



The effect of synovial fluid  
from injured knee joints on  
*in vitro* chondrogenesis

# Chapter 07





## Abstract

**Goal:** Synovial fluid (SF) plays a major role in the nutrition and metabolic status of articular cartilage, and, therefore, may play a significant role in the detrimental effect of a disturbed joint homeostasis on cartilage regeneration. The goal of the current study was to investigate the effect of human SF from injured knee joints on *in vitro* chondrogenesis by expanded human chondrocytes.

**Methods:** Chondrocytes isolated from healthy cartilage biopsies were expanded in monolayer under GFSEC for 2 passages and were subsequently redifferentiated on collagen-coated filters for 14 days either in the presence or absence of 10% SF from injured knee joints.

**Results:** SF supplementation resulted in a significant downregulation of final proteoglycan (PG) content ( $p = 0.0001$ ), PG content normalized to DNA ( $p < 0.05$ ), collagen type II mRNA levels ( $p < 0.001$ ), and differentiation index ( $p < 0.001$ ) as compared to control culture conditions. Additionally, SF-supplemented media resulted in significantly increased cellularity compared with control media ( $p < 0.0001$ ). Morphology, and collagen type I, X, and aggrecan mRNA levels were not significantly affected.

**Conclusion:** This study demonstrates that SF from injured human knee joints significantly affects *in vitro* chondrogenesis and therefore may provide a viable target for future improvement of ACI by refinement of culture techniques, patient selection, or pretreatment of affected joints to restore joint homeostasis.



## Introduction

Synovial fluid (SF) contains factors that are essential for nutrition and metabolic chondrocyte activity. SF is a dialysate of blood plasma with the addition of components synthesized by synovial tissue and catabolic products from the surrounding tissues, e.g. PG and collagen breakdown products<sup>270</sup>. Furthermore, SF contains growth factors and, dependent on the status of the joint, pro- and anti-inflammatory cytokines<sup>60,271,272</sup>. Therefore, synovial fluid may also affect chondrogenesis in joints with cartilage defects. This has consequences for regenerative medicine since the SF may affect the quality of the biopsy region prior to *in vitro* expansion, but may also play an important role in the detrimental environment the cells encounter on reimplantation in joints with prolonged cartilage defects<sup>37,38</sup>. However, relatively little is known about the role of SF in cartilage turnover and differentiation, in particular during cartilage regeneration after trauma.

The goal of this study was to investigate the effect of human SF from injured knee joints on *in vitro* chondrogenesis by expanded human chondrocytes in a previously validated culture model<sup>273</sup>. Collagen types I and II and aggrecan mRNA levels were determined to evaluate the effect of SF on the degree of re-differentiation. Collagen type X mRNA levels were measured to detect hypertrophic changes<sup>248-251</sup>. Tissue morphology, PG content, and cell content of the extracellular matrix (ECM) were determined to assess the effect of SF on the final tissue quality.

## Materials and methods

### Synovial fluid collection

Synovial fluid (SF) samples were collected from 12 patients, either during arthroscopy or in the outpatient clinic. Demographic and clinical data of these samples are summarized in table 1. Defect ages were determined from patient history. Diagnoses were determined either by MRI or arthroscopically. The donors had no clinical history of inflammatory or degenerative joint disorders and did not use corticosteroids (systemically or intra-articularly). After aspiration, the samples were spun at 300 x g to remove debris and stored in small aliquots at -80°C until further use.

### Culture methods

Articular cartilage was harvested from a femoral condyle of a 48 year-old human female donor. The chondrocytes were expanded for 2 passages under growth factor supplemented expansion conditions (GFSEC). After the chondrocytes were expanded for 2 passages, the chondrocytes were redifferentiated on collagen type II-coated filters in two groups: (A) Control group: "standard" redifferentiation culture as described in chapter 4, or (B) Identical to control condition, but with 10% synovial fluid supplemented to the culture media.

The culture media were renewed at 7 and 10 days of culture. The samples were



harvested after 14 days of culture. For each analysis method, 3 samples per SF donor were cultured. The results of the present study were confirmed by repeating the experiment with chondrocytes isolated from a 51 year-old human male donor. Results that were not consistent with results observed in the first experiment are mentioned explicitly in the results section.

