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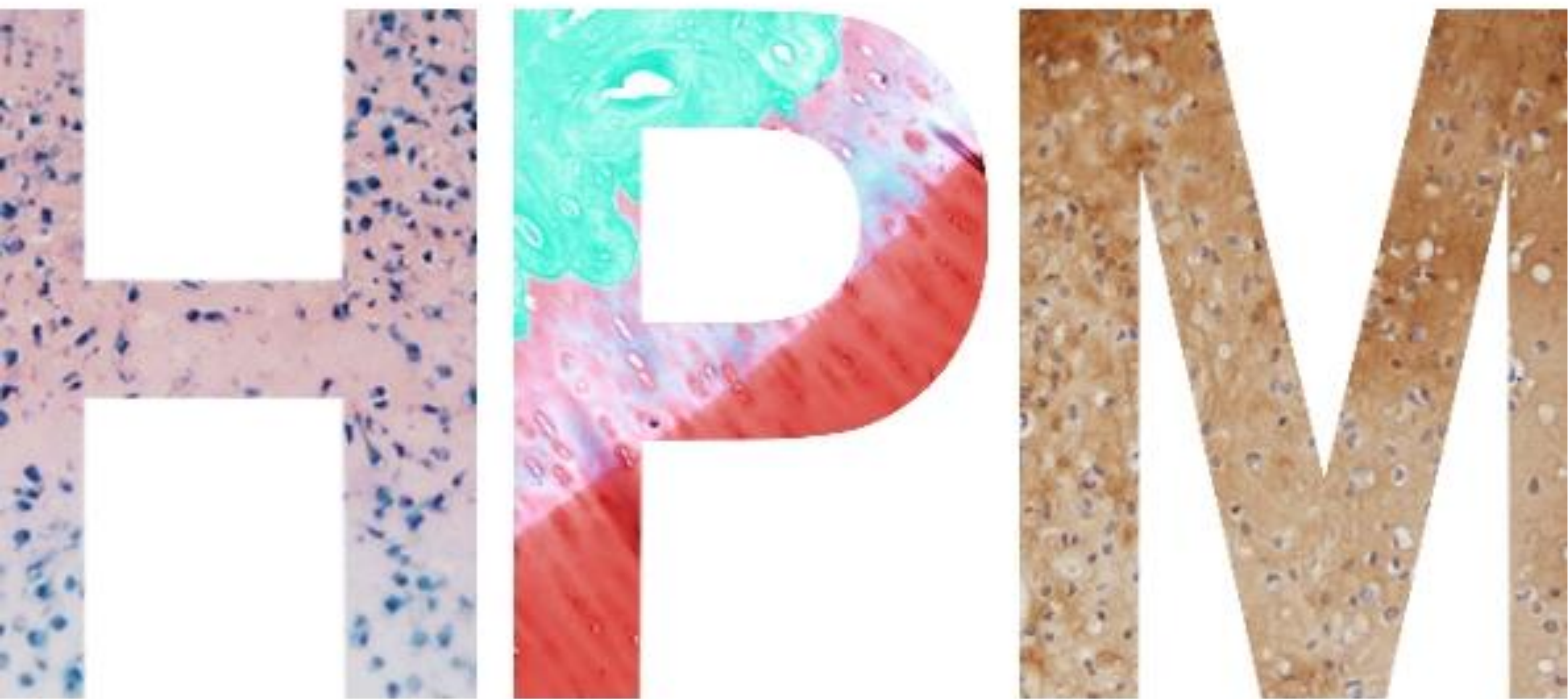
The role of joint homeostasis in equine articular cartilage repair



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Contents

Summary		6
Chapter 1	General introduction	7
Chapter 2	Effects of synovial fluid on healthy and osteoarthritic chondrocytes in 3D culture	17
Chapter 3	Effects of synovial fluid obtained from joints with different stages of joint damage on *in vitro* chondrogenesis	36
Chapter 4	Differences in chondrocyte behaviour between horse breeds and pony breeds	52
Chapter 5	General discussion	64
Appendix I	Overview experimental designs	68
Appendix II	Courses	69

Summary

Osteoarthritis (OA) has a great impact on the mobility of both animals and man. In OA development, joint homeostasis becomes disturbed, which is reflected in the composition of the synovial fluid (SF). This altered joint environment might challenge the regenerative capacity of cartilage even more and might influence the effectiveness of new regenerative therapies. This thesis focussed on investigating the influence of SF obtained from equine joints with different stages of osteoarthritic changes on the regenerative capacity of cartilage in vitro.

