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# Enhanced cell-induced articular cartilage regeneration by chondrons; the influence of joint damage and harvest site

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SUMMARY

*Objective:* Interactions between chondrocytes and their native pericellular matrix provide optimal circumstances for regeneration of cartilage. However, cartilage diseases such as osteoarthritis change the pericellular matrix, causing doubt to them as a cell source for autologous cell therapy.
*Methods:* Chondrons and chondrocytes were isolated from stifle joints of goats in which cartilage damage was surgically induced in the right knee. After 4 weeks of regeneration culture, DNA content and proteoglycan and collagen content and release were determined.
*Results:* The cartilage regenerated by chondrons isolated from the damaged joint contained less proteoglycans and collagen compared to chondrons from the same harvest site in the nonoperated knee ($P < 0.01$). Besides, chondrons still reflected whether they were isolated from a damaged joint, even if they where isolated from the opposing or adjacent condyle. Although chondrocytes did not reflect this diseased status of the joint, chondrons always outperformed chondrocytes, even when isolated from the damaged joints ($P < 0.0001$). Besides increased cartilage production, the chondrons showed less collagenase activity compared to the chondrocytes.
*Conclusion:* Chondrons still outperform chondrocytes when they were isolated from a damaged joint and they might be a superior cell source for articular cartilage repair and cell-induced cartilage formation.
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## Introduction

Hyaline articular cartilage is a highly specialized tissue, which lines the bones within synovial joints such as the knee. It allows for smooth movement and is able to withstand compressive and shear forces and distribute them onto the subchondral bone[1–3]. Cartilage consists of an extracellular matrix (ECM) and a relatively small number of cells i.e., chondrocytes. This ECM is primarily composed of collagens (mainly type II collagen), proteoglycans, glycosaminoglycans, and glycoproteins[4].

When injured, articular cartilage has a limited potential of endogenous repair. Due to this characteristic, especially in weight bearing areas, damage results in an on-going process of progressive tissue damage. Untreated cartilage defects eventually lead to osteoarthritis[1–3,5].

Currently, autologous chondrocyte implantation (ACI) is increasingly being considered the standard of care for larger cartilage lesions[5,6]. In an ACI procedure, chondrocytes are traditionally harvested from a non-load bearing site in the affected knee during an arthroscopy. The chondrocytes are released from the matrix by enzymatic digestion, expanded and subsequently implanted in the focal cartilage defect. Although new tissue is formed in the defect and clinical results appear to be good, the quality of the regenerated tissue leaves room for improvement[7,8]. The repair site often contains fibrocartilaginous tissue, which is inferior to hyaline cartilage. This fibrous tissue has suboptimal mechanical properties compared to hyaline cartilage and is therefore more prone to degeneration.

Several studies have shown that chondrons produce cartilage of better hyaline quality as compared to fully isolated chondrocytes *in vitro*. Chondrons are defined as chondrocytes with their

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pericellular matrix[9–11]. This pericellular matrix consists of relatively high levels of type VI collagen compared to the interchondron ECM, but also contains type II, III, IX and XI collagen, hyaluronan, proteoglycans, glycosaminoglycans and glycoproteins[12–18].

It has been hypothesized that the profound interactions between chondrocytes and their native pericellular matrix provides a more favorable condition for cartilage matrix production, than chondrocytes without such a cell-matrix interaction[11]. In addition, it has been shown that the pericellular matrix protects chondrocytes from collagen-induced collagenase (MMP13) expression, an enzyme that can efficiently degrade collagen from the cartilage ECM, and thus leads to matrix collagen degradation[19,20]. From that point of view, it would be preferable to use chondrocytes with their pericellular matrix (chondrons) for cartilage repair instead of fully isolated chondrocytes.

Recently, there has been considerable debate on the optimal location to harvest cells for an ACI. Where it is not ideal to harvest from intact tissue as performed now, it may be that the affected cartilage from the rim of a defect has different regenerative capacities[21]. Besides, changes in the pericellular matrix have been suggested to represent one of the earliest identifiable matrix changes associated with cartilage diseases[20,22–26]. This would contra-indicate the use of chondrons from affected cartilage for articular repair strategies. Hence, the question is whether chondrons from damaged articular cartilage are still able to outperform isolated chondrocytes from these areas and maybe even from healthy areas.

In this study, we investigated whether chondrons obtained from damaged articular cartilage still have a higher regenerative capacity when compared to chondrocytes without their pericellular matrix. Chondrons and chondrocytes were isolated from cartilage of the stifle joints of goats 20 weeks after surgical induction of cartilage damage of the medial femoral condyle of the right knee (according to the Groove model)[27–29]. The left knee was left untouched. Chondrons and chondrocytes isolated from different locations (affected and non-affected) were compared.