#### **Analytical methods**

After 14 days of redifferentiation culture, proteoglycan content, cell content and tissue morphology (Safranin O / fast green) were determined in order to assess the outcome of the two culture conditions. The collagen type I, II, X and aggrecan mRNA levels were determined by real-time PCR. Since collagen type II is one of the typical structural proteins synthesized by differentiated chondrocytes in hyaline cartilage, as compared to collagen type I that is normally synthesized by dedifferentiated chondrocytes, we defined the ratio of collagen type II and collagen type I mRNA levels as "differentiation index".

#### **Statistical analysis**

The effect of synovial fluid supplementation compared to no supplementation was analyzed using a separate-variance T-test. If non-normal distribution or unequal variance was observed among the treatment groups, that specific variable was analyzed by a Wilcoxon Rank Sum test. The graphs show average values in either treatment group; p-values lower than 0.05 were considered statistically significant. Statistical calculations were done with the JMP (Cary, NC, USA) version 5.0 software package.

For further details of the materials and methods used, please refer to appendix B and C.



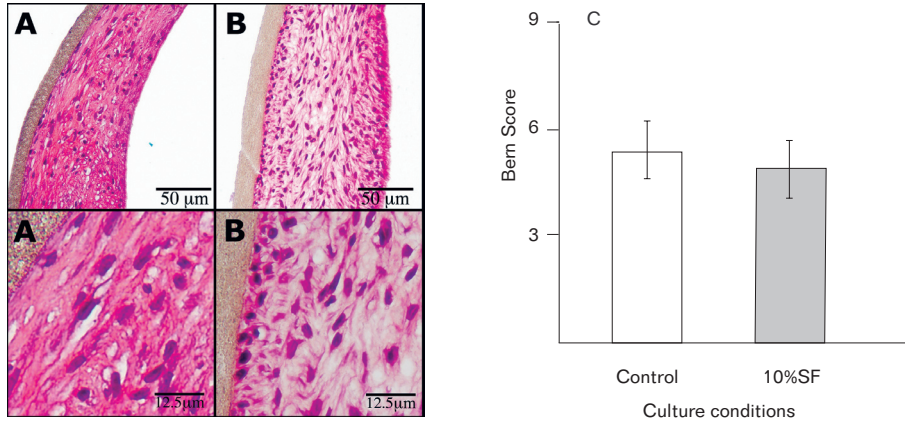
**Table 1**  
Demographic data of synovial fluid samples

Patient No.	Patient age [Years]	Defect age [Days]	Diagnosis
1	27	5	Knee distortion with pain and swelling: no further tissue damage demonstrated
2	43	7	Knee distortion with pain and swelling: no further tissue damage demonstrated
3	23	8	Cartilage defect: Medial Femoral condyle
4	47	9	Knee distortion with pain and swelling: no further tissue damage demonstrated
5	28	14	Medial meniscus rupture
6	36	14	Cartilage defect: Medial Femoral condyle
7	20	31	Cartilage defect: Medial Femoral condyle and anterior cruciate ligament rupture
8	33	39	Cartilage defect: Medial Femoral condyle
9	46	254	Cartilage defect: Medial Femoral condyle
10	46	341	Cartilage defect: Medial Femoral condyle and medial meniscus rupture
11	44	885	Cartilage defect: Medial Femoral condyle
12	46	>1000	Cartilage defect: Medial Femoral condyle

## Results

### Tissue morphology

Both culture with and without SF supplementation resulted in the synthesis of fibrocartilaginous tissue by chondrocytes. The extracellular matrix stained positive with Safranin O in all samples and most chondrocytes had a rounded appearance, although a substantial part of the cells had a fibroblastic appearance and lacked lacunae, typical for native chondrocytes in articular cartilage (Figure 1A and B). No differences in tissue morphology between both treatment groups were observed. In order to objectify this finding, three blinded observers scored the morphological quality of the tissue synthesized according to the "Bern score". No statistically significant differences were found between both treatment groups, tissue synthesized under control conditions scoring  $4.9 \pm 0.7$  points versus tissue synthesized in SF supplement culture media, which scored  $4.6 \pm 0.8$  points (Figure 1C).



