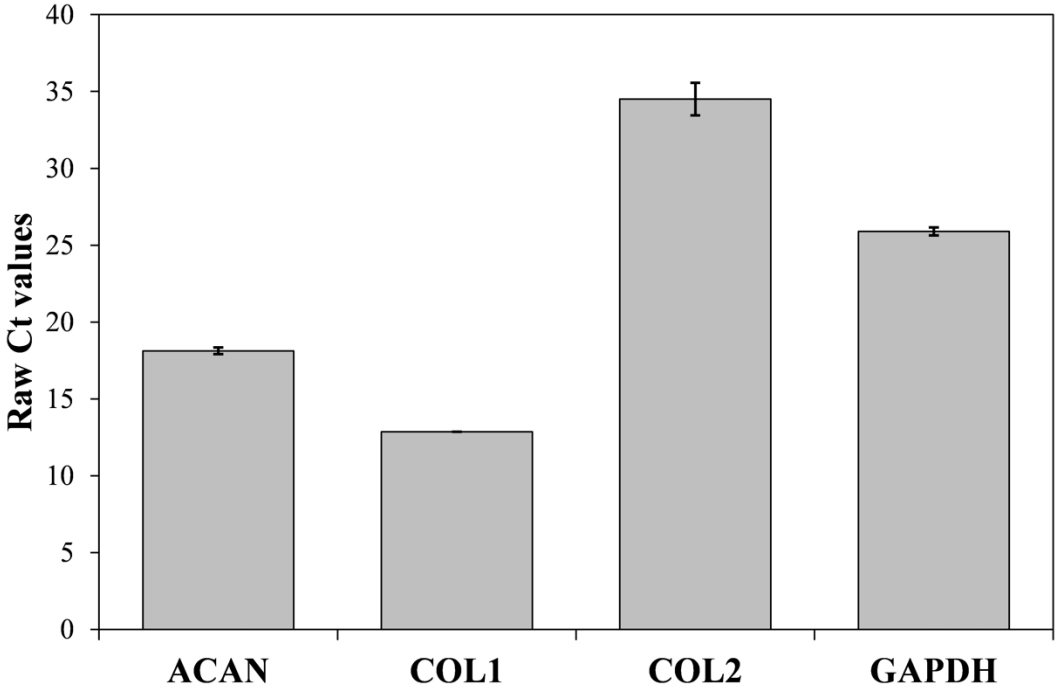

Fig. 5: Results of qPCR analysis presented as absolute Ct values of target genes expression (ACAN, COL1, COL2 and GADPH). The results are presented as average values with the standard errors of a triplicate.

4 DISCUSSION

The development of novel solutions related to any tissue engineering application consumes a huge amount of cells to prove safety and efficiency (Groeber et al. 2012; Maver et al. 2015; Mohd Hilmi & Halim 2015; Rodriguez-Vazquez et al. 2015). Cartilage tissue engineering is no exception, and hence large scale expansion of chondrocytes is required either for novel scaffold testing, determination of potential cytotoxic effects of medical devices and implants for orthopaedic use

(Bomer et al. 2016; Camarero-Espinosa et al. 2016; Makris et al. 2015). Cultivation of such high cell counts is a demanding task, especially considering the low number of obtained cells in the primary culture, and an often limited amount of available tissue. As a consequence, further expansion and consecutive passages are needed, which on the other hand can lead to dedifferentiation (Mirando et al. 2014; Shortkroff & Spector 1999; Thirion & Berenbaum 2004). The latter is evident by morphological changes of the cells from polygonal to more elongated, as well as through a reduction in the growth rate (Cetinkaya et al. 2011; Haudenschild et al. 2001; Otero et al. 2012). For example, development of novel scaffolds for cartilage tissue engineering often requires a million cells per experiment (the number depends on the size of the scaffold to be tested), exposing the high demand for cells and at the same time one of the major bottle necks in development of novel tissue engineering solutions. At later passages, the quality of chondrocytes gradually decreases and is characterized with many of the phenotypic traits of fibroblast like cells and an increased synthesis of collagen type 1, rather than type 2 (Bonaventure et al. 1994; Diekman et al. 2009; Schnabel et al. 2002b).