In experiment 1, pellets were formed out of P0 and P1 chondrocytes harvested from the carpal joint and fetlock joint. Culture medium was supplemented with 25% and 50% healthy SF (HSF). In experiment 2, pellets were formed out of OA chondrocytes and healthy chondrocytes. After 1 day of pre-culture, chondrogenic medium was replaced or supplemented by 25% HSF, 25% LPSSF (SF obtained 24 hours after intra-articular LPS injection), 25% OASF (SF obtained from OA joints), or 10 ng/ml TGF-β1. A prolonged pre-culture period of 2 weeks was tested in experiment 3 and 4. After pre-culture, pellets were cultured in chondrogenic medium supplemented with 25% HSF, 25% OASF, or 10 ng/ml TGF-β1. To elucidate differences in chondrocyte performance between donors from different breeds, chondrocytes derived from 4 pony breed donors and 3 horse breed donors were cultured in chondrogenic medium supplemented with 10 ng/ml TGF-β1 in experiment 5. Biochemical and histological analyses as well as mRNA expression of matrix components and cartilage marker genes were used as outcome measures in all experiments.

In experiment 1, replacement of 25% and 50% of culture medium with SF was found sufficient to differentiate effects of SF on P1 pellets cultured in chondrogenic medium. Experiment 2 demonstrated that pellets formed out of OA chondrocytes showed less safranin-O staining, but more collagen II staining compared to healthy chondrocytes under every condition. Histological examination showed collagen II deposition after addition of OASF, but not after addition of LPSSF or HSF. Additionally, GAG/DNA amounts appeared to be higher in the OASF and LPSSF group compared to the HSF group. On gene expression, the ratio between collagen II and collagen I/III was lower after addition of LPSSF in culture, compared to OASF and HSF. In experiment 4, statistically significant higher GAG amounts were found at 3 weeks of culture in the OASF group compared to the HSF group. However, GAG amounts formed as of 2 weeks of culture were extremely low under each condition. Results from experiment 5 presented no obvious differences between horse and pony breeds, but major individual donor differences in collagen II and GAG production became apparent.

We can conclude that addition of HSF, OASF and LPSSF to culture medium all have positive effects on the matrix production of chondrocytes in vitro compared with chondrogenic medium. Under influence of OASF and LPSSF, matrix production was stimulated to a higher degree compared with HSF. However, addition of 10 ng/ml TGF-β1 resulted in more matrix production compared to all SF conditions. On the other hand, SF from an acutely inflamed joint induced higher expression of cartilage dedifferentiation markers. Finally, clues were found with respect to the importance and selection of well-performing individual donors. A major limitation in this project is that results were obtained assessing pellets with relatively low matrix production, due to poor performing cell donors. Therefore, the relevance and interpretation of differences between HSF, OASF and LPSSF that were found need to be considered carefully.

Chapter 1

General introduction

Osteoarthritis (OA) is one of the most common and impeding degenerative joint diseases in both men and horses. Surveys suggest that 60 percent of the lameness in horses finds its origin in OA (1). Osteoarthritis is defined as a 'whole joint disease', with interactions between all joint components present. Meaning that in OA not only cartilage surfaces are damaged, but other joint tissues including subchondral bone and the synovial membrane are also affected. This results in a disturbed joint homeostasis. A disturbed joint homeostasis is reflected by changing biomarker profiles in the synovial fluid (SF) (2) that fills up the joint cavity, and interacts with all other joint tissues. This thesis focusses on effects of SF from equine osteoarthritic joints on cell viability and matrix characteristics of 3D cultured articular cartilage cells (chondrocytes). To make an optimal translation from a whole joint disease in a living subject to a mimicked situation *in vitro*, insight in the structure and function of healthy articular cartilage as well as underlying processes in OA development is imperative.

Healthy articular cartilage composition

The synovial joint consists of two cartilage layers, one at each outer part of the long bones. In between these cartilage layers, SF acts as lubricant for the joint. Subchondral bone lays adjoined to the lower part of the cartilage layers. The whole joint is enclosed by a capsule consisting of a fibrous layer which is lined with a synovial membrane (Fig. 1A), and the joint is rigidized by tendons and ligaments. Therefore, joints are able to bear a lot of load in several directions (3).

Three types of cartilage in the body can be distinguished, namely elastic cartilage, hyaline cartilage, and fibrocartilage. Synovial joints consist of hyaline type cartilage, while for instance cartilage repair tissue is often recognized as fibrocartilage. In cartilage, chondrocytes are responsible for the synthesis and turn-over of extracellular matrix (ECM) structures. This ECM consists mainly of collagen, proteoglycans and glycoproteins (4,5). Not all cartilage types, however, are composed in a similar fashion. Where collagen II is ascendant in hyaline type cartilage, collagen I is often predominant in fibrocartilage (6).