**Figure 1**

5 µm histological sections with safranin O / fast green staining after 14 days of culture of tissue synthesized by chondrocytes redifferentiated in (A) control media and (B) culture media supplemented with 10% synovial fluid. Original magnification: 40X. (C) "Bern" histological score after 14 days of culture. Bars represent average ± SD.

### Proteoglycan synthesis and DNA content

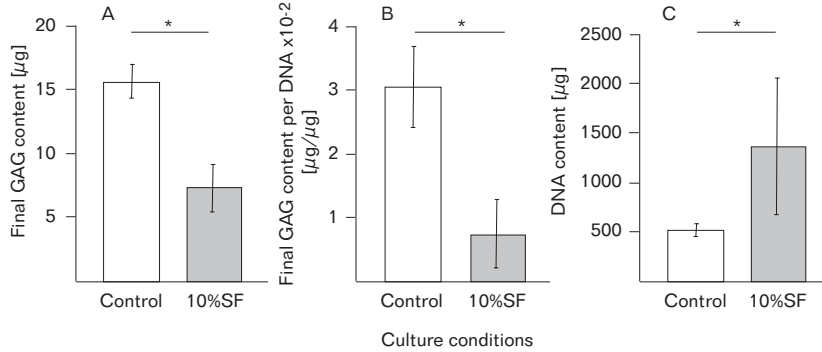
Extra-cellular matrix (ECM) synthesis as measured by the final PG content of the tissue was significantly affected by the supplementation of SF to the culture media. Chondrocyte redifferentiation culture under control conditions resulted in a significantly higher amount of PGs synthesized after 14 days of culture compared to SF-supplemented culture conditions ( $15.6 \pm 1.3$  mg versus  $7.3 \pm 1.8$  mg respectively;  $p = 0.0001$ ; Figure 2A). PG content normalized to DNA content showed a similar significant downregulation by SF-supplemented culture conditions compared to control culture conditions ( $0.7 \pm 0.5$  mg/µg versus  $3.0 \pm 0.6$  mg/µg respectively;  $p < 0.05$ ; Figure 2B).

Chondrocyte redifferentiation under SF-supplemented culture conditions resulted in a significantly higher DNA content after 14 days of culture compared to control media ( $1369 \pm 683$  µg versus  $514 \pm 72$  µg respectively;  $p < 0.0001$ ; Figure 2C).

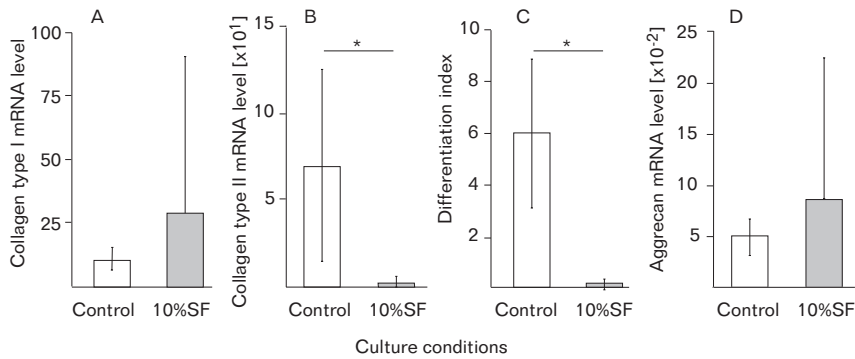
### Post-redifferentiation mRNA levels

SF supplementation resulted in significant downregulation of relative collagen type II mRNA levels ( $0.2 \pm 0.3$  versus  $7.0 \pm 5.6$  respectively) and the differentiation index (the collagen type II/I mRNA ratio;  $0.1 \pm 0.2$  versus  $6.0 \pm 2.9$  respectively) as compared to control media (Figure 3B-C).

SF-supplementation appeared to result in upregulation of collagen type I mRNA levels in both donors, although this effect was not consistently significant (Donor 1:  $28.3 \pm 61.7$  versus  $10.6 \pm 3.9$  respectively;  $p = 0.31$  and Donor 2:  $10.2 \pm 9.1$  versus  $4.5 \pm 1.7$  respectively;  $p = 0.03$ ; Figure 3A). Aggrecan mRNA levels were not significantly different between culture conditions (Control  $5.1 \pm 1.7$  versus 10% SF  $8.6 \pm 13.8$ ; Figure 3D). Collagen type X was below detection level for all samples.