## Materials and methods

### *Animals and model*

Nine skeletally mature milk goats (72.9 ± 2.9 kg, age 2.3 ± 0.2 years, all female) were obtained from a commercial Dutch breeder. During the entire experiment the animals were housed in two groups of four and five animals each and were allowed to walk freely in pens of approximately 20 square meters. There were no dietary restrictions and the goats had access to water ad libitum. The Utrecht University Medical Ethical Committee for animal studies approved the experiment (DEC2009.III.01.002). After a few weeks of acclimation, cartilage damage was introduced on the medial femoral condyle of the right stifle joints according to the Groove model[27–29]. Under general anesthesia, surgery was performed through a 3–5 cm medial incision close to the ligamentum patellae. Cartilage of the weight bearing area of the medial femoral condyle was surgically damaged. A maximum of 10 diagonal and longitudinal grooves were made with a K-wire that was bended at 0.4 mm of the sharp triangular tip, preventing damage of the underlying subchondral bone. Grooves were made under visual control in utmost flexion of the knee assuring no harm was done to the opposing tibial plateau. Afterwards, the synovial tissue, joint capsule and skin were sutured separately according to their anatomical layers. Pain medication and antibiotics were supplied until 3days post-operatively. One day postoperative, all animals were fully active and showed normal behavior. The contralateral stifle joints were left untouched and served as paired inter-articular controls. Twenty weeks (5 months) later the goats were euthanized and both hind limbs amputated. By scoring stained sections[30] according to the Osteoarthritis Research Society International (OARSI) criteria[31], it was verified that the medial femoral condyle of the right knee showed clear signs of cartilage damage and the opposing tibial plateau showed less severe damage, while both the lateral compartments and non-operated knees showed no damage[32].

### *Isolation and culture of cells*

Directly after removal of both hind limbs from the goats, the joints were opened under laminar flow conditions and full thickness cartilage samples, excluding the underlying bone, were taken from predefined locations. Articular cartilage samples were taken from the medial femoral condyles of both legs, lateral femoral condyles of both legs, medial sides of the tibial plateaus of both legs and lateral sides of the tibial plateaus. Samples from the respective joint locations were analyzed separately. Subsequently, one half from each location (randomly taken) was used for chondrocyte isolation and the other half for chondron isolation.

For chondrocyte isolation, the tissue fragments were subjected to sequential treatments of Dulbecco's modified Eagle's medium (DMEM, Gibco, Paisley, UK) supplemented with 1% fetal bovine serum (FBS, HyClone, Logan, UT), 100 U/ml penicillin, 100 μg/ml streptomycin (all from Gibco) and 2.5% (w/v) Pronase E (Sigma, St. Louis, MO) for 1 h, then with DMEM supplemented with 25% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin and 0.125% (w/v) collagenase (CLS-2, Worthington, Lakewood, NJ) for 16 h at 37°C.

For chondron isolation, minced cartilage was digested with 0.3% (w/v) dispase (Gibco) plus 0.2% (w/v) collagenase in phosphate buffered saline (PBS, Gibco) supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin for 5 h at 37°C[33].

The cells were filtered through a 100 μm cell strainer (BD Biosciences, San Diego, CA) and washed. The cells that were not directly processed for analysis were seeded at a density of $1.6 \times 10^6$ cells/cm$^2$ on Millicell filters (0.4 μm polytetrafluoroethylene (PFTE) (Millipore, Bedford MA)) that were precoated with type II collagen (type II collagen from chicken sternal cartilage (Sigma))[34,35]. The filters were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin and 50 μg/ml ascorbate-2-phosphate (Sigma).

Cultures were incubated at 37°C in 5% $CO_2$ and culture media were renewed every 3 days.

### *Pericellular matrix staining*

Cytospin slides were prepared using freshly harvested chondrons and chondrocytes by cytocentrifugation (500 rpm, 5 min) (Thermo Fisher Scientific Inc., Waltham, MA) and fixed for 30 min with a 4% buffered formaldehyde solution. The cytospins were stained using Safranin O-fast green[30].

### *Papain digestion*

After 4 weeks of culture, samples were digested at 60°C for 18 h in a papain enzyme solution consisting of 5 mM L-cysteine, 50 mM $Na_2$EDTA, 0.1 M NaAc, pH 5.53 with 2% (v/v) papain (Sigma).

### *Proteoglycan analysis*

To analyze the proteoglycan content of the regenerated cartilage tissue, a dimethylmethylene blue (DMMB) spectrophotometric analysis was performed[36]. The papain digest or medium sample was mixed with the DMMB solution and the absorbance was read

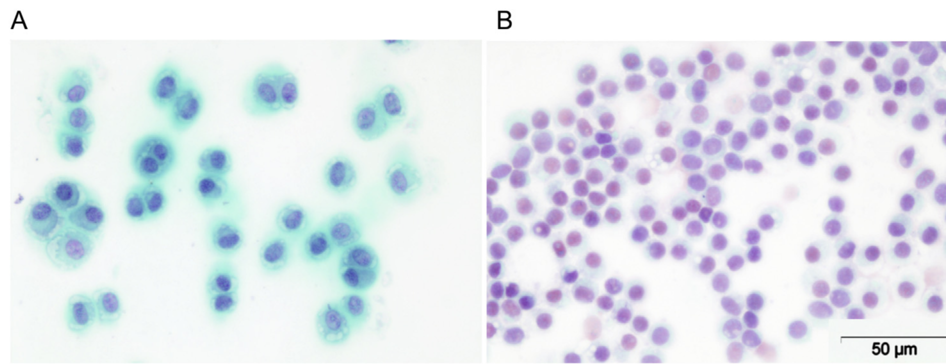


**Fig. 1.** Safranin-O staining of the pericellular matrix of isolated chondrons (A) and chondrocytes (B). Magnification ×200.