Focussing on hyaline type of cartilage, several additional ECM molecules are present next to collagen II. Aggrecan is the largest and most present proteoglycan in hyaline cartilage. Other proteins contributing to ECM structure are for example cartilage oligomeric matrix protein (COMP) and fibronectin (7). Notable is that the turn-over rate of collagen II is very low, while proteoglycans undergo replacement more frequently due to external stimuli (8). In addition to ECM molecules, a large fluid phase of which the majority consists of water is present in cartilage. Interaction between fluids and low permeable matrix components in cartilage provides comprehensive resilience, which is important in the transmission of forces to the subchondral bone. In cartilage, glycosaminoglycans (GAGs) are attached as chains to proteoglycans. The negatively charged GAGs cause water absorption into the cartilage, which proves the role of GAGs in this resistance to mechanical loading (9).

Looking closer to articular cartilage composition, three layers can be distinguished in cartilage (Fig. 1B). The upper part is the superficial zone, which has the least amounts of GAGs, but highest amounts of collagen II. In the middle and deep zone GAG production is increased. Chondrocytes present in cartilage become more round and organized in columns in the deep zone. Between the deep zone and the underlying subchondral bone, a calcified layer is present.

The calcified layer is derived from remnants of the growth plate, and serves as a mechanical buffer. Histologically, a tidemark is recognizable as boundary between this mineralized layer and the deep zone (4,5,10). Chondrocytes exist in relatively limited amounts in cartilage compared to the great amount of matrix. Recent research has found that also a population of articular cartilage progenitor cells are present in mature cartilage (11,12).

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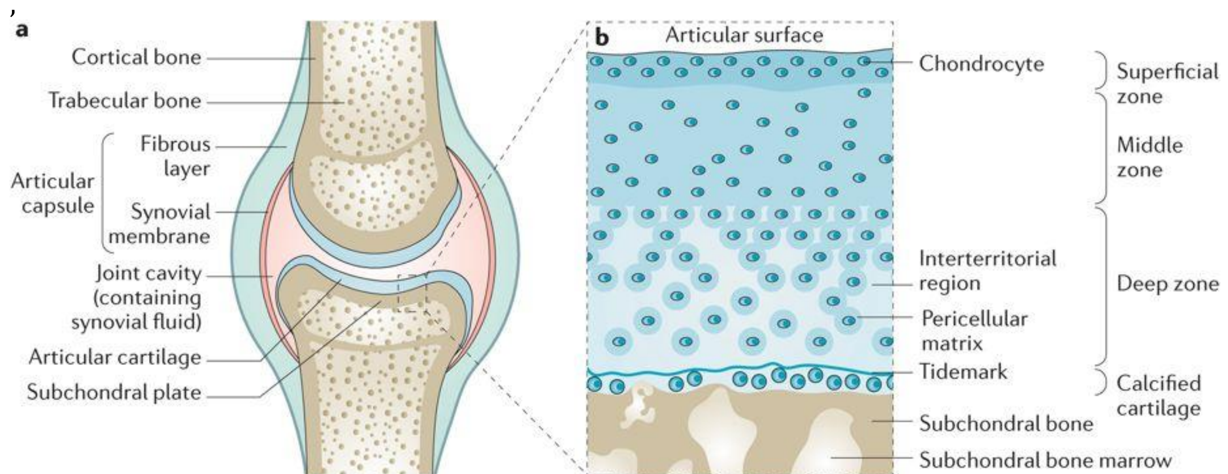

Figure 1 An overview of synovial joint composition **(a)**, with a magnification of the articular surface **(b)** (10).

Synovial membrane and synovial fluid in healthy joints

Since cartilage itself contains no neurons and is not vascularised, cells are dependent on nutrients present in SF. Synovial fluid is a derivate of the blood plasma, supplemented with products synthesized by synoviocytes residing in the synovial membrane. The synovial membrane contains two types of synoviocytes, a macrophage type and fibroblast type. The synovial fibroblasts are responsible for producing hyaluronic acid (HA) and lubricin, two major components of SF important for lubrication (13). Macrophage type synoviocytes play a role in phagocytoses of cell debris and waste present in the joint cavity (14). Additionally, proteins released by chondrocytes can be found in SF. Therefore, SF is considered the ‘mirror of joint homeostasis’ (2), and biomarker profiles of SF can provide clues about joint condition (13,15).

Chondrogenesis and differentiation during embryonic development

Understanding embryonic development of articular chondrocytes is needed for understanding OA pathophysiology, since various stages in embryonic development of cartilage and bone formation can give information about markers reflecting certain chondrocyte stages. Embryonic development of both articular cartilage and long bone formation finds it origin in mesenchymal (progenitor) cells (16). Mesenchymal cells that become chondrocytes in ossification centres and growth plates eventually will reach apoptosis and be replaced by new bone tissue. On the other hand, chondrocytes at the outer layer of long bones, in a region called the interzone, will differentiate to persistent hyaline type cartilage. The interzone is a region discriminating between two long bones, intended to form the joint cavity. Mesenchymal cells in the interzone eventually form whole joints including cartilage, synovial membrane and ligaments (Fig. 2).