**Figure 2**  
 (A) Final GAG content. (B) Final GAG content normalized to DNA content. (C) Final DNA content. Bars represent average  $\pm$ SD. Statistically significant differences are indicated by \* ( $p < 0.05$ ). Note that SF supplementation to redifferentiation media significantly inhibits the final proteoglycan content of the tissue synthesized and stimulates the cellularity compared to control redifferentiation conditions.



**Figure 3**  
 Relative mRNA levels of (A) collagen type I, (B) collagen type II, (C) the collagen type II/I ratio (differentiation index), and (D) aggrecan in tissue synthesized by expanded chondrocytes that were redifferentiated on collagen coated filters for 14 days. The mRNA levels shown in these graphs are relative expression levels after normalization to GAPDH as endogenous control. Statistically significant differences are indicated by \* ( $p < 0.05$ ). Note that SF supplementation results in significantly decreased collagen type II mRNA levels and differentiation index compared to control redifferentiation conditions.



## Discussion

In order to optimize the quality of tissue formed after clinically applied ACI, cartilage regeneration has been studied extensively, both *in vitro* and *in vivo*. However, few studies have aimed at elucidating the effect of the *in vivo* environment on chondrogenesis. The current study clearly demonstrates that *in vitro* chondrogenesis is significantly affected by supplementation of culture media with SF obtained from injured knee joints. After 14 days of culture, SF supplementation resulted in a significant decrease of final PG content, significant increase of final cellularity, and a significant inhibition of chondrocyte redifferentiation at the mRNA level. These results suggest that SF will influence the intra-articular environment of chondrocytes at reimplantation and, in addition, may affect the “normal” cartilage taken for culture expansion at the first step in current ACI.

The observed decrease in final PG content by chondrocyte redifferentiation in SF supplemented culture media may be caused either by a higher anabolic activity of chondrocytes cultured in control media, a higher catabolic activity of chondrocytes cultured in SF supplemented culture media, or a combination of these two factors. Either way, these results in combination with decreased redifferentiation suggest that SF from injured knee joints is detrimental to *in vitro* chondrogenesis. Possible factors responsible are chemokines, inflammatory cytokines and matrix metalloproteinases (MMPs). These mediators have been described to be induced as a physiological reaction to damage in a variety of tissues and are known to be crucial for e.g. cutaneous wound healing<sup>274-277</sup>. Likewise, joint injuries might result in the induction of such mediators<sup>278,279</sup>. However, in the context of cartilage biology, these mediators are suggested to be involved in osteoarthritis development and to be detrimental to the ECM of articular cartilage<sup>60,62</sup>. Therefore, they might be responsible for the decreased final PG content in SF-supplemented cultures by inducing increased PG degeneration rate. Future studies should aim at elucidating the factors responsible for the observed effects, as these might provide a possible target for improvement of clinically applied ACI, e.g. by desensitizing chondrocytes for specific cytokines using siRNA, or restoring a beneficial joint homeostasis for chondrogenesis by pre-treatment of joints. Similar treatments have been studied in patients with rheumatoid arthritis, for example tumor necrosis factor-alpha (TNF- $\alpha$ ) inhibitors<sup>280,281</sup> and are now a successful example of modern early intervention that has changed the course of the disease<sup>282</sup>

In contrast with our results, Hegewald *et al* demonstrated that autologous SF obtained from healthy equine joints is supportive for *in vitro* chondrogenic differentiation and PG synthesis by equine mesenchymal stem cells<sup>283</sup>. Furthermore, Skoog *et al* presented similar supportive effects for *in vitro* chondrogenesis from perichondral tissue harvested from rabbit rib cartilage<sup>284</sup>. These discrepancies may be explained by the fact that, in the current study, SF samples were harvested from human injured knee joints in contrast to above described studies, which used SF obtained from healthy joints. This hypothesis appears supported by the fact that SF from “chronically” injured knee joints has indeed been demonstrated to inhibit





the PG synthesis rate in a chick limb bud assay<sup>38</sup>. However, in the same study, PG synthesis rate was stimulated by synovial fluid from “acutely” injured knee joints. Likewise, periosteal transplantation in joints with longer existing cartilage defects has been demonstrated to result in regeneration tissue with significantly worse morphology and to affect PG turnover of the treated joint negatively compared to periosteal transplantation in joints with “fresh” cartilage defects<sup>37</sup>. These studies suggest that the effect of SF on chondrogenesis is related to the age-of-injury of the joint from which the SF samples are harvested. Such trauma-age related effects were not observed in the current study. This may be due to various factors, such as the culture model used, differences in chondrocyte species, the limited number of SF samples, or the outcome parameter used, e.g. the above-described studies determined proteoglycan turnover parameters rather than final proteoglycan content, while the latter appears more relevant as this has a direct relation to mechanical properties of the tissue. These studies do confirm that joint injury may result in disturbed joint homeostasis, which may be detrimental for the quality of regenerating tissue after ACI.

Interestingly, SF supplementation resulted in significantly higher cellularity of tissue formed after 14 days of culture. These results are in accordance with a recently published study in which cartilage injuries were demonstrated to result in basic Fibroblastic Growth Factor (bFGF) release<sup>285</sup>. Besides being a potent mitogen, bFGF is known to be a potent inhibitor of the anabolic effects of both IGF-1 and OP-1, i.e. bFGF significantly down-regulated the PG synthesis rate of culture chondrocytes induced by these growth factors<sup>286</sup>. A similar mechanism might be responsible for the observed effects of SF supplementation in the current study. However, in an animal study Fukuda *et al* demonstrated that treatment of osteochondral defects by matrix induced autologous chondrocyte implantation (MACI) resulted in macroscopically, morphologically and mechanically improved tissue regeneration when scaffolds were impregnated with bFGF<sup>287</sup>. These apparently contrasting results may be explained if bFGF-induced proliferation precedes redifferentiation rather than occurring simultaneously. A similar process was previously suggested to occur during *in vitro* periosteal chondrogenesis<sup>231</sup>. Although this hypothesis seems to be supported by the downregulation of redifferentiation parameters in our study, future experiments with extended culture periods should investigate whether this hypothesis holds true. Joint-injury-induced upregulation of intra-articular bFGF levels might actually result in increased amounts of regeneration tissue, rather than to be detrimental as the results of the current study suggest.

Altogether, various parameters of ECM quality and chondrocyte redifferentiation appear to be strongly affected by SF supplementation to redifferentiation media. However, pinpointing the exact factors involved in the effects found is difficult, as various limitations should be considered in this study. All SF tested were obtained from injured knee joints. Therefore, the observed effects cannot be assigned with certainty to the fact that the joints, from which the SF samples were harvested,





were injured. Unfortunately, human SF samples from “healthy “ joints are sparsely available. Furthermore, tissue quality was determined at only one time point, limiting the possibility of temporal interpretations of the current data. Whether decreased PG content combined with increased cellularity actually represent detrimental effects induced by SF supplementation, or rather reflects increased amounts of tissue that may be synthesized, remains to be elucidated in future studies with extended culture periods. Finally, SF samples that were supplemented to the redifferentiation media were obtained from other donors than the chondrocytes, which suggests that the adverse effects induced by SF supplementation may be due to an immunological rejection. However, the significantly increased final cellularity induced by SF supplementation contradicts this hypothesis. In conclusion, synovial fluid supplementation to culture media significantly affects *in vitro* chondrogenesis, suggesting that caution is warranted when investigating *in vitro* chondrocyte redifferentiation without SF supplementation to culture media. Moreover, this study suggests that SF from injured knee joints has a detrimental effect on redifferentiation and extracellular matrix synthesis. Therefore, factors present in the synovial fluid of injured joints may, once identified, provide a viable target for future improvement of ACT by refinement of culture techniques, patient selection or pre-treatment of affected joints in order to restore joint homeostasis.



The effect of synovial fluid from injured knee joints on *in vitro* chondrogenesis