at 540 nm and 595 nm. As reference, chondroitin sulfate C (Sigma) was used. Total proteoglycan was defined as the amount of proteoglycans found in the papain digest and the total amount of proteoglycans released into the culture medium.

### DNA analysis

Total DNA was quantified in papain digest using Quant-iT Picogreen (Invitrogen) according to the manufacturer's instructions. Picogreen reagens was added to papain digest. This was incubated at ambient temperature for 5 min, protected from light. The fluorescence was measured at ~480 nm excitation and ~520 nm emission and DNA content determined using lambda DNA as standard.

### Collagen analysis

To analyze the collagen content, hydroxyproline content was determined in papain digest or medium samples using a modified colorimetric assay[37]. In short, freeze-dried papaine digests or medium samples were hydrolyzed and the free hydroxyprolines were oxidized with Chloramine-T for the production of pyrroles. The addition of Ehrlich's reagent resulted in the formation of chromophores that were measured at 550 nm and collagen content was determined using gelatin as standard. The total amount of collagens is defined as the amount of collagens found in the papain digest and the amount of collagens released into the culture medium.

### Collagenase activity assay

To analyze collagenase activity, the Enzcheck Gelatinase/Collagenase Assay Kit (Invitrogen) was used according to the manufacturer's instructions. DQ Collagen Fluorescein conjugate was added to 100 times in reaction buffer (kit component) diluted conditioned medium. This was incubated for 4 h at ambient temperature, protected from light. The fluorescence was measured at ~480 nm excitation and ~520 nm emission and collagenase activity was determined using collagenase type IV from Clostridium histolyticum (kit component) as standard.

### Statistical analysis

Data are expressed as mean ± 95% confidence interval (CI). Data were analyzed statistically using SPSS version 22.0.0. Normal distribution of the data was verified by plotting a frequency distribution histogram and a Shapiro–Wilk test. Differences were tested by a repeated-measures analysis of variance (ANOVA).

Differences in ratios were tested with a paired Student's $t$ test after log transformation (correction for skewing).

## Results

### Pericellular matrix staining

To verify how much pericellular matrix was left after the chondrocyte and chondron isolation, a safranin-O staining was performed on cytospins made of the isolated cells. The chondrons showed a clear green staining around the cells [Fig. 1(A)], indicating the presence of collagen pericellularly. The chondrocytes isolated from the articular cartilage showed no clear staining around the cells [Fig. 1(B)].

### Chondrons produce tissue with a higher proteoglycan and collagen content

The tissue that was produced by the chondrons over a period of 4 weeks in culture contained significantly more proteoglycans and

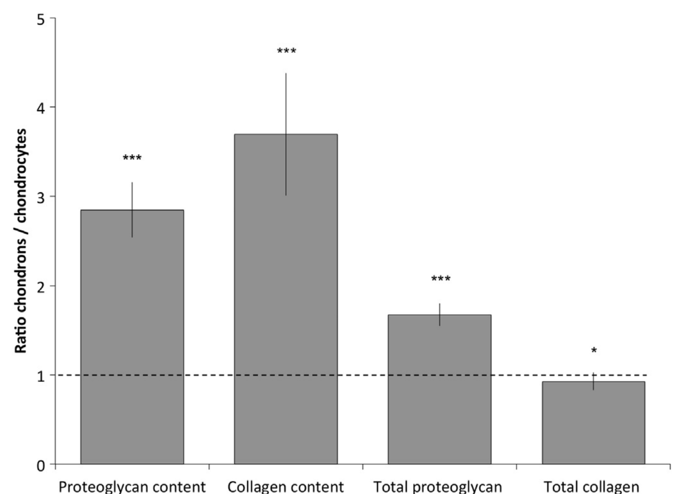


**Fig. 2.** Regeneration cultures containing chondrons and chondrocytes isolated from the medial and lateral condyles from a grooved right and non-operated left knee from the femoral and tibial plateaus of nine goats were digested and analyzed for proteoglycan (determined as glycosaminoglycans, normalized for DNA) and collagen content (determined as hydroxyproline, normalized for DNA), and total amount of proteoglycans and collagen. Results are presented as the mean ± 95% CI of the ratio of the amount of chondrons vs chondrocytes. Per harvest location[8] from each goat[9], the amount of the chondrons was divided by the amount of the chondrocytes before calculating the mean, resulting in $N = 72$. *: $P = 0.020$; ***: $P < 0.0001$. Absolute values of the various harvest locations can be found in Table I.

collagen compared to the tissue produced by the chondrocytes (Fig. 2). Also the total amount of proteoglycans produced, adding up proteoglycans released in the medium to those retained in the tissue, and likewise the total amount of collagen produced by the chondrons was higher compared to the chondrocytes (Fig. 2).

### *Chondrons reflect whether they were isolated from a healthy or damaged joint*

The matrix that was produced by the chondrons isolated from the surgically damaged location, the medial femoral condyle of the right knee, contained less proteoglycans and collagen compared to the chondrons isolated from the same location in the left knee [Fig. 3(A) and (B)]. Chondrons from the surgically untouched lateral femoral condyle of the right knee produced more proteoglycans and collagen compared to the 'grooved' medial femoral surface chondrons, but less compared to the chondrons from the lateral femoral condyle of the nonoperated left knee [Fig. 3(A) and (B)]. For chondrocytes, no differences were observed in proteoglycan and collagen content between the different locations from the femoral condyle including the grooved and the non-operated knee [Fig. 3(A) and (B)].

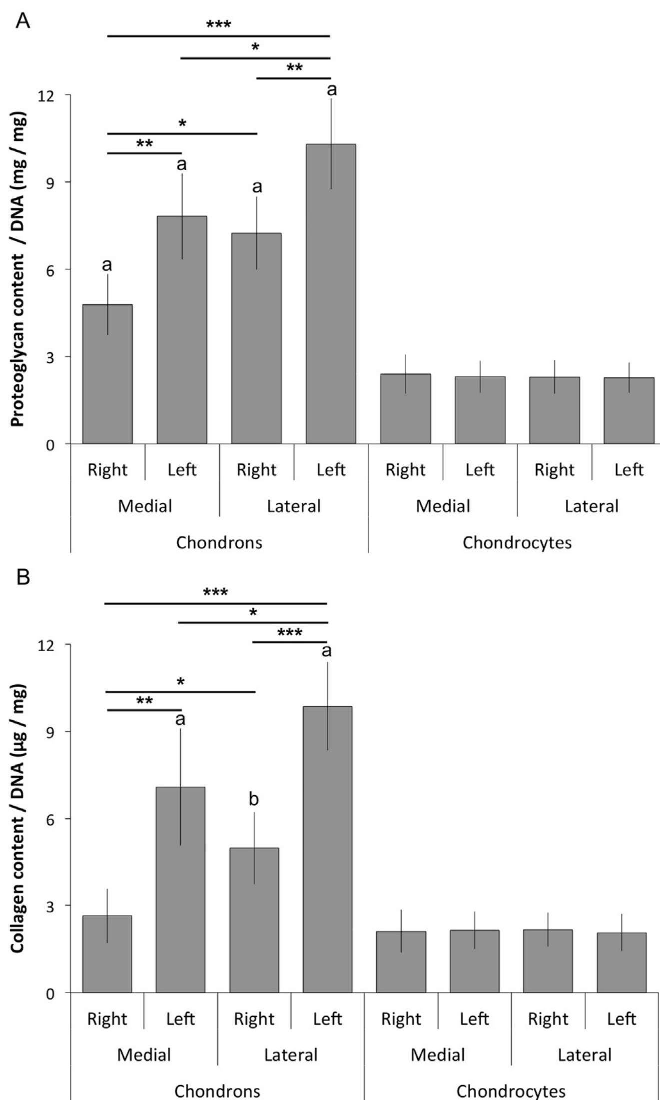


**Fig. 3.** Regeneration cultures containing chondrons and chondrocytes isolated from the medial and lateral condyles from an grooved right and non-operated left knee from the femoral condyles were digested and analyzed for proteoglycan content (A, determined as glycosaminoglycans, normalized for DNA) and collagen content (B, determined as hydroxyproline, normalized for DNA). Data are shown as mean ± 95% CI. $N = 9$. *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$; b: $P < 0.01$ compared to chondrocytes; a: $P < 0.001$ compared to chondrocytes.

Tissue regenerated by chondrons from the damaged medial femoral surface of the right knee contained more proteoglycans ($P = 0.009$) and collagen ($P = 0.034$) compared to tissue from chondrocytes from the medial femoral surface of the left knee. The proteoglycan content of the produced tissue was higher for the chondrons from the damaged location compared to chondrocytes from a healthy location ($P = 0.002$), but no differences in collagen content were observed ($P = 0.313$) [Fig. 3(A) and (B), Table I].

For the opposing tibial plateau, the same pattern was observed (Table I); the proteoglycan ($P = 0.0005$) and collagen content ($P = 0.004$) of chondron-derived tissues was higher for the non-operated left knee compared to the operated right knee. In addition, proteoglycan and collagen content from tissue produced by chondrons isolated from the lateral condyle were higher compared to those from the medial condyle ($P = 0.029$ and $P = 0.024$), while chondrocytes showed no differences at all between harvest locations.

### *Collagen release is higher by chondrocytes*

Collagenase activity and collagen release was higher in the culture medium of the chondrocytes compared to that of the chondrons [Fig. 4(A) and (B)]. For the various compartments, no differences were found in the collagenase activity in the chondrocyte medium. However, the activity was higher in the chondrocyte culture medium from the femoral condyles compared to that of the tibial plateau ($P < 0.0001$) (Table I). Only the culture medium of the chondrons that were isolated from the damaged location on the medial femoral condyle of the right knee showed higher collagenase activity compared to the other locations [Fig. 4(A)], albeit lower than that of the chondrocytes from the same location.

The collagen release into the culture medium shows the same pattern as the collagenase activity in the culture medium [Fig. 4(B)]. The collagen assay measures both intact collagen as well as degraded collagen fragments. The amount of collagen released into the culture medium of the chondrocytes was higher compared to the amount found in the medium of the chondrons. However, the culture medium of the chondrons from the damaged location on the medial femoral condyle of the right knee revealed a higher collagen release compared to the other locations [Fig. 4(B)].

No differences in proteoglycan release into the culture medium were found (Table I).

### *Differences between harvest locations independent of cartilage damage*

The matrix produced by the chondrons contained more proteoglycans and collagen when the chondrons were isolated from a lateral condyle compared to a medial condyle [Fig. 5(A)]. This also goes for the total amounts of proteoglycan and collagen [Fig. 5(A)]. No differences were found between proteoglycan and collagen content, neither in total proteoglycan and collagen, by chondrocytes isolated from a lateral or medial condyle [Fig. 5(A)].

For tissue produced by chondrons and chondrocytes, the proteoglycan and collagen content, and the total amount of proteoglycan and collagen was higher when they were isolated from a femoral plateau compared to a tibial plateau [Fig. 5(B)].

**Table I**
Absolute values of proteoglycan, collagen and collagenase measurements normalized for DNA

| | | | | PG content | PG release | Total PG | Col content | Col release | Total Col | CLG activity |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Mean ± 95% CI | Mean ± 95% CI | Mean ± 95% CI | Mean ± 95% CI | Mean ± 95% CI | Mean ± 95% CI | Mean ± 95% CI |
| Chondrons | Femur | Medial | Right | 4.78 ± 1.05 | 8.01 ± 1.62 | 12.79 ± 2.56 | 2.64 ± 0.93 | 8.69 ± 1.41 | 11.33 ± 1.99 | 0.29 ± 0.09 |
| | | | Left | 7.82 ± 1.48 | 6.48 ± 1.04 | 14.30 ± 1.68 | 7.09 ± 2.02 | 5.07 ± 0.75 | 12.16 ± 2.18 | 0.16 ± 0.08 |
| | | Lateral | Right | 7.24 ± 1.25 | 8.06 ± 1.07 | 15.29 ± 2.01 | 4.98 ± 1.25 | 5.50 ± 0.64 | 10.48 ± 1.84 | 0.15 ± 0.05 |
| | | | Left | 10.30 ± 1.56 | 7.04 ± 1.33 | 17.34 ± 2.48 | 9.86 ± 1.53 | 5.51 ± 0.86 | 15.37 ± 2.32 | 0.16 ± 0.06 |
| | Tibia | Medial | Right | 1.98 ± 0.31 | 4.64 ± 0.38 | 6.61 ± 0.51 | 0.37 ± 0.25 | 0.70 ± 0.13 | 1.06 ± 0.25 | 0.05 ± 0.01 |
| | | | Left | 3.13 ± 0.35 | 4.17 ± 0.49 | 7.30 ± 0.77 | 1.01 ± 0.29 | 0.82 ± 0.13 | 1.83 ± 0.36 | 0.06 ± 0.01 |
| | | Lateral | Right | 2.59 ± 0.56 | 4.07 ± 0.71 | 6.66 ± 1.05 | 0.63 ± 0.36 | 0.70 ± 0.26 | 1.33 ± 0.46 | 0.05 ± 0.02 |
| | | | Left | 3.91 ± 0.98 | 4.05 ± 0.72 | 7.96 ± 1.23 | 1.53 ± 0.48 | 0.83 ± 0.28 | 2.36 ± 0.58 | 0.05 ± 0.02 |
| Chondrocytes | Femur | Medial | Right | 2.39 ± 0.67 | 7.07 ± 1.44 | 9.47 ± 1.60 | 2.11 ± 0.74 | 11.81 ± 1.20 | 13.93 ± 1.90 | 0.50 ± 0.09 |
| | | | Left | 2.30 ± 0.56 | 6.41 ± 1.13 | 8.71 ± 1.07 | 2.15 ± 0.65 | 12.40 ± 1.48 | 14.55 ± 2.05 | 0.50 ± 0.10 |
| | | Lateral | Right | 2.30 ± 0.57 | 6.65 ± 1.48 | 8.95 ± 1.25 | 2.18 ± 0.59 | 13.54 ± 0.84 | 15.71 ± 1.35 | 0.53 ± 0.15 |
| | | | Left | 2.27 ± 0.52 | 6.13 ± 1.43 | 8.40 ± 1.45 | 2.07 ± 0.64 | 12.89 ± 1.24 | 14.96 ± 1.82 | 0.52 ± 0.15 |
| | Tibia | Medial | Right | 1.38 ± 0.33 | 3.31 ± 0.88 | 4.69 ± 1.06 | 0.22 ± 0.07 | 1.74 ± 0.60 | 1.96 ± 0.67 | 0.09 ± 0.05 |
| | | | Left | 1.49 ± 0.32 | 3.36 ± 0.85 | 4.86 ± 1.06 | 0.24 ± 0.10 | 1.42 ± 0.22 | 1.67 ± 0.23 | 0.08 ± 0.01 |
| | | Lateral | Right | 1.41 ± 0.45 | 3.31 ± 0.80 | 4.71 ± 1.06 | 0.26 ± 0.14 | 1.62 ± 0.33 | 1.88 ± 0.45 | 0.09 ± 0.04 |
| | | | Left | 1.39 ± 0.47 | 3.39 ± 1.19 | 4.78 ± 1.34 | 0.24 ± 0.07 | 1.64 ± 0.32 | 1.88 ± 0.38 | 0.08 ± 0.01 |

PG: proteoglycan; Col: collagen; CLG: collagenase.

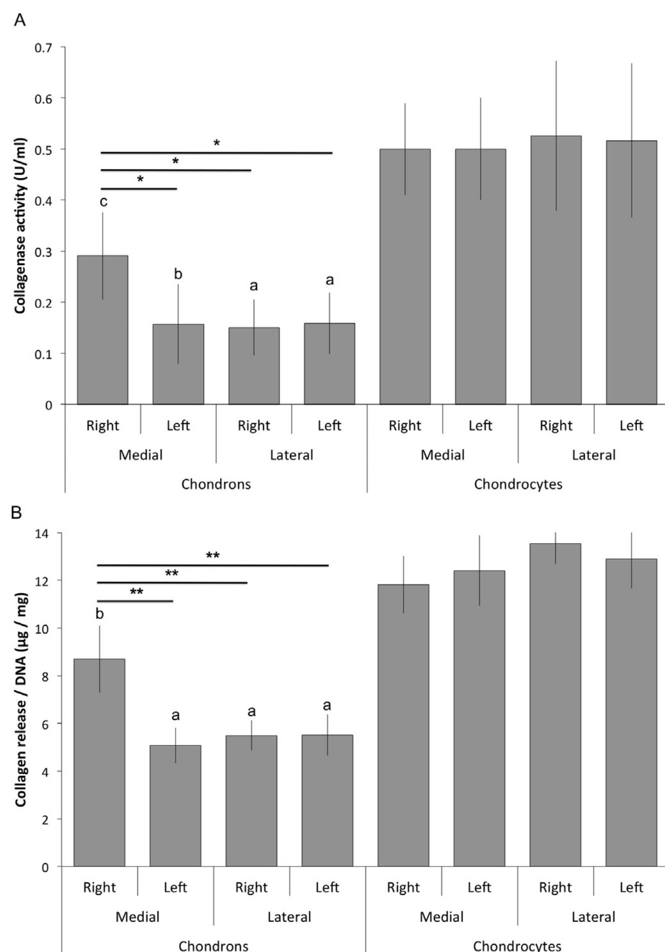


**Fig. 4.** Collagenase activity (A) and collagen released (B, determined as hydroxyproline, normalized for DNA) in the culture medium of regeneration cultures containing chondrons and chondrocytes isolated from the medial and lateral femoral condyles from an grooved right and non-operated left knee were measured. Data are shown as mean ± 95% CI. $N = 9$. *: $P < 0.05$; **: $P < 0.01$; c: $P < 0.05$ compared to chondrocytes; b: $P < 0.01$ compared to chondrocytes; a: $P < 0.001$ compared to chondrocytes.

## Discussion

In this study we have shown that chondrons perform better in hyaline cartilage production on a cell-by-cell basis *in vitro* compared to chondrocytes, even when they are isolated from damaged cartilage. Chondrons isolated from damaged cartilage were even shown to perform better compared to chondrocytes isolated from healthy cartilage. It was already known from previous studies that chondrons produce better hyaline cartilage compared to chondrocytes[9–11]. However, this is the first study that also demonstrates superior regeneration for chondrons isolated from affected cartilage. Since many, if not all joints treated by ACI have some form of disturbed joint homeostasis[5,38], an improved regenerative capacity under these conditions may improve outcome.

The present study is part of a research line that focuses on developing a one-stage ACI in which primary, and not cultured, chondrocytes are used, whether or not co-implanted with off-the-shelf allogeneic mesenchymal stromal cells[5]. From our present study, it seems that for such an approach chondrons may outperform primary chondrocytes harvested from non-weightbearing locations, and confirms that damaged (debrided) tissue can be used.

However, tissue production by chondrons does reflect whether they were isolated from the grooved or the non-operated knee. This was not only the case for the chondrons isolated from the grooved location on the medial condyle of the right knee, but also for chondrons from the other locations of the operated right knee.

Notwithstanding, the regenerative capacity of chondrons isolated from damaged areas was less than from none-damaged areas. In this respect, inferior regenerative capacity will be the result of the disturbed tissue homeostasis of these damaged areas[5,38]. It is striking however, that this was only reflected by the chondrons and not by the chondrocytes, suggesting the pericellular matrix plays an important role in this impaired activity. One of the roles of the pericellular matrix is to function as a storage unit for newly formed proteins[15–17]. In disturbed tissue homeostasis, the expression of cytokines and cartilage degrading enzymes is altered[5,38]. The pericellular matrix of chondrons isolated from the damaged cartilage locations may still contain these cytokines and enzymes, which subsequently affected the *ex vivo* cultures. Changes to the pericellular matrix itself[20,22–26] might have also altered the cartilage metabolism *in vitro*.

The differences found in the proteoglycan content of the regenerated tissue are due to differences in proteoglycan production,

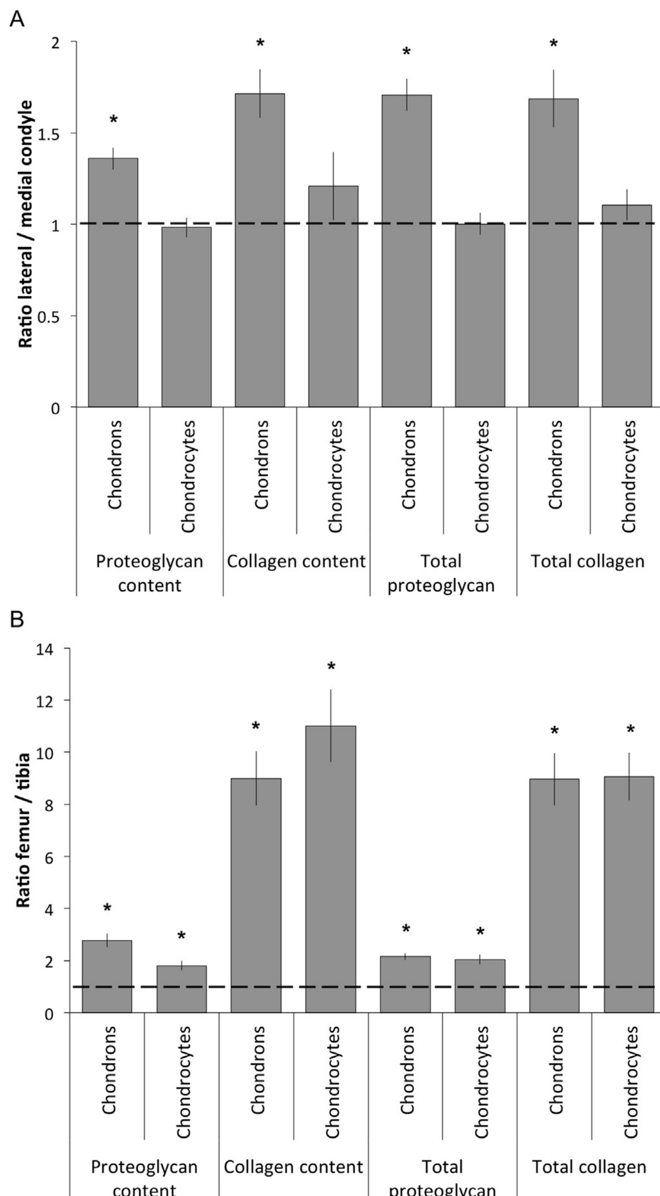


**Fig. 5.** Regeneration cultures containing chondrons and chondrocytes isolated from the medial and lateral condyles from a grooved right and non-operated left knee from the femoral and tibial plateaus of nine goats were digested and analyzed for proteoglycan (determined as glycosaminoglycans, normalized for DNA) and collagen content (determined as hydroxyproline, normalized for DNA), and total amount of proteoglycans and collagen. Results are presented as the mean ± 95% CI of the ratio of the amount of the lateral locations vs tibial locations (A) and femoral vs tibial plateaus (B). Per harvest location[4] from each goat[9], the amount of the lateral locations was divided by the amount of medial locations (A) and the amount of the femoral condyles was divided by the amount of the opposing tibial plateaus (B) before calculating the mean, resulting in $N = 36$. *: $P < 0.0001$. Absolute values of the various locations can be found in Table I.

not to differences in proteoglycan release into the medium. As no differences were observed in the proteoglycan release, it is likely that grooving the cartilage surface has no effect on chondrocyte mediated proteoglycan degradation, at least not when cells are isolated from this cartilage and cultured *in vitro*. *In vivo*, the damage demonstrated enhanced proteoglycan release, which might be directed by mechanical and cellular processes[32]. When chondrons are isolated from a damaged joint, such as in the grooved cartilage surface model used here[27–29], the total proteoglycan production is lower compared to isolation from a healthy joint. However, this production remains higher than that of chondrocytes.

For collagen, differences were found in production and collagen release between chondrocytes and chondrons and between the various harvest locations. The initial amounts of collagen and proteoglycan of chondrons and chondrocytes at the time of seeding were not taken into account in this study as it has been shown that for up to 4 million cells, no differences in the amounts of collagen and proteoglycans can be detected[9–11]. Here, the initial seeding density was 720,000 chondrons or chondrocytes per collagen coated filter. The filters were coated with 3.125 μg type II collagen and unfortunately it was not possible to discriminate between the collagen used for the coating and the collagen produced by the cells. However, the lowest amount of tissue collagen (uncorrected for DNA) found in the cultures was 25 μg. So although we cannot distinguish between total collagen produced and collagen coating, more than 85% of the total collagen was produced by the cells.

The relatively high collagenase activity measured in the culture medium of the chondrocytes is reflected by the relatively high collagen release into the culture medium. This was also showed for chondrons isolated from the grooved location on the medial condyle of the right knee, suggesting that grooving has an effect on collagenase activity. However, this phenomenon was not seen in the cultures of the chondrons from the other locations of the grooved right knee.

That in general, chondrons show a lower collagenase activity than chondrocytes can be explained by the finding that pericellular matrix prevents collagen-induced collagen degradation by protecting against recognition by the integrin alpha 1 and discoidin domain receptor 2[19,20]. In line with this we found that the chondrocytes released more collagen in the culture medium and had a higher collagenase activity. Although chondrons reflected whether they were isolated from the grooved location or not, they do outperform chondrocytes in terms of cartilage production.

Next to being isolated from normal or damaged cartilage, chondrons also display a difference between isolation from the medial and the lateral portions. Again, the proteoglycan and collagen contents were higher for the chondrons isolated from the lateral locations compared to the medial ones. However, this difference was not reflected by the collagenase activity and the proteoglycan and collagen release. It is unknown why this difference between the lateral and medial location is observed. As most studies performed with chondrocytes and chondrocytes do not look at the harvest site, this phenomenon may not have been observed previously. It is known that, just as bone and muscle, cartilage adapts to mechanical loading. Excessive or low mechanical loading decreases proteoglycan and collagen content of cartilage, whereas moderate mechanical loading can strengthen the cartilaginous tissue[39–41]. In ovine knee joints, the medial-lateral load distribution across the tibial condyles is approximately 75% on the medial condyle[42]. Given the anatomical characteristics of the ovine and goat knee joint, it is likely that in goats, there is also predominantly medial loading. The cell cultures in the current study were not loaded at all. Since the biomechanical load in medial compartments is higher *in vivo*, the chondrons may have responded stronger to the absence of loading compared to the chondrons from the lateral compartments, resulting in lower proteoglycan and collagen content. In line with this, the chondrons from the tibial locations might also have responded stronger to the absence of loading compared to the femoral chondrons as *in vivo*, at least in humans, the compression is higher and more constant in tibial cartilage[43,44]. This suggests that for *in vitro* tissue engineering purposes, chondrons should preferably be isolated from the lateral femoral condyle. For transplantation purposes it is hard to predict, since *in vivo* mechanical loading is present. It might be worth to

investigate *in vitro* cartilage formation by isolated chondrons from surgically induced cartilage damage to the lateral condyle with or without mechanical loading.

Since chondrons outperform chondrocytes, they seem the preferred cells for a cell-based cartilage repair treatment. However, the number of cells that can be obtained remains a challenge. In fact, it seems impossible to expand chondrons because they will lose their pericellular matrix, thus becoming chondrocytes. The newer generation of ACI uses a three-dimensional matrix to assist the implantation of expanded chondrocytes[5,45]. A single-step procedure in which isolated cells are used immediately after isolation would be more desirable, also with respect to the burden to the patient. Still, the number of chondrons that can be isolated seems to be the limiting factor. A possible approach to overcome this challenge would be mixing chondrogenic cells such as chondrons with cells with multilineage potential[5,46,47]. The first as such clinical trial has recently been started; the IMPACT trial (NCT 02037204, www.clinicaltrials.gov) uses autologous chondrons derived from cartilage harvested from the defect rim combined with allogeneic mesenchymal stromal cells[5].

In conclusion, although chondrons that were isolated from damaged cartilage show more catabolic and less anabolic activities compared to chondrons from healthy cartilage, they still outperform chondrocytes isolated from healthy areas.

## Author contributions

Conception and design: LV, LC, DS.
Goat surgeries: FL, SM.
Collection and assembly of data: LV, TW, AK, MB.
Analysis and interpretation of the data: LV, TW, LC, DS.
Drafting of the article and reviewing: LV, TW, WD, FL, LC, DS.
Final approval of submitted version: LV, TW, AK, MB, SM, WD, FL, LC, DS.

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The funding source had no role in study design, collection, analysis or interpretation of data, in writing the manuscript or in submitting the manuscript.

## Conflict of interest

The authors declare that they have no competing interests.

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