Two critical pathways in early differentiation of mesenchymal cells to articular cartilage cells are activation of Sox9 expression (17) and canonical Wnt signalling, with β-catenin as key mediator (Fig. 2). Especially Wnt-14 inhibits chondrogenesis, and is therefore important in maintaining the mesenchymal interzone (18). Maintenance of the interzone assists in forming the joint cavity. On the other hand, Wnt signalling plays a role in stimulating endochondral ossification.

Other factors contributing to chondrogenic development are members of the transforming growth factor beta (TGFβ) superfamily, among which several bone morphogenetic proteins (BMPs), growth differentiation factor 5 (GDF5), and TGFβ (Fig 2). Members of the BMP family are originally found to have a function in chondrocyte maturation and endochondral bone formation (19). Moreover, BMP expression also seems to play a role in chondrogenesis (20). Not all BMPs serve the same function, BMP4 for instance accelerates chondrocyte maturation toward hypertrophy, while BMP14 (GDF5) is more involved in recruitment and differentiation of chondroprogenitor cells (21). TGFβ signalling occurs through Smad2 and Smad3 activation, and is important for withholding chondrocyte maturation (22,23). In Smad3 deficient chondrocytes, a higher BMP signalling via Smad1/5/8 has been observed, which causes accelerated chondrocyte maturation (24). These findings indicate that several pathways in chondrogenesis are tightly regulated and show great similarity with pathways involved in inducing chondrocyte hypertrophy and subsequently endochondral ossification. Therefore, small disturbances may lead to cartilage changes directing towards OA.

Of note, differences in ECM production occur in different stages of chondrocyte formation and should be considered as well. Whereas in early differentiation stages collagen I is an important marker, later collagen II, collagen IX, and collagen XI become more crucial. Ultimately, synthesis of COMP, aggrecan and hyaluronan initiates, and these proteins start to become the major components of mature cartilage ECM. Chondrocytes that will eventually be replaced by bone have as main characteristic that they become hypertrophic before apoptosis occurs. In hyperthropic cells articular cartilage ECM markers like collagen II disappear, and collagen X becomes more prominent (4,25).

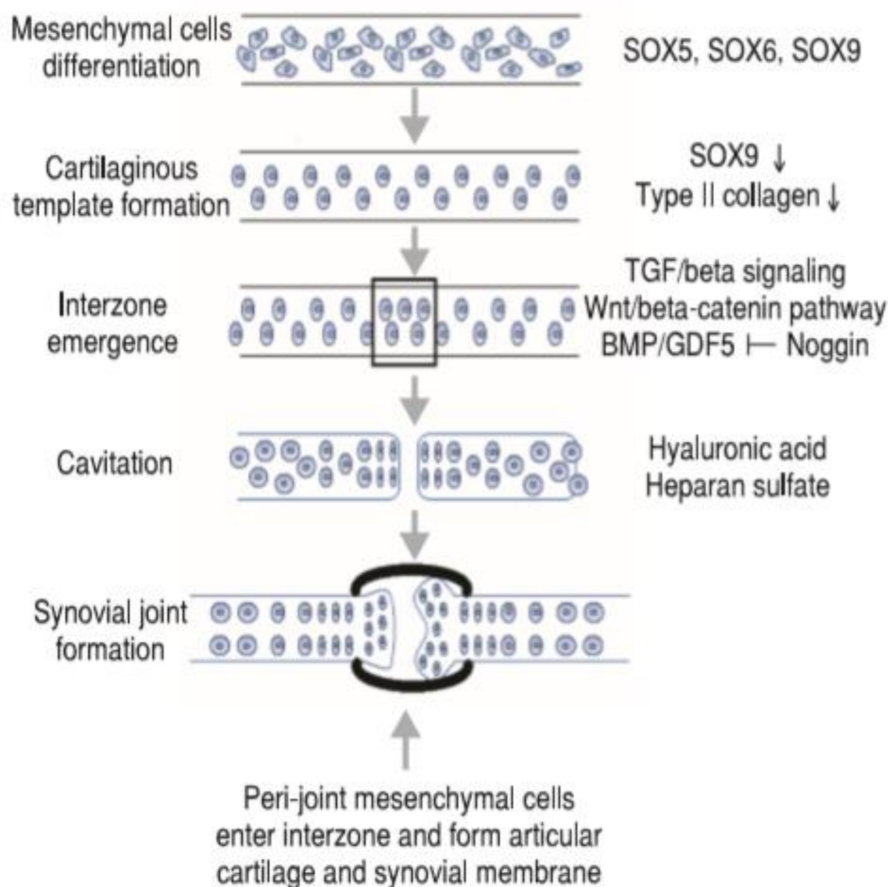

Figure 2 Synovial joint formation in steps (26).

Articular cartilage damage and disturbed joint homeostasis in OA development

Articular cartilage maintains a certain homeostasis, which is regulated by several mediating proteins and pathways that are also involved in embryonic development. For instance, a loss of TGFβ-induced Smad2/3 signalling with ageing might contribute to OA development (27). Upregulated Wnt pathway may result in terminal chondrocyte differentiation and degradation of cartilage (28). Additionally, a number of other factors are described to be of importance for cartilage homeostasis. For example, the pericellular matrix around chondrocytes has been found to play a role in maintaining homeostasis by preventing interaction between the cell and ECM components (29). Mechanical loading is crucial in joint homeostasis, because it causes fluid movement between SF and cartilage, which is important for nutrition and mediating cell signalling (30,31). A hypoxic environment is crucial for maintaining homeostasis, because it supresses proteinases matrix metalloproteinase (MMP) 3 and MMP13 production, and stimulates anabolic pathways (32). Additionally, upcoming research focusses on the effect of extracellular vesicles in cartilage and SF. Extracellular vesicles are transporters of bioactive signalling molecules, and have been found to be important in preserving joint homeostasis (33,34).

Consequently, when this homeostasis is disturbed, there is a great risk for developing OA. Small changes in the integrity of the cartilage cause an imbalance, characterized by an excessive catabolism relative to anabolism. These changes can be caused by trauma induced cartilage damage or inflammation. Additionally, factors such as aging, obesity, genetics, and mechanical forces give higher risks for developing OA (35).

In early stages after cartilage injury, anabolic pathways are initiated, and result in an increased matrix production attempting to restore the damage site. Growth factors like BMP and TGFβ are concerned in stimulating anabolic pathways (36). Activated chondrocytes adjust their low turn-over state into an anabolic state, visible as a heightened degree of newly synthesized matrix components released to SF (37). However, when OA progresses, chondrocytes are stimulated to synthesize collagenases and proteinases. Important matrix remodelling proteins are ADAMTS4/5 for aggrecan degradation (38) and MMP3 for collagen degradation. MMP13 is present in greater amounts than MMP3, and causes the degradation of both collagen II and aggrecan (39,40). These proteinases cause an increase of matrix degeneration particles in the joint, which activate pro-inflammatory mediators like cytokines interleukin-1 (IL1), tumour necrosis factor alfa (TNFα), nitric oxide (NO), prostaglandin E2 (PGE2), and proteins involved in complement system (41,42). These mediators act in a paracrine or autocrine fashion on chondrocytes. Consequently, these mediators provoke further deregulation of chondrocyte activity, and activate expression of catabolic genes. In advanced OA a modulation in phenotype occurs, resembling terminal differentiated chondrocytes, which is reflected by hypertrophic cell markers like collagen X (43). Hence, in these cells endochondral ossification can be activated. Eventually, this results in apoptosis and will be visible as increased bone formation in subchondral bone and the calcified layer.

Synovial membrane and synovial fluid in OA

An interesting finding in OA research is that not only cartilage is involved in OA. Because of close interaction between cartilage and adjacent tissue, cartilage damage can also induce synovitis. Vice versa, primary synovitis can induce OA. Cartilage degradation particles and pro-inflammatory cytokines released by chondrocytes are mediators for inflammation. In response of inflammation, synoviocytes produce pro-inflammatory cytokines. Studies on SF components of OA patients observed increased levels of complement proteins, pro-inflammatory cytokines, like IL-1 and other inflammatory mediators (44). Additionally, TGFβ is found to be higher in SF with cartilage defects, which stimulates regeneration. In excessive amounts, however, TGFβ stimulates endochondral ossification and leads to osteophyte formation (27,45). Matrix

components such as GAGs are also present in SF, although previous research shows contradictive results regarding the correlation between severity of joint damage and GAG amounts in SF (46,47). An additional effect seen in OA patients is that SF becomes less viscous due to a decrease in concentration and size of hyaluronic acid molecules. This results in less efficient lubrication and eventually increased cartilage wear (48).

In vitro **models to study the role of joint homeostasis in cartilage repair**

A major challenge in joint regeneration is the limited repair capacity of articular cartilage. Current therapies of OA in horses is limited to medical treatment (49) and several (emerging) surgical techniques (50), but complete joint repair is not possible yet. An upcoming surgical therapy in cartilage regeneration is for instance the use of (cell seeded) 3D scaffolds (51,52). Equine studies are often used for testing these new therapies, since equine OA models are representable for human OA (53,54). However, *in vivo* testing of these new therapies is often performed in artificial cartilage defects in healthy horses with a normal joint homeostasis. In contrast, joint homeostasis in patients suffering from cartilage injury, is likely to be disturbed. *In vitro* and *in vivo* studies showed that in the presence of a disturbed joint homeostasis, especially in chronic situations, chondrogenesis is significantly impaired, possibly interfering with new regenerative therapies (55,56). Testing of new regenerative therapies could be improved by more specialized *in vitro* research, as this enables to mimic parts of the altered joint environment.

Aim and outline of this thesis

The aim of this thesis was to investigate the influence of SF obtained from equine joints with different stages of OA, on the vitality, matrix production and metabolic profile of chondrocytes in 3D cultures. To this end, a series of experiments (experiment 1-5) were performed. Biochemical and histological analyses as well as mRNA expression of matrix components and markers for chondrocyte characteristics were used as outcome measures. Seven sub-questions that were either defined at the start of the project or that became important after interim data analysis are dealt with in three different chapters.

Chapter 2 describes how the experimental design was optimized by testing different cell stages for 3D culture and assessing the impact of various concentrations of SF added to culture medium (experiment 1). Furthermore, effects on healthy chondrocytes were compared with effects on osteoarthritic chondrocytes (experiment 2).
In Chapter 3, the influence of SF from joints with acute synovitis on chondrocyte pellets was compared with that of SF from joints with severe (chronic) cartilage degeneration (experiment 2 and 4). Additionally, a prolonged pre-culture period was tested in order to create a stable starting pellet prior to the addition of different medium conditions (experiment 4).
Chapter 4 elucidates differences in chondrocyte performance in the previous experiments. Performance of donors from different breeds (ponies versus horses) were tested individually (experiment 5).

The general discussion (Chapter 5) deals with the major findings that were obtained by each of the experiments with regard to the main aim of this thesis. Limitations and pitfalls are discussed and ideas for future experiments are presented.

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Chapter 2

Effects of synovial fluid on healthy and osteoarthritic chondrocytes in 3D culture

Abstract

BACKGROUND: Chondrocytes have the tendency to lose their original phenotype and dedifferentiation occurs when cultured in monolayer. This causes difficulties when effects of synovial fluid (SF) on chondrogenesis are tested. Besides, differences between osteoarthritic (OA) chondrocytes and healthy chondrocytes might diminish due to this dedifferentiation. To overcome this problem, redifferentiation in 3D culture is a possible solution. The aim of the experiments described in this chapter was to find an optimal *in vitro* design for culturing chondrocytes in medium enriched with SF.

MATERIALS AND METHODS: Chondrocytes obtained from middle carpal (MC) and metacarpophalangeal (MCP) joints were formed into pellets without passaging (P0), or after expansion (P1), and were cultured for 1 week in non-chondrogenic or chondrogenic medium enriched with 25% HSF (healthy SF), 50% HSF, 10 ng/ml TGF-β1, or 10 ng/ml LPS, in experiment 1. Collagen II and glycosaminoglycan (GAG) formation was evaluated on histology. Additionally, mRNA expression levels of cartilage markers were tested. In experiment 2, P1 OA chondrocyte and healthy chondrocyte pellets were cultured for 1 week in chondrogenic medium enriched with 25% HSF or 10 ng/ml TGF-β1. In this experiment histology and mRNA expression analyses were extended with biochemical measurements for GAG and DNA content, and GAGs released to medium.

RESULTS: Our results demonstrated that chondrocytes after passaging had higher expression levels of cartilage marker genes, and formed histologically better pellets compared to primary chondrocytes. Replacement of 25% and 50% of culture medium with SF resulted in improved histological GAG and collagen II production compared to plain medium. Gene expression levels of both anabolic and catabolic cartilage marker genes increased progressively with a higher concentration of added SF. Pellets formed out of OA chondrocytes showed less safranin-O staining under all conditions, however more collagen II staining was present compared to healthy chondrocytes. Additionally, lower GAG levels were found in OA chondrocytes compared to healthy chondrocytes.

CONCLUSION: Pellet culture with P1 chondrocytes is a suitable method for culturing in chondrogenic medium enriched with SF. Addition of 25% to culture medium is sufficient to achieve improved chondrogenesis and matrix production in pellet culture. When redifferentiating OA cells in pellet culture, differences in chondrocyte behaviour compared to healthy chondrocytes remain visible.

Introduction

Culturing chondrocytes has been a great deal of interest for researching joint disease pathophysiology, but also for developing regenerative therapies where cultured chondrocyte function as replacement of damaged cartilage such as autologous chondrocyte implantation (ACI) (1). One of the main challenges in chondrocyte culture is that chondrocytes have the tendency to lose their original phenotype and dedifferentiate when cultured in monolayer (2). To overcome this problem, several culture methods have been developed. When culturing in monolayer, chondrocyte phenotype can be maintained by using a high seeding density (3). Researching matrix production, however, remains difficult in 2D culturing. An alternative technique which allows studying matrix production, is to redifferentiate chondrocytes in a 3D culture. Expanded chondrocytes can for instance be embedded in constructs containing alginate, collagen or other supportive structures (4–6). Chondrocytes are also able to form pellets and produce matrix, without the use of these cohesive support structures (6). Therefore, pellet culture is a relative manageable and often used culture method for studying chondrocytes.

These culture possibilities provide several options for researching chondrocyte behaviour. An interesting extension of these culturing methods is the addition of synovial fluid (SF) to culture media, since SF represents the natural environment for chondrocyte growth. Culture medium enriched with SF can be used for improved articular cartilage engineering, but also for researching effects of SF derived from healthy or damaged joints on chondrocyte performance. However, culturing in SF has a practical challenge due to its viscosity. Besides, only limited amounts are available per donor. A new design proposed a 3D culture method, where chondrocytes were cultured in up to 100% SF (7). Nevertheless, effects on chondrocyte activity were already visible at 30% SF. In several other studies that used SF in culture medium, effects of SF were already visible when culture medium was enriched with 20% SF (8,9). This indicates that 3D pellet cultures are feasible for studying effects of SF on chondrocyte performance, and that enrichment of medium with relatively low percentages of SF is sufficient.

Although redifferentiation of expanded chondrocytes in pellets is achievable, initial expansion might influence the chondrocytes original phenotypic characteristics. When chondrocytes in expansion have been passaged more often, redifferentiation is more difficult to achieve (10). Chondrocytes from OA patients *in vivo* show characteristics of hyperthropic cells and have different gene expression compared to healthy chondrocytes (11). Studying OA chondrocytes *in vitro* is specifically challenging, because of potential dedifferentiation and thus loss of their OA related characteristics. Previous research concerning OA phenotype lost is conflicting. Dehne et al. found that OA chondrocytes and healthy chondrocytes are almost completely comparable in gene expression in scaffold culture, and have analogues chondrogenic capacity (12). In contrast, other studies demonstrated remaining differences between OA chondrocytes and healthy chondrocytes after de- and redifferentiation (13,14). When investigating effects of SF on chondrocytes derived from joints with a certain stage of degeneration, phenotype loss might be an obstacle. Therefore, more insight is desired in the suitability of pellet culture for investigating effects of SF on both healthy chondrocytes and OA chondrocytes.

The aim of the experiments described in this chapter was to find an optimal *in vitro* design for culturing equine chondrocytes in medium enriched with SF, giving a reliable reflection of the *in vivo* situation. To achieve this, different cell passages will be tested, and we hypothesized that chondrocytes with a lower passage number are easier to redifferentiate. Subsequently, effects of supplemented SF will be better visible on these low passage number pellets. Additionally, effects of several concentrations of SF added to culture medium on matrix production and metabolic profile will be investigated. With this knowledge we can determine the minimum concentration of SF that is required to differentiate effects of SF from chondrogenic medium only. We hypothesized that differences between SF-enriched and plain medium will be most pronounced

for higher concentrations of SF, but that a concentration between 20 and 30% will be sufficient. Additionally, a 3D pellet culture will be tested with chondrocytes derived from both healthy horses and horses with OA. We expected find differences in behaviour of OA cells in pellet culture compared to healthy cells.

Materials and methods

Experimental designs

All variables and fixed factors per experiment are summarized in Appendix I (Table 1). The current chapter describes experiment 1 and experiment 2.

Experiment 1

Chondrocytes were harvested from the carpal joint and fetlock joint, and were pooled for both donors per joint (Table 1). Pellets were formed out of primary chondrocytes (P0), and after expansion (P1). These pellets were pre-cultured for 1 day in chondrogenic medium and non-chondrogenic medium. Then, chondrogenic medium and non-chondrogenic medium were partly replaced or supplemented by 25% healthy SF (HSF), 50% HSF, 10 ng/ml human recombinant transforming growth factor β1 (TGF-β1, R&D Systems, Minneapolis, Minnesota, Unites States) TGF-β1, or 10 ng/ml Lipopolysaccharides (LPS, from *Escherichia coli* O55:B5, Sigma-Aldrich). Addition of TGF-β1 functioned as positive control, while addition of LPS was used as negative control. Pellets were harvested 1 week after conditions were applied. Histology (safranin-O), immunohistochemistry (collagen II) and qPCR analyses were performed. For each analysis n=2 pellets were used.

Experiment 2

Two cell donors were pooled for healthy P1 chondrocyte pellets, and two different cell donors were pooled for P1 OA chondrocyte pellets (Table 1). After 1 day of pre-culture, chondrogenic medium was replaced or supplemented by 25% HSF, 25% SF derived from joints injected with LPS (LPSSF), 25% SF derived from osteoarthritic joints (OASF), or 10 ng/ml TGF-β1. Addition of TGF-β1 functioned as positive control, while chondrogenic medium without additives was used as negative control. After 1 week of culture pellets were harvested for histological examination (safranin-O), immunohistochemistry (collagen II), GAG and DNA amounts in pellets, and for qPCR measurements. Additionally, GAG amounts released to culture medium were measured. For each analysis n=2 pellets were used, except for GAGs released to medium n=6 samples were used. In the current chapter only results of the addition of 25% HSF and 10 ng/ml TGF-β1 are evaluated. Effects of other medium conditions are evaluated in Chapter 3.

Synovial fluid collection

Previously obtained and freezer-stored SF from metacarpophalangeal (MCP) and middle carpal (MP) joints from horses at a local abattoir and from patients euthanized at the clinic (with owner consent) was used as healthy SF (HSF). For each condition 2 or more donors (aged >2years) were pooled, and in each experiment different SF donors were used. Punctures were performed using a 10 ml syringe with an 18 gauge needle. Joints were clipped and sterile prepared with 70% ethanol (EtOH) and chlorhexidine preparatory to puncture. Obtained SF was centrifuged for 5 minutes at 2520 g to remove cells and debris. The supernatant was stored at -80°C.

Table 1 Overview characteristics of the donors used for cell culture in each experiment.

Exp. nr.	Donor	Breed	Age	Sex	Joint	Cell type	Cell stage
1	Pony 1	Shetland pony	6	Gelding	MCP, MC	Chondrocyte	P0, P1
	Pony 4	Shetland pony	8	Mare	MCP, MC	Chondrocyte	P0, P1
2	Pony 1	Shetland pony	6	Gelding	MCP	Chondrocyte	P1
	Pony 4	Shetland pony	8	Mare	MCP	Chondrocyte	P1

Cell stage represents the stage of the cells the pellets were formed with. Age is in years. MCP = metacarpophalangeal joint, MC = middle carpal joint.

Cell isolation and expansion

Chondrocytes were harvested within 24 hours post mortem with a sterile scalpel knife, and cartilage pieces were kept in DMEM (high glucose, GlutaMAX, pyruvate) (31966, Gibco, Dublin, Ireland) + 1% p/s (penicillin/streptomycin, P11-010, Gibco) at 37°C. Within 24 hours after harvest, cartilage pieces were washed in sterile HBSS (Hanks Balanced Salt Solution, Gibco) + 1% p/s, and minced cartilage pieces were digested overnight on a roller plate at 37°C in 30 ml 0.15% collagenase II (Worthington, Lakewood, United States) solution in DMEM + 1% p/s. After digestion, cells were strained through a 70 µm cell strainer (Greiner bio-one, Alphen aan de Rijn, The Netherlands) to remove debris. Cells were stored in liquid nitrogen.

Cell culture experimental conditions

Cells were expanded using expansion medium in T175 flasks (Cellstar, Greiner bio-one) in a density of approximately 5000 cells/cm2. Expansion medium contained DMEM (high glucose, GlutaMAX, pyruvate) (31966, Gibco) + 1% p/s + 1.25 µg/ml Fungizone (Amphothericin, Gibco) + 10% FBS (Foetal Bovine Serum, Gibco,) + 0.5% 0.1mM ASAP (Ascorbic acid 2 – phosphate, Sigma-Aldrich) + 1 ng/ml βFGF (Basic fibroblast growth factor, AbD Serotec, Bio-Rad, Hercules, Californië, United States). When confluent, cells were trypsinized with 1x TripLE expres (Gibco) and counted with TC20 cell counter (Bio-Rad). Cells were resuspended in chondrogenic medium containing DMEM/F-12 (HEPES, no phenol red, L-glutamine, 11039, Gibco) + 1% ITS+ premix (Insulin Transferrin Selenium, Corning Life Sciences, Corning, United States) + 1% p/s + 1.25 µg/ml Fungizone + 5µl /ml 20mM ASAP + 1.5 mg/ml BSA (Bovine Serum Albumin, Sigma-Aldrich), or non-chondrogenic medium without BSA and ITS+ premix for experiment 1. In experiment 2 only chondrogenic medium was used. Cells were plated out in an ultra-low cell attachment 96 wells plate with a round bottom (Costar 7007, Corning Life Sciences) in a cell density of $2x10^5$ cells per well and centrifuged for 5 minutes at 300 g to form pellets. Medium was refreshed each 3 or 4 days and stored in Micronic tubes (MICRONIC, