



In the quest of the optimal tissue source (porcine male and female articular, tracheal and auricular cartilage) for the development of collagen sponges for articular cartilage

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ABSTRACT

Background: In the quest of the ideal material for scaffold fabrication for cartilage engineering, the use of collagen type II has been advocated, as it is the major constituent of cartilage tissue. Considering though the high batch-to-batch variability of animal-derived products, it is essential to identify suitable collagen type II sources.

Methods: Herein, we ventured to assess the influence of biological sex (male, female) and tissue (articular, tracheal, auricular) on the biophysical, biochemical and biological properties of pepsin extracted porcine collagen type II scaffolds.

Results: Articular cartilage resulted in pure collagen type II preparations, whilst tracheal and auricular cartilage preparations were contaminated with collagen type I. Pore size, porosity and denaturation temperature were not affected ($p > 0.05$) as a function of tissue and biological sex. Articular cartilage derived sponges exhibited significantly ($p < 0.05$) higher resistance to enzymatic degradation and biomechanical properties in comparison to tracheal and auricular cartilage sponges. Biological analysis using human adipose derived stem cells revealed no significant ($p > 0.05$) differences between the groups in cell viability, DNA concentration, metabolic activity and Alcian blue staining. In general, the articular cartilage groups induced the highest ($p < 0.05$) sulphated glycosaminoglycans synthesis and aggrecan and collagen type II mRNA expression (fold change ≥ 2.0).

Conclusions: Our data indicate a tissue memory of collagen type II and indicate that for cartilage engineering, articular cartilage derived collagen type II scaffolds may be more suitable for effective chondrogenesis.

1. Introduction

Articular cartilage is a hydrated connective tissue with low coefficient of friction that covers the bone ends of articulating joints, enabling smooth and efficient weight-bearing. Since articular cartilage is an avascular, alymphatic and aneural tissue with low metabolic activity, its self-healing capacity is minimal [1,2]. As a result of tissue degradation,

due to pathophysiology or injury, osteoarthritis is manifested, which currently affects over 240 million people globally [3]. Osteoarthritis is characterised as the second most expensive health condition to treat in US hospitals with total direct expenditures of US\$ 41.7 billion in 2011 [4] and is projected to increase further proportionally to the increase of population aging and obesity [5, 6]. The state of the art in cartilage repair and regeneration includes microfracture [7–9], tissue grafting

Abbreviations: TNBSA, 2,4,6-trinitrobenzene sulfonic acid; PEG-4SG, 4-arm polyethylene glycol succinimidyl glutarate; ACAN, aggrecan; alpha-MEM, alpha Minimum Essential Medium; COMP, cartilage oligomeric matrix protein; COL1A1, collagen type I $\alpha 1$; COL2A1, collagen type II $\alpha 1$; COL3A1, collagen type III $\alpha 1$; COL10A1, collagen type X $\alpha 1$; DSC, differential scanning calorimeter; DMEM, Dulbecco's Modified Eagle's medium; ECM, extracellular matrix; FARC, female articular cartilage; FAUC, female auricular cartilage; FTRC, female tracheal cartilage; HBSS, Hanks' balanced salt solution; hADSCs, human adipose derived stem cells; HPRT1, hypoxanthine phosphoribosyltransferase 1; ITS, insulin-transferrin-selenium; MARC, male articular cartilage; MAUC, male auricular cartilage; MTRC, male tracheal cartilage; PBS, phosphate buffered saline; PUM1, pumilio homolog 1; RPLP0, ribosomal protein lateral stalk subunit P0; SEM, scanning electron microscope; SOX9, SRY-box 9; sGAG, sulphated glycosaminoglycans; TCP, tissue culture plastic; TGF- $\beta 3$, transforming growth factor beta 3.

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[10–12], scaffold or scaffold-free, autologous or allogeneic, chondrocyte or stem cell transplantation [13–15], molecular delivery [16–18] and various acellular biomaterial [19,20] approaches. Unfortunately, there is still no widely accepted therapeutic treatment available. It is therefore imperative and timely to develop functional therapeutic interventions for cartilage repair and regeneration.

As conventional 2D monolayer cell culture systems, where cells are grown on flat surfaces, result in loss of chondrogenic phenotype, substantial research and development efforts are directed towards the development of 3D scaffolds that would mimic native cartilage tissue and therefore induce effective chondrogenesis [21–25]. Among the diverse range of natural and synthetic materials, the use of collagen and in particular collagen type II (as opposed to collagen type I) has been advocated, as it constitutes the major extracellular matrix (ECM) protein of cartilage [26–28] and has shown repeatedly to maintain chondrocyte phenotype *in vitro* [29, 30]. Collagen type II has also been shown to induce chondrogenic differentiation of stem cells [31] by inducing a round cell shape via the integrin β 1-mediated Rho A/Rock signalling pathway [32] and to suppresses articular chondrocyte hypertrophy and osteoarthritis progression via promotion of integrin β 1-Smad1 interactions [33]. It is also worth noting that collagen type II matrices have been shown to promote superficial cartilage-like-tissue formation in rabbit full thickness articular cartilage defects, compared to collagen type I [34], and collagen type I scaffolds have failed to demonstrate efficiency in human osteochondral repair [35] and in human large cartilage defects [36]. Further, collagen type II scaffolds from porcine cartilage (with autologous articular cartilage chondrocytes) [30,37] and from bovine trachea (with autologous rabbit bone marrow stem cells) [38] have been shown to induce hyaline neocartilage formation in chondral canine defect and osteochondral rabbit defect animal models, respectively. Considering though the inherent variability of animal-derived products, it is imperative to identify an optimal porcine collagen type II tissue source.

Herein, we ventured to assess the influence of biological sex (male, female) and tissue (articular, tracheal, auricular) on the biophysical, biochemical and biological properties of pepsin extracted porcine collagen type II scaffolds. Pepsin extraction was chosen as it has been shown to increase yield and to reduce immune responses [39–41] and 4-arm polyethylene glycol succinimidyl glutarate was selected to crosslink the produced scaffolds as its stabilisation and cytocompatibility efficiency have been demonstrated repeatedly in the literature [42–44].

2. Materials and Methods

2.1. Materials

All chemicals were of analytical grade. Porcine tissues (articular cartilage, tracheal cartilage, auricular cartilage) were obtained from a local slaughterhouse. All tissue samples were obtained freshly and stored at -20 °C until analysis. 4-arm polyethylene glycol (PEG) succinimidyl glutarate (4SG, Mw 10,000) was purchased from JenKem Technology (USA). All other materials and reagents were purchased from Sigma-Aldrich (Ireland) unless otherwise stated.

2.2. Type II collagen isolation

Extraction of pepsin soluble collagen was performed as previously described with slight modifications [40]. The selective cleavage of the N- and C- terminal regions of the collagen molecules was achieved by enzymatic digestion with pepsin (3,200–4,500 units per mg protein, cat. no. P6887) [45]. Porcine tissues were thawed at room temperature and washed thoroughly under running tap water. Cartilage was manually dissected using a surgical scalpel, incubated in 0.2 M NaOH solution for 12 h and washed with absolute ethanol. Cartilage samples were manually cut into small pieces, homogenised in liquid nitrogen using a CryoMill (SPEX SamplePrep 6870, Germany) and suspended in tissue to 1 M acetic acid ratio of 1 to 1 (g/l) under stirring for 48 h at 4 °C. Then pepsin

was added at tissue to pepsin 10 to 1 (w/w) ratio at room temperature and the solutions were kept under constant stirring for 48 h at 4 °C. Collagen solution was filtered through sieve and filter mesh; insoluble matter was discarded. Purified collagen solutions were obtained after repeated [first in collagen to 1 M acetic acid ratio of 1 to 1 (g/l) and second in 1 M acetic acid at collagen to acetic acid ratio of 1.5 to 1 (g/ml)] salt precipitation (0.9 M NaCl) / centrifugation (20 min, 8,000 rpm, 4–8 °C). Both supernatants were discarded. The precipitates from the second salt precipitation were dissolved in minimum amount of 1 M acetic acid to obtain highly concentrated collagen solutions. Subsequently, the highly concentrated collagen solutions were dialysed against 0.001 M acetic acid for 48 h at 4 °C. The final collagen solutions were stored at 4 °C until use.