A sufficient amount of cells can be ensured either through significant expenses (purchase of cells from different cell banks) or isolation of desired cells from tissues. While the first scenario requires sufficient funds, the latter requires appropriate tissue sources, an approval of respective Committees of Medical Ethics, and a rigorous final analysis to confirm the isolation of the desired cell type only. Since we are a small laboratory relying on limited available funds, yet work in the close proximity and in tight collaboration with the local University Medical Centre, the second scenario was more convenient. Our goal was to prepare an efficient and relatively less expensive protocol, which would include the isolation of primary chondrocytes from full-thickness cartilage that is surgically removed from the femoral condyle of an arthritic knee during total knee arthroplasty (TKA). As a preliminary prove of the protocols' efficiency, we considered gene expression analysis as the best option, since it is affordable and the required instrumentation (PCR, inverted optical microscope) is most likely available in most cell biology laboratories.

In general, the chondrocyte isolation protocol can be divided into different stages: isolation, seeding and chondrocytes grow in culture, although description and number of steps can vary (Gosset et al. 2008; Thirion & Berenbaum 2004). After initial plating of the primary cultures, the chondrocytes spread out after 2-3 days and after 4–7 days the sufficient amounts of total RNA may be extracted. Primary cartilage phenotype (often confirmed by evaluating the presence of COL2 and ACAN mRNAs) may be initially preserved, but the expression of nonspecific collagens (e.g. COL1) begins to appear already 7 days after isolation (Otero et al. 2005). Moreover, adult articular chondrocytes are strongly contact-inhibited and undergo a rapid change in phenotype and gene expression, termed "dedifferentiation", when isolated from cartilage tissue and cultured on culturing plastics (Haudenschild et al. 2001). Therefore, primary chondrocyte cultures should be used for experimental analyses immediately before or just after confluence is reached to assure optimal matrix synthesis and cellular responsiveness (Schneevoigt et al. 2016).

The protocol of chondrocyte isolation described in this article led to successful chondrocyte growth and proliferation up to the second passage, yielding suitable number of cells for further studies. The characterization of primary human chondrocytes by molecular analysis showed the expression

of cartilage specific genes (COL2 and ACAN), as well as a sign of dedifferentiation towards fibrocartilage for the second passage (indicated by the expression of COL1).

The purpose of our study was not to revolutionize the chondrocyte isolation procedures, but rather to push the evolution of cartilage tissue engineering. As such, our desire was to present an alternative, affordable and relatively simple approach of chondrocyte isolation, especially suitable for laboratories working closely together with orthopaedic clinics. Such laboratories have the unique opportunity to use surgical waste materials, occurring during TKA. Since TKA is a very common surgery (considering the present demographics, the incidence will only increase (Peterson et al. 2015)), this approach could make cartilage related studies far more available also for laboratories with limited resources, and hence push the overall development of this field towards novel and cheaper therapeutic solutions. Based on our results, we can claim that the combination of the use of surgical waste occurring during TKA, and analysis by inverted optical microscopy and chondrocyte specific gene expression, indeed results in an alternative and affordable means to boost cartilage related research in the future.

5 CONCLUSION

In this study we describe an efficient, simple and affordable procedure of isolation and cultivation of human articular chondrocytes with a preserved chondrogenic phenotype to the second passage. As the source material we propose the surgical waster occurring during total knee arthroplasty (TKA). Chondrocyte cells are crucial not only for development of therapeutic approaches in cartilage repair (e.g. autologous chondrocyte implantation – ACI), but are necessary in cartilage tissue engineering to allow the development of functional cell models and novel scaffolds. For this purpose, chondrocytes have to be isolated in sufficient quantities and their phenotype should be preserved. Since all mentioned is related to very high costs, we propose alternative isolation and testing protocols that are cheaper and could especially boost the preliminary studies related to cartilage research.

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