2.3. Sodium dodecyl sulphate poly-acrylamide gel electrophoresis

To assess the purity of the extracted collagen from the various tissues, sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions was conducted [46] using a Mini-Protean 3 electrophoresis system (Bio-Rad Laboratories, UK). 3 % running gel and 5 % separation gel were used. Collagen samples were freeze dried in freezer-dryer (FreeZone Plus 4.5 L Console Freeze Dry System, Labconco, USA) and then dissolved at 1 mg/ml concentration in 0.5 M acetic acid. Collagen solutions were neutralised with 1 N NaOH, followed by the addition of 5x sample buffer (bromophenol blue / SDS). The sample-buffer mixture was heated at 95 °C for 5 min and a 10 μ l aliquot of the mixture was loaded onto each well of the running gel. High purity soluble collagen type I (Symatase, France) was used as negative control and soluble collagen type II isolated from chicken sternal cartilage (C9301, Sigma-Aldrich) was used as positive control, both at 1 mg/ml concentration in 0.5 M acetic acid. Electrophoresis was carried out by first applying 50 V constant voltage until the samples reach the end of the running gel and then 120 V constant voltage was applied until the samples reached the end of the separation gel. The gels were silver stained using SilverQuest™ kit (Invitrogen, USA), according to the manufacturer's protocol.

2.4. Collagen crosslinking and scaffold fabrication

Collagen type II preparations were freeze dried, re-dissolved in 0.05 M acetic acid to form solutions of 5 mg/ml concentration, crosslinked with 1 mM 4-arm polyethylene glycol succinimidyl glutarate (PEG-4SG, Mw 10,000) in phosphate buffered saline (PBS) [43] and frozen at -80 °C for 12 h. Non-crosslinked collagens were prepared using PBS only. Frozen solutions were freeze dried to obtain collagen type II sponges. All the collagen scaffolds were produced on the same day and under the same freeze-drying condition to avoid variabilities.

2.5. Ultrastructural assessment

Collagen sponges were sectioned in the dry state to expose the inner porous structure, mounted onto a carbon disk, gold sputter coated (Emitech K-550X Sputter Coater, Emitech, UK) and imaged with a Hitachi S-4700 scanning electron microscope (SEM, Hitachi, High-Technologies Europe GmbH, Germany). Three micrographs of different areas were obtained from each collagen sponge and average pore size and porosity of non-crosslinked and crosslinked collagen sponges were determined using ImageJ software (National Institutes of Health, USA). The images were binarised using thresholding procedure, a window showing pore size distribution was emerged and 80-100 pores were measured per image. The average pore size and porosity were assessed after proper thresholding [47].

2.6. Biomechanical assessment

The mechanical properties of the collagen sponges were assessed via uniaxial compression using a material testing machine (Z2.5,

Zwick/Roell, Germany) loaded with a 10 N load cell. Uniaxial constant loading was performed on dry collagen sponges with approximately 4 mm height and 9 mm in diameter. Scaffolds were placed between two loading cells and compressed until 70% deformation was reached, with a compression rate of 10 mm/min. Force, strain and elastic modulus were determined by plotting stress versus strain curves. Both compression strength and elastic modulus were determined within the linear area of the curves at the position of 30% deformation and elastic modulus was calculated using the linear equation of trend-lines at the position of 30% of the deformation [48].

2.7. Denaturation temperature assessment

The denaturation temperature of collagen sponges was assessed using a differential scanning calorimeter (DSC, DSC-60, Shimadzu, Japan) in wet state [49]. In brief, collagen sponges were hydrated in 1x PBS for 12 h at 4 °C. The samples were then blotted with filter paper to remove excess PBS and hermetically sealed in aluminium crucibles. Collagen samples of 10–15 mg were used for DSC analysis. An empty aluminium crucible was used as reference. Heating was carried out at a constant temperature ramp of 10 °C/min with a temperature range of 20–100 °C. Thermal denaturation, the endothermic transition, was recorded as typical peak and the peak (temperature of maximum power absorption during denaturation) temperature was recorded.

2.8. Free amines assessment

Crosslinking efficiency was quantified using the 2,4,6-trinitrobenzene sulfonic acid (TNBSA) assay (Thermo Fisher Scientific, Ireland). Collagen free amine groups react with TNBSA to form highly chromogenic derivatives that can be measured at 335 nm. Briefly, collagen sponges (~3 mg) were incubated with TNBSA at 37 °C for 2 h. The reaction was stopped by adding 10% sodium dodecyl sulphate (SDS) and 1 M hydrochloric acid. The mixtures were subsequently heated at 95 °C for 15 min in order to hydrolyse the collagen samples. The absorbance of each sample was read at 335 nm (Varioskan Flash Multimode Reader, Thermo Fisher Scientific, Ireland) and values were normalised to the standard curve, which was generated with a series of a known glycine solution at different concentrations (0.005, 0.01, 0.02, 0.03, 0.04 and 0.05 mg/ml).

2.9. Resistance to enzymatic degradation assessment

Enzymatic stability of the collagen sponges was quantified with the collagenase assay [50]. Briefly, collagen sponges were weighed and hydrated for 2 h in 0.1 M Tris-HCl and 500 mM CaCl₂ at pH 7.4. The sponges were then incubated in 50 U/ml bacterial collagenase type II (MMP-8; Gibco, cat. no. 17101-015, Ireland). After incubation at different time points (3 h, 6 h, 9 h, 12 h and 24 h) at 37 °C, centrifugation was carried out at 10,000 g for 5 min, the supernatants were removed and the remaining collagen sponges were freeze-dried and weighed. The degree of enzymatic degradation was quantified using the weight difference approach $[(W_o - W_t) / W_o] \times 100$, where W_o is the original weight and W_t is the remaining weight.

2.10. Cell culture

Human adipose derived stem cells (hADSCs) from female healthy donor (cat. no. PT-5006) were purchased from Lonza (Switzerland) and cultured in alpha Minimum Essential Medium (alpha-MEM) with GlutaMAXTM (Gibco Life Technologies, Ireland) supplemented with 10% foetal bovine serum and 1% penicillin-streptomycin at 37 °C in 95% humidified atmosphere of 5% CO₂. At passages 5, cells were resuspended in fresh medium and 100 µl of cell suspension was seeded onto each collagen sponge at a density of 200,000 cells/sponge. As a negative control

group, human adipose derived stem cells were cultured on tissue culture plastic (TCP) at 50,000 cells/cm². Cells were left to attach to the sponge for 2 h before the chondrogenic induction medium was added. Expansion medium was changed to chondrogenic induction medium the next day to allow for the cells to attach and spread evenly throughout the scaffold. The chondrogenic induction medium was composed of high glucose Dulbecco's Modified Eagle's medium (DMEM), 100 nM dexamethasone, 50 mg/ml bovine serum albumin, 50 µg/ml ascorbic acid-2-phosphate, 5 ml insulin-transferrin-selenium (ITS) liquid media supplement, 10 ng/ml transforming growth factor beta 3 (TGF-β3, R&D Systems, USA). Cells were cultured for 7, 14 and 21 days and media were changed every 2 days.

2.11. Cell viability assessment

Cell viability was analysed using the Live/Dead[®] assay (Life Technologies, Ireland) as per manufacturer's protocol. Briefly, at the end of each timepoint, the collagen sponges were washed three times with Hanks' balanced salt solution (HBSS) and incubated with calcein AM and ethidium homodimer 1 solution (2 µM calcein-AM and 4 µM ethidium homodimer-1) in HBSS at 37 °C, 5 % CO₂ and 95% relative humidity for 30 min. The collagen sponges were washed with fresh HBSS to remove excess dye. Subsequently, images were acquired using a Fluoview 1000 confocal laser microscope (FV1000 Shackelton, Olympus, Japan). Live (green) and dead (red) cells were analysed using ImageJ Software (National Institutes of Health, USA).

2.12. Cell proliferation assessment

Cell proliferation was assessed using the Quant-iTTM PicoGreen[®] ds-DNA kit (Invitrogen, USA), according to the manufacturer's guidelines. Collagen sponges were washed three times with HBSS at the end of each timepoint, 200 µl DNase free water was added and frozen at -80 °C until analysis. Collagen sponges were freeze-thawed at least three times in order to lyse the cells. Subsequently, PicoGreen[®] working solution was added to the collagen sponges and incubated at room temperature for 5–10 min, protected from light. Fluorescence was measured at excitation and emission wavelengths of 480 nm and 520 nm using a VarioskanTM Flash Multimode Reader (Thermo Fisher Scientific, Ireland). The obtained values were normalised to the standard curve, which has been generated with a series of known DNA stock solutions at different concentrations (0, 5, 10, 25, 50, 100, 500 and 1,000 ng/ml).

2.13. Cell metabolic activity assessment

Cell metabolic activity was analysed using an alamarBlue[®] assay (Invitrogen, USA), as per manufacturer's protocol. Briefly, collagen sponges were washed three times with HBSS at the end of each time point, 10% alamarBlue[®] was added to each collagen sponge and incubated at 37 °C, 5% CO₂ and 95% relative humidity for 3 h. Absorbance was measured at 570 nm and 600 nm using a VarioskanTM Flash Multimode Reader (Thermo Fisher Scientific, Ireland).

2.14. Sulphated glycosaminoglycan assessment

The amount of sulphated glycosaminoglycans (sGAG) produced by the hADSCs was quantified using the BlyscanTM sGAG assay kit (Bicolor, UK) according to the manufacturer's protocol. Briefly, collagen sponges were washed three times with HBSS at the end of each timepoint, followed by papain extraction. Then, 1 ml BlyscanTM dye reagent was added to each sample and the samples were mixed gently using an incubated orbital shaker (Thermo Fisher Scientific, Ireland) for 30 min. The samples were then centrifuged at 17,000 g for 10 min. Unbound dye solution was carefully drained and 0.5 ml dissociation reagent was

Table 1
Genes and their primer 1 (forward), probe, and primer 2 (reverse) sequences.

Genes	Primer 1 (5'-3')	Probe (5'-3')	Primer 2 (5'-3')
HPRT1	GTA TTC ATT ATA GTC AAG GGC ATA TCC	/56-FAM/TGG TGA AAA /ZEN/GGA CCC CAC GAA GT/3IABkFQ/	AGA TGG TCA AGG TCG CAA G
PUM1	GAG CAG CAG AGA TGT ATC TTC C	GAC CAG GAC ATT CAC AGA CAC	GAC CAG GAC ATT CAC AGA CAC
RPLP0	CAG ACA GAC ACT GGC AAC A	/56-FAM/CCT GAA GTG /ZEN/CTT GAT ATC ACA GAG GAA ACT /3IABkFQ/	ACA TCT CCC CCT TCT CCT T
COL1A1	GGT TGA TTT CTC ATC ATA GCC AT	/56-FAM/AGG AAA CTT /ZEN/TGC TCC CCA GCT GT/3IABkFQ/	CTG GAC AGC CTG GAC TTC
COL2A1	GTT TTC CAG CTT CAC CAT CAT C	/56-FAM/TGG GAC CAG /ZEN/AGA CAC CAG GTT CA/3IABkFQ/	CCT CAA GGA TTT CAA GGC AAT
COL3A1	TTGGCATGGTCTGGCTT	/56-FAM/TGGGAACAT/ZEN/CCTCCTCAACAGCTTC/3IABkFQ/	CTACTTCTCGCTGCTTCATC
COL10A1	GTA CCT TGC TCT CCT CTT ACT G	/56-FAM/CCA AGA CAC /ZEN/AGT TCT TCA TTC CCT ACA CC/3IABkFQ/	CAT AAA AGG CCC ACT ACC CA
SOX9	CGT TCT TCA CCG ACT TCC TC	/56-FAM/AAG GGC CGC /ZEN/TTC TCG CTC T/3IABkFQ/	CTG GGC AAG CTC TGG AG
COMP	ACAGGCATCCCCTATACCAT	/56-FAM/ACCCAATC/ZEN/AGACCAGAAGGACAGT/3IABkFQ/	GACAAAAGGACACAGACCA
ACAN	AGA TTC ACA GAA CTC CAG TGC	/56-FAM/CGA AGA ACA /ZEN/CCT CCC CCT CAA GTC /3IABkFQ/	ACC TAC GAT GTC TAC TGC TTT G

added to the remaining sGAG droplets. 200 µl of each sample was transferred to individual wells of a 96 micro well plate and the absorbance was measured at 656 nm using a Varioskan™ Flash Multimode Reader (Thermo Fisher Scientific, Ireland). Standard aliquots containing 1, 2, 3, 4 and 5 µg GAGs were used as reference standards for standard curve. The sGAG concentration of each collagen sponge was obtained after normalisation to the standard curve.

2.15. Histology assessment

Collagen sponges were collected at each time point, washed with 1x PBS and immersion-fixed with 4% paraformaldehyde for 30 min at room temperature. Collagen sponges were washed with 1x PBS and cryoprotected in PBS containing 15% and 30% sucrose, consecutively. Collagen sponges were embedded in optimal cutting temperature compound (Tissue-Tek, USA) and cryo-sectioned using a Cryostat (Leica, CM1850, Germany). Serial sections of 5–10 µm in thickness were collected on adhesion glass slides (Superfrost Plus, Thermo Fisher Scientific, Austria), air-dried for 1 h at room temperature and stored at -20 °C until further processing. Sections were stained using haematoxylin and eosin (H&E) stain. Nuclei were stained with Mayer's Haemalum solution (Carl Roth, Germany) for 5 min, slides were washed in 0.5% hydrogen chloride-ethanol solution to destain the connective tissue and subsequently rinsed in 1% acetic acid to stop the staining reaction. Samples were washed with running tap water for 10 min and sections were counterstained with an alcoholic Eosin Y solution (50% of ethanol and 0.15% of eosin) for 30 s. After rinsing in 1% acetic acid, samples were dehydrated through graded ethanol and xylene and mounted with distyrene-dibutyl phthalate-xylene (DPX) mounting medium. Images were captured under a light microscope (Olympus, Japan) and analysed using ImageJ Software (National Institutes of Health, USA).

The detection of sulphated GAGs was carried out through Alcian blue staining. Serial sections of collagen sponges were stained with 3% Alcian blue staining solution for 30 min, followed by a rinse in 1% acetic acid and then counterstained with nuclear fast red solution (Merck, USA) for 5 min. Then, the slides were briefly rinsed in distilled water, dehydrated with 100% ethanol and mounted with DPX mounting medium. Images were captured under a light microscope (Olympus, Japan).

2.16. RNA isolation

Total RNA was isolated from hADSC-seeded collagen sponges of all groups after 21 days of *in vitro* culture in chondrogenic media and 3 collagen sponges from each group were pooled for each sample. Collagen sponges were homogenised using surgical scalpels before TRI-Reagent® was added. Two consecutive extraction steps using phenol-chloroform were performed. Total RNA was precipitated for 30 min at -20 °C with an equal volume of ice-cold isopropanol, followed by centrifugation for 30 min at 13,000 g at 4 °C. RNA pellets were washed in 75% ethanol, air dried and resuspended in the Ambion® RNA Storage Solution (Thermo Fisher Scientific, Ireland) supplemented with RiboSafe RNase inhibitor

(Bioline, UK) and stored at -80 °C. RNA yield and purity were determined using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Ireland). An OD 260/280 value between 1.8 and 2.0 was defined as pure RNA. RNA integrity was verified by the Agilent 2100 Bioanalyzer Electrophoresis System (USA). RNA with RNA quality indicator > 8 was defined as intact RNA.

2.17. Quantitative real-time PCR

1 µg of total RNA was reverse-transcribed using iScript™ RT-PCR Supermix (BioRad, Ireland). 5 ng cDNA per reaction were subsequently analysed by quantitative PCR using TaqMan® Gene Expression Master Mix (Applied Biosystems, Ireland) and TaqMan® PrimeTime Pre-designed qPCR assays (Integrated DNA Technologies, USA) for collagen type I $\alpha 1$ (COL1A1), collagen type II $\alpha 1$ (COL2A1), collagen type III $\alpha 1$ (COL3A1), collagen type X $\alpha 1$ (COL10A1), aggrecan (ACAN), cartilage oligomeric matrix protein (COMP), SRY-box 9 (SOX9), hypoxanthine phosphoribosyltransferase 1 (HPRT1), pumilio homolog 1 (PUM1) and ribosomal protein lateral stalk subunit P0 (RPLP0). Table 1 lists the genes and the sequence of their respective primers. Default amplification conditions were as follows: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min using Applied Biosystems (Ireland) StepOnePlus™ Real Time PCR System. Cq values were analysed using qBase+ (Biogazelle, Belgium) and normalised relative quantities (NRQs) were calculated by normalising the data to the expression of 3 validated endogenous control genes (HPRT1, PUM1, RPLP0). Upregulated or downregulated mRNA expression was defined as a fold change ≥ 2.0 , no difference in mRNA expression was defined as a fold change ≤ 2.0 .

2.18. Statistical analysis

Numerical data are expressed as mean \pm standard deviation. Statistical analysis was performed using SPSS Statistics (USA). Analysis was performed using One-way analysis of variance (ANOVA) for multiple comparisons and 2-sample t-test for pair wise comparisons after confirming the following assumptions of parametric analysis: the distribution from which each of the samples was derived was normal (Shapiro-Wilk normality test) and the variances of the population of the samples were equal to one another (Levene's test for equal variances). Nonparametric statistics were used when either or both of the above assumptions were violated and consequently Kruskal-Wallis test for multiple comparisons or Mann-Whitney test for 2-samples were carried out. Statistical significance was accepted at $p < 0.05$.

3. Results

3.1. Purity assessment

SDS-PAGE (Fig. 1) revealed a typical electrophoretic mobility of collagen type II, although collagen type I (presence of $\alpha 2$ chain) contami-

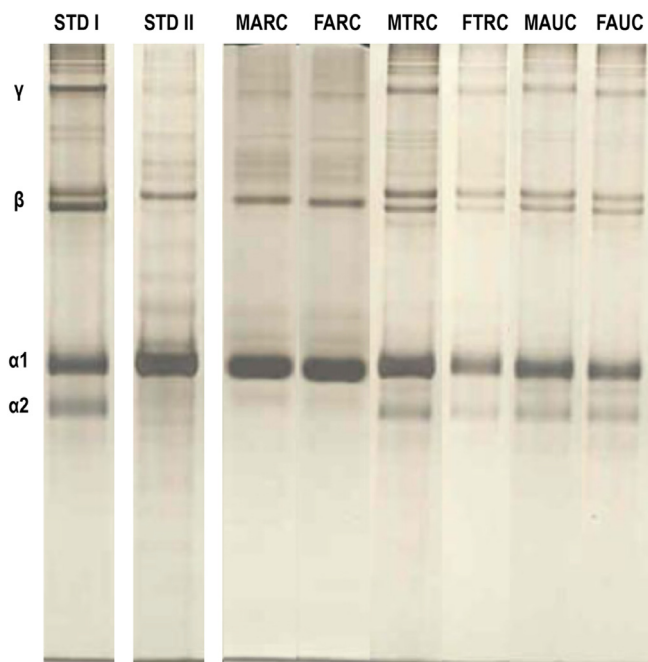


Fig. 1. SDS-PAGE analysis made apparent that purified collagen type II was extracted from articular cartilage tissues, but some collagen type I impurities were evidenced in tracheal and auricular cartilage preparations. STD I: Standard collagen type I. STD II: Standard collagen type II. MARC: Male articular cartilage. FARC: Female articular cartilage. MTRC: Male tracheal cartilage. FTRC: Female tracheal cartilage. MAUC: Male auricular cartilage. FAUC: Female auricular cartilage.

nation was evidenced in tracheal and auricular cartilage preparations. No clear differences were observed as a function of biological sex.

3.2. Ultrastructural assessment

All materials exhibited a porous structure, as revealed by SEM analysis, and subsequent quantitative pore size and porosity analyses of the

produced sponges revealed no apparent differences ($p > 0.05$) as function of tissue, biological sex and crosslinking (Fig. 2).

3.3. Biomechanical assessment

Biomechanical assessment (Table 2) made apparent that among non-crosslinked and crosslinked groups, the MARC and FARC groups exhibited higher ($p < 0.005$) compression stress and elastic modulus, whilst no significant ($p > 0.05$) differences were observed as a function of biological sex within a tissue group. Crosslinking significantly ($p < 0.005$) increased the compression stress and elastic modulus in all groups.

3.4. Thermal properties assessment

DSC analysis (Table 2) revealed that crosslinked samples exhibited significantly ($p < 0.005$) higher denaturation temperature than their non-crosslinked counterparts and that no significant ($p > 0.05$) differences in denaturation temperature were observed as a function of tissue and biological sex within the non-crosslinked and crosslinked groups.

3.5. Free amines assessment

Free amine quantification (Table 2) revealed that within the male and female non-crosslinked and crosslinked groups, the MARC and FARC groups, respectively, had the highest ($p < 0.0005$)% of free amines. Crosslinking significantly ($p < 0.005$) reduced the % of free amines of each group. Within a tissue, no significant ($p > 0.05$) differences were observed in free amines as a function of biological sex.

3.6. Resistance to enzymatic degradation assessment

Collagenase digestion analysis (Fig. 3) revealed that all non-crosslinked samples were completely degraded ($< 3\%$ remaining mass) within 12 h of collagenase digestion, whilst crosslinked samples were more resistant to collagenase digestion ($\sim 30\%$ remaining mass after 24 h of collagenase digestion). Within the non-crosslinked male and female groups, the MARC and FARC groups were significantly ($p < 0.0005$) more resistant to collagenase digestion than the MTRC and MAUC and FTRC and FAUC groups, respectively, for the first 6 h. Within non-crosslinked tissues, no significant ($p > 0.05$) differences in

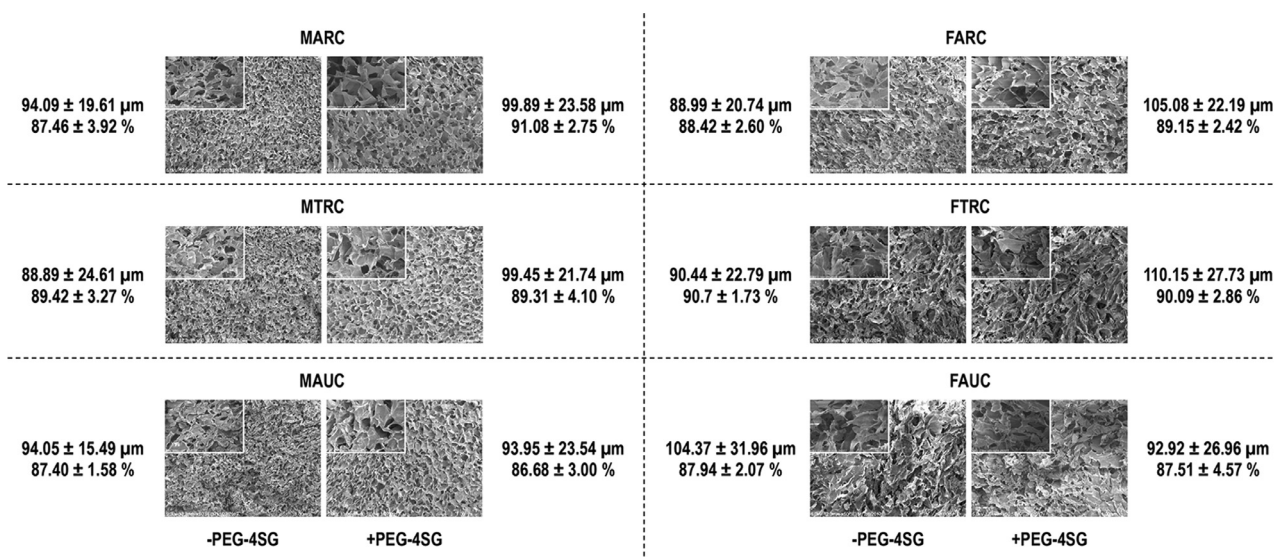


Fig. 2. SEM and complementary pore size (μm) and porosity (%) analyses did not reveal any significant ($p > 0.05$) differences between the produced scaffolds. MARC: Male articular cartilage. FARC: Female articular cartilage. MTRC: Male tracheal cartilage. FTRC: Female tracheal cartilage. MAUC: Male auricular cartilage. FAUC: Female auricular cartilage. -PEG-4SG: No crosslinked groups. +PEG-4SG: Crosslinked groups.

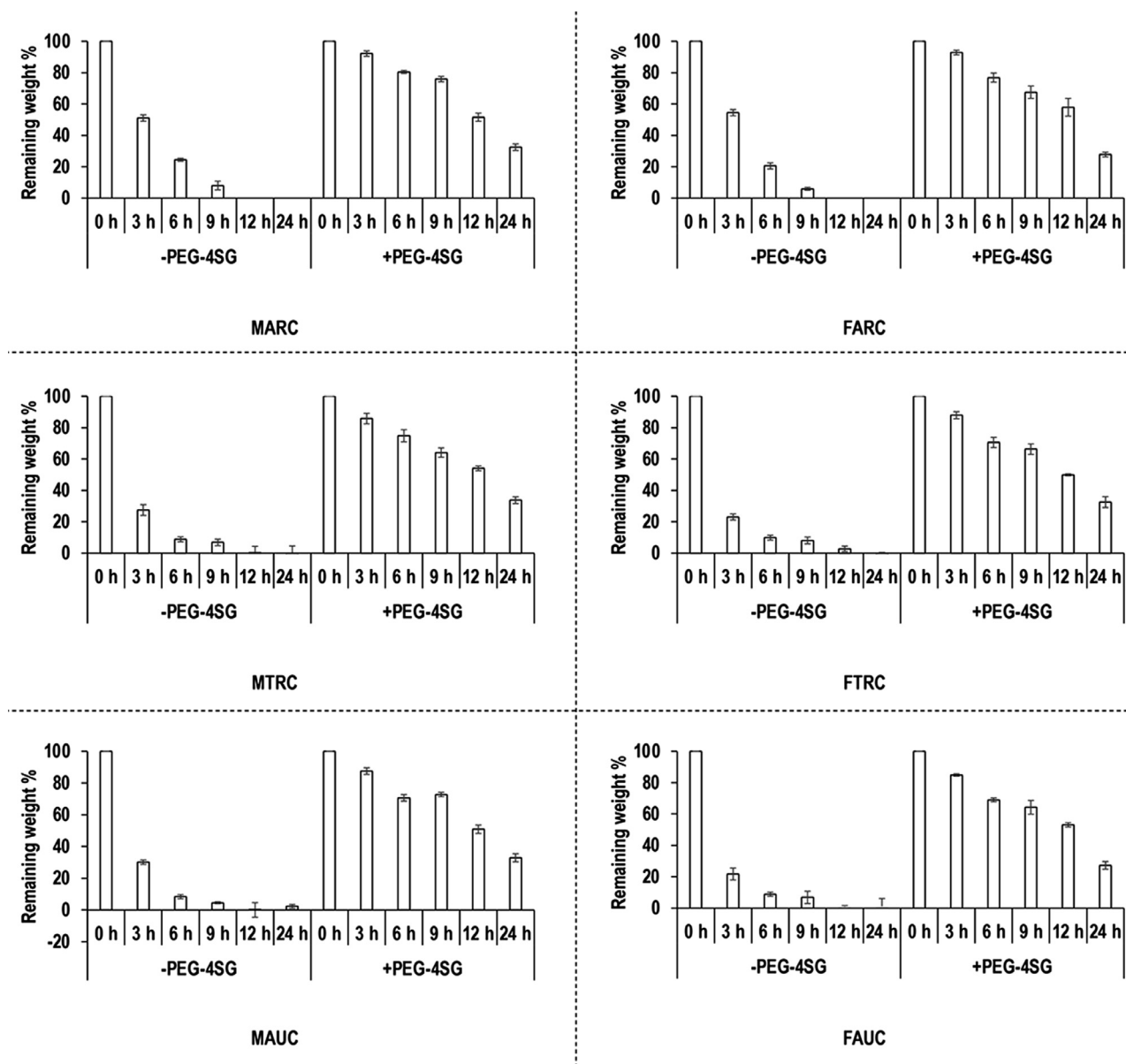


Fig. 3. Non-crosslinked male and female articular cartilage scaffolds were significantly ($p < 0.0005$) more resistant to enzymatic degradation than the MTRC and MAUC and FTRC and FAUC groups, respectively, for the first 6 h. No significant ($p > 0.05$) differences in susceptibility to collagenase digestion were observed as a function of biological sex within non-crosslinked tissues. Crosslinked groups MARC and FARC groups were significantly ($p < 0.005$) more resistant to collagenase digestion than the MTRC and MAUC and FTRC and FAUC groups, respectively, for the first 3 h. Crosslinking significantly ($p < 0.0005$) increased the resistance to collagenase digestion of each group. MARC: Male articular cartilage. FARC: Female articular cartilage. MTRC: Male tracheal cartilage. FTRC: Female tracheal cartilage. MAUC: Male auricular cartilage. FAUC: Female auricular cartilage. -PEG-4SG: Non-crosslinked groups. +PEG-4SG: Crosslinked groups.

susceptibility to collagenase digestion were observed as a function of biological sex. Within the crosslinked male and female groups, the MARC and FARC groups were significantly ($p < 0.005$) more resistant to collagenase digestion than the MTRC and MAUC and FTRC and FAUC groups, respectively, for the first 3 h. Crosslinking significantly ($p < 0.0005$) increased the resistance to collagenase digestion of each group.

3.7. Basic cellular function assessment

Cell viability (Fig. 4A), DNA concentration (Fig. 4B) and metabolic activity (Fig. 4C) analyses indicated that all scaffolds equally ($p > 0.05$) supported hADSCs attachment, proliferation and growth up to 21 days in culture (longest timepoint assessed) independently of the tissue and the biological sex. Qualitative cell infiltration and ECM synthesis analysis via H&E staining (Fig. 4D) revealed a homogeneous cell distribution

throughout the scaffolds and increased ECM synthesis as a function of time in culture independently of the tissue and the biological sex.

3.8. Chondrogenic potential assessment

Alcian blue staining (Fig. 5A) and sGAG quantification (Fig. 5B) revealed no differences ($p > 0.05$) as a function of biological sex at any timepoint, but a significant ($p < 0.0005$) increase in sGAG content from day 14 to day 21 for all groups, with the articular cartilage derived scaffolds to induce the highest ($p < 0.0005$) increase.

Gene expression analysis after 21 days of chondrogenic induction (Fig. 6) revealed no differences (fold change ≤ 2.0) in COL1A1, COL3A1, COL10A1 and COMP mRNA levels between the experimental groups and all experimental groups showed higher (fold change ≥ 2.0) COL3A1, COL10A1 and COMP mRNA levels than TCP. With respect to SOX9

Table 2

Among the non-crosslinked and crosslinked groups, the MARC and FARC groups exhibited higher ($p < 0.005$) compression stress and elastic modulus, whilst no significant ($p > 0.05$) differences were observed as a function of biological sex within a tissue group. Crosslinking significantly ($p < 0.005$) increased the compression stress and elastic modulus in all groups. Crosslinking significantly ($p < 0.005$) increased the denaturation temperature of each group and no differences ($p > 0.05$) in denaturation temperature were observed as a function of tissue and biological sex within the non-crosslinked and crosslinked groups. Articular cartilage groups ($p < 0.0005$) and non-crosslinked groups ($p < 0.005$) exhibited significantly higher % of free amines than their respectively counterparts. MARC: Male articular cartilage. FARC: Female articular cartilage. MTRC: Male tracheal cartilage. FTRC: Female tracheal cartilage. MAUC: Male auricular cartilage. FAUC: Female auricular cartilage. -PEG-4SG: No crosslinked groups. +PEG-4SG: Crosslinked groups.

	MARC		FARC		MTRC		FTRC		MAUC		FAUC	
	-PEG-	+PEG-	-PEG-	+PEG-	-PEG-	+PEG-	-PEG-	+PEG-	-PEG-	+PEG-	-PEG-	+PEG-
	4SG	4SG	4SG	4SG	4SG	4SG	4SG	4SG	4SG	4SG	4SG	4SG
Stress (kPa)	2.38 ± 0.26	4.76 ± 0.5	2.55 ± 0.21	4.75 ± 0.37	1.43 ± 0.4	3.41 ± 0.61	1.59 ± 0.08	3.44 ± 0.42	1.64 ± 0.21	3.28 ± 0.31	1.69 ± 0.22	3.36 ± 0.28
Modulus (kPa)	7.89 ± 0.83	15.75 ± 1.71	8.41 ± 0.76	15.67 ± 1.17	4.78 ± 1.34	11.24 ± 2.03	5.25 ± 0.28	11.35 ± 1.43	5.43 ± 0.69	10.83 ± 1.00	5.58 ± 0.69	11.07 ± 0.97
Denaturation Temperature (°C)	46.68 ± 0.19	54.27 ± 1.52	46.34 ± 0.63	53.74 ± 0.60	47.43 ± 0.72	53.55 ± 1.11	47.18 ± 0.84	53.44 ± 1.72	47.10 ± 0.73	54.26 ± 0.95	46.58 ± 0.22	53.96 ± 0.41
Free Amines (%)	10.67 ± 0.64	7.43 ± 0.29	11.67 ± 0.69	6.59 ± 0.98	6.79 ± 0.16	4.90 ± 0.25	6.79 ± 0.32	4.83 ± 0.34	6.84 ± 0.37	5.25 ± 0.55	6.98 ± 0.44	4.93 ± 0.19

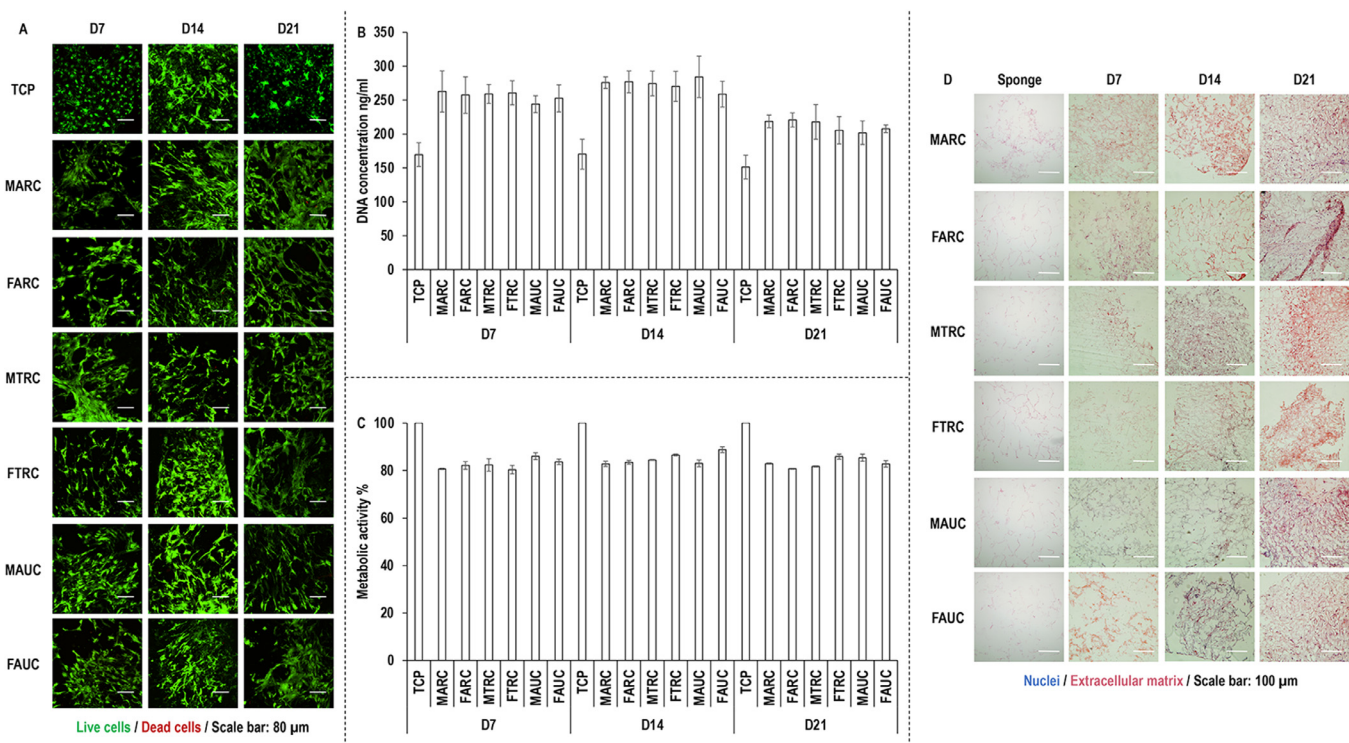


Fig. 4. No apparent differences ($p > 0.05$) in hADSC viability (A), DNA concentration (B), metabolic activity (C) and infiltration and ECM synthesis (D) were detected as a function of tissue and biological sex for up to 21 days in culture (longest timepoint assessed). TCP: Tissue culture plastic. D: Day. MARC: Male articular cartilage. FARC: Female articular cartilage. MTRC: Male tracheal cartilage. FTRC: Female tracheal cartilage. MAUC: Male auricular cartilage. FAUC: Female auricular cartilage.

mRNA levels, the male articular cartilage showed higher (fold change ≥ 2.0) SOX9 mRNA expression compared to the female tracheal cartilage and all experimental groups showed higher (fold change ≥ 2.0) SOX9 mRNA levels than TCP. Both male and female articular cartilage derived scaffolds exhibited higher (fold change ≥ 2.0) ACAN mRNA expression compared to the female auricular cartilage scaffolds and the female articular cartilage scaffolds showed higher (fold change ≥ 2.0) ACAN mRNA expression than male and female tracheal cartilage scaffolds and TCP. COL2A1 expression was only detected in male articular cartilage, female tracheal cartilage and female auricular cartilage groups and among them, the male articular cartilage derived scaffolds showed the highest (fold change ≥ 2.0) mRNA expression of COL2A1.

4. Discussion

Among the various natural and synthetic materials available for scaffold fabrication for cartilage repair and regeneration, collagen type II has shown great potential, considering that it is the main ECM component of cartilage [51] and collagen type II derived scaffolds have been shown to maintain chondrocyte phenotype [52,53], to drive stem cells towards chondrogenic lineage [54,55] and to induce hyaline cartilage in preclinical models [30,56]. Considering though the batch-to-batch variability of animal extracted biomolecules, it is imperative to standardise the process for consistency purposes. To this end, herein, we assessed the influence of biological sex (male, female), tissue (articular, tracheal, auricular) and crosslinking (no crosslinked and 4-arm polyethylene gly-

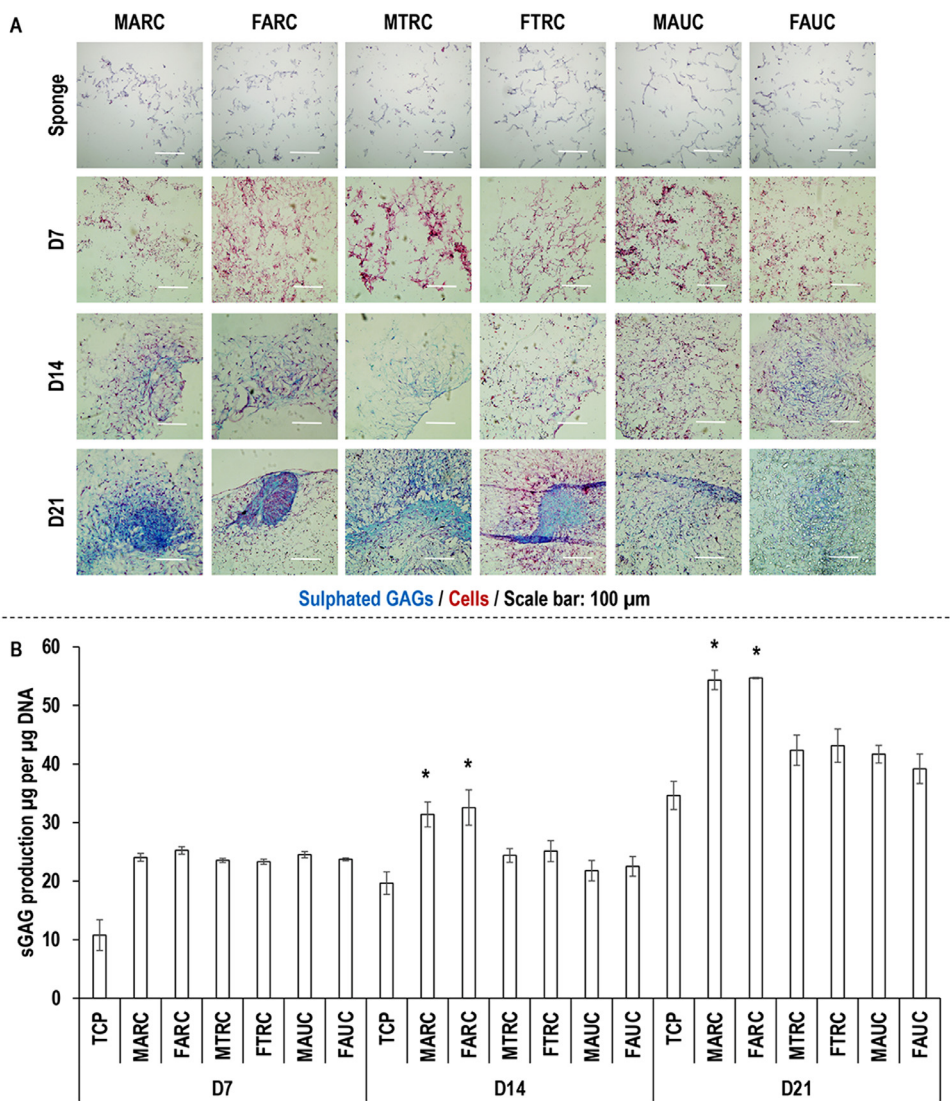


Fig. 5. Alcian blue staining (A) and sGAG quantification (B) revealed that the articular cartilage derived scaffolds induce the highest ($p < 0.0005$; indicated with * in the graph) increase in sGAG content at day 14 and day 21. TCP: Tissue culture plastic. D: Day. MARC: Male articular cartilage. FARC: Female articular cartilage. MTRC: Male tracheal cartilage. FTRC: Female tracheal cartilage. MAUC: Male auricular cartilage. FAUC: Female auricular cartilage.

col succinimidyl glutarate crosslinked) on the biophysical, biochemical and biological properties of pepsin extracted porcine collagen type II scaffolds.

Pure collagen type II was extracted from male and female articular cartilage, as evidenced by a typical collagen type II electrophoretic mobility, corresponding to three $\alpha 1(\text{II})$ chains as has been shown before from various articular cartilage collagen type II preparations [28,57–59]. In contrast, collagen extracted from male and female tracheal and auricular cartilage presented a mixture of collagen type I and collagen type II collagen, shown by the presence of $\alpha 2(\text{I})$ chain of collagen type I [60,61]. Type I collagen contamination most probably resulted from the perichondrium surrounding tracheal and auricular cartilage, but not articular cartilage, which mainly consists of collagen type I [62].

Cartilage tissue source, biological sex and crosslinking did not significantly affect the macrostructure of the fabricated collagen type II scaffolds. All scaffolds had pore size and porosity range of 80–110 µm and 80–95%, respectively, that has been shown to induce chondrogenic differentiation in human stem cell cultures [63–65].

Collagen scaffolds from male and female articular cartilage exhibited significantly higher stress and modulus values than their tracheal and auricular cartilage counterparts, with no significant differences as a function of biological sex. This can be explained considering the mechanical properties of the tissue from which collagen was extracted from (modulus of porcine articular cartilage 7.2 MPa [66], tracheal cartilage

1.78 MPa [67], auricular cartilage 0.25 MPa [68]) and therefore our data suggest that collagen may retain tissue memory. It is worth noting that a previous publication showed that the addition of collagen type II to collagen type I gels significantly decreased their stiffness, but this was attributed to the reduction of the total collagen in the system [69], as opposed to the difference in mechanical properties between the different collagen types (it is also worth noting that the collagen type I was from rat tail tendon and the collagen type II was chicken sterna and therefore direct comparison cannot be conducted). In accordance with previous publications [70], crosslinking significantly increases the mechanical properties of the produced collagen sponges.

Collagen extracted from porcine male and female articular, tracheal and auricular cartilage exhibited comparable denaturation temperatures of approximately 47 °C, indicating that thermal stability is independent of tissue source and biological sex and crosslinking increased the denaturation temperature of all scaffolds to approximately 54 °C. Previous studies have shown similar denaturation temperature values for non-crosslinked collagen type II scaffolds [71,72]. The observed increase in denaturation temperature as a function of crosslinking is also well established in the literature for collagen type II scaffolds [71].

With respect to free amine content and resistance to collagenase digestion of the non-crosslinked collagen type II scaffolds, no differences were observed as function of biological sex within the different tissues and among the different tissues, the articular cartilage derived colla-

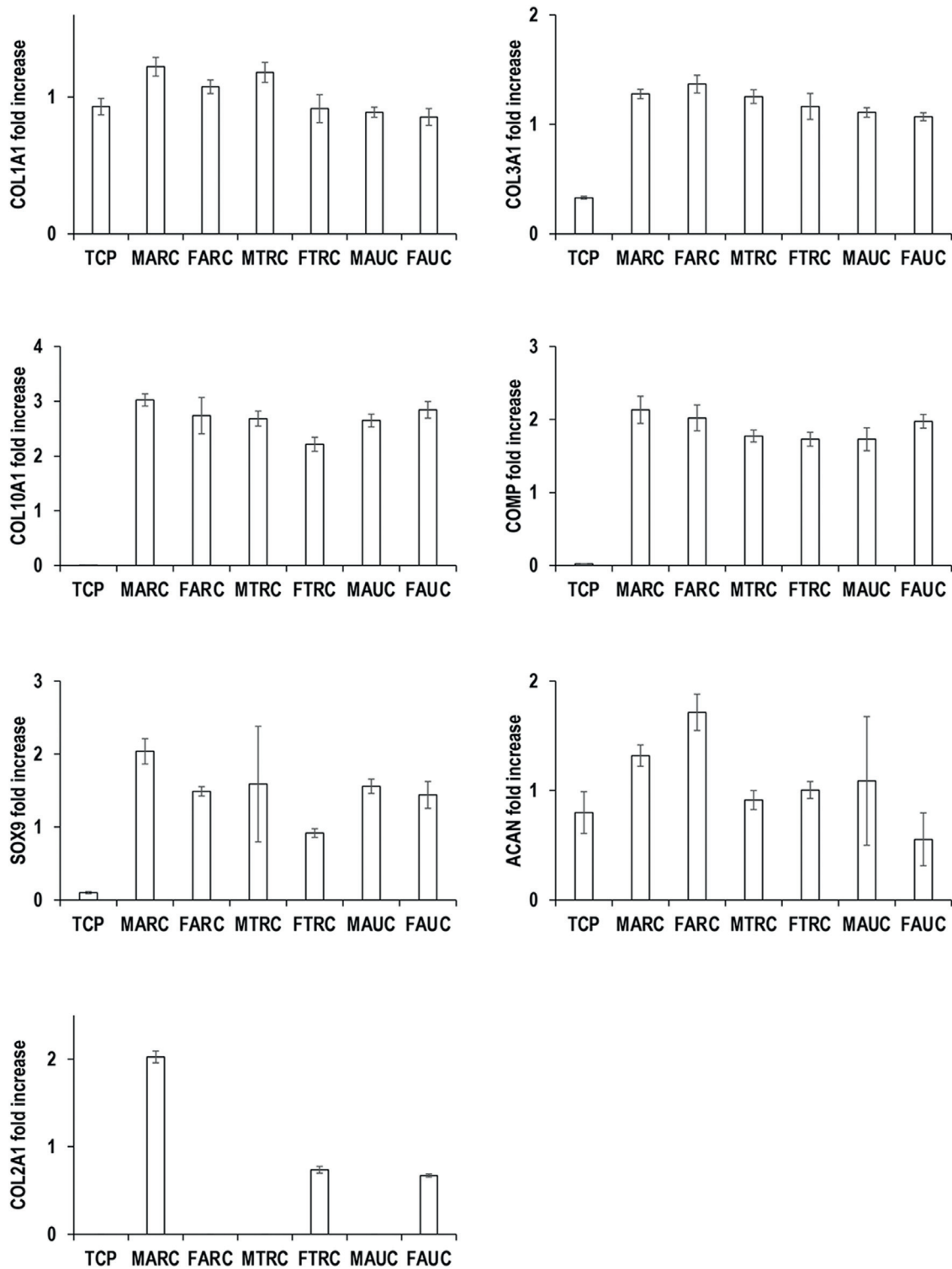


Fig. 6. Gene expression analysis after 21 days of chondrogenic induction revealed no difference (fold change ≤ 2.0) in COL1A1, COL3A1, COL10A1 and COMP mRNA levels between the experimental groups. The male articular cartilage scaffold induced higher (fold change ≥ 2.0) SOX9 expression than the female tracheal cartilage scaffold. The female tracheal scaffolds exhibited higher (fold change ≥ 2.0) mRNA expression than the male and female tracheal and female auricular scaffolds. The male articular cartilage scaffolds exhibited the highest (fold change ≥ 2.0) COL2A1 mRNA expression. TCP: Tissue culture plastic. MARC: Male articular cartilage. FARC: Female articular cartilage. MTRC: Male tracheal cartilage. FTRC: Female tracheal cartilage. MAUC: Male auricular cartilage. FAUC: Female auricular cartilage.

gen type II scaffolds (the male within the male and the female within the female groups of the different tissues) had the highest free amine content and resistance to collagenase digestion. Obviously, these observations are rather contradictory, considering that higher free amine content is associated with less crosslinking and lower resistance to enzymatic degradation [73]. As the tissues were from the same animals and the same extraction and freeze-drying protocols were used for all groups, we rule out the age of the animals and the process, which can affect the crosslinking density [40,74]. We therefore speculate that the remnant collagen type I in the tracheal and auricular cartilage preparations may be the reason for these unusual results; possibly electrostatic interactions between collagen type I and collagen type II reduced the free amines, without affecting the mechanical properties. The observed differences were diminished under crosslinking and, in accordance to previous works [75], crosslinking increased the resistance to collagenase degradation and reduced the free amine content.

Biological analysis using hADSCs did not reveal any differences as a function of tissue origin, biological sex and crosslinking, which in agreement with previous publications that have been shown collagen type II scaffolds to support physiological cell growth [76] and 4-arm polyethylene glycol succinimidyl glutarate to be cytocompatible [43]. Histological analysis also demonstrated equal cell and ECM distribution throughout the scaffolds independently of the tissue and the biological sex of the extracted collagen, further confirming that the pore size and porosity were suitable to allow cell migration and colonisation, as has been shown before [71]. Similarly to mechanical properties, Alcian blue staining and sGAG quantification revealed a tissue (but not biological sex) dependent chondrogenesis. Specifically, both male and female articular cartilage sponges were significantly more chondrogenic than their tracheal and auricular cartilage counterparts. This can be attributed to the lack of collagen type I in the articular cartilage derived sponges. To substantiate this one should consider that a previous study has shown that collagen type I / collagen type II blends (3 to 1 ratio) had greater chondrogenic potential than pure collagen type I scaffolds [77] and other studies have shown higher chondrogenic potential of collagen type II scaffolds as opposed to collagen type I scaffolds in human bone marrow stem cells [54], bovine meniscus cells [78] and canine chondrocytes [52]. With respect to gene analysis, in general all collagen scaffolds were significantly more chondrogenic than the TCP, which can be attributed to their (three-) dimensionality; it is after all well established in the field the need of a three-dimensional environment for effective chondrogenic induction [79,80]. Between the groups, no differences were observed in mRNA levels of COL1A1, COL3A1, COL10A1 and COMP. It is interesting to note that male articular cartilage sponges induced higher SOX9 mRNA expression than female tracheal cartilage sponges, female articular cartilage sponges induced the highest ACAN mRNA expression and the male articular cartilage sponges induced the highest COL2A1 mRNA expression. Although it is clear that the male cartilage sponges follow the same trend as the mechanical properties and the histology with respect to tissue memory, we are not clear why the female articular cartilage sponges did not also follow the same trend with respect to COL2A1. One possible explanation could be the endpoint; although 21 days are traditionally used for chondrogenesis [81–83], other studies advocate longer timepoints to engineer mature cartilage-like tissue (e.g. 28 days [84–87], 42 days [88], 49 days [89], 56 days [90,91], 84 days [92]). Of course, the high variability between donors cannot be excluded.

One should also note that despite the positive data that have been obtained over the years with collagen type II scaffolds, their commercialisation has been jeopardised by early studies that showed injections of native collagen type II from human, chick, murine and bovine cartilage to induce inflammatory arthritis in rats [93–95]; native collagen type II from foetal bovine cartilage to induce arthritis in non-human primates [96]; and antibodies of native and denatured collagen type II to be present in patients with early rheumatoid arthritis and chronic gouty arthritis [97–99]. However, it has been shown that effectively

crosslinked collagen type II, even from bovine nasal septal cartilage, does not induce arthritis in rats [100]. Porcine collagen type II may be the ideal scaffold material for cartilage engineering, as *per os* administered porcine collagen type II has been shown to reduce pain in a rat osteoarthritis model [101] and porcine collagen type II scaffolds have been shown to maintain chondrogenic phenotype of rabbit [102], human [103] and ovine chondrocytes [104] and to promote the formation of neocartilage [105]. Based on data obtained from this study and previous studies of our groups with collagen type I, we feel that pepsin extracted (40) and 4-arm polyethylene glycol succinimidyl glutarate [42] stabilised articular cartilage derived collagen type II devices will have reduced immunogenicity and appropriate mechanical resilience, cytocompatibility and chondrogenic potential for cartilage engineering.

5. Conclusions

The use of collagen type II scaffolds for cartilage engineering has been supported, considering that cartilage is primarily comprised of collagen type II. As animal-derived products are of high variability, it is imperative to identify suitable collagen type II sources. This study suggests an extracted articular collagen type II memory of the tissue from which it was extracted. Specifically, our data advocate the use of male / female articular cartilage (as opposed to tracheal and auricular cartilage) derived collagen type II scaffolds for cartilage engineering applications, as judged by resistance to enzymatic degradation, biomechanical properties and chondrogenic differentiation potential.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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Authors' contributions

Concept: S.H.K., D.I.Z.; Methodology: Z.W., S.H.K., D.I.Z.; Experiments, Analysis: Z.W., S.H.K.; Writing, Reviewing, Editing: Z.W., S.H.K., D.I.Z.; Resources: A.M.M., I.S., A.T., D.I.Z.; Funding: A.M.M., D.I.Z.; Supervision: S.H.K., D.I.Z.; Discussion & Approving Manuscript: All Authors.

Declarations of Competing interest

The authors declare that they have no competing interests.

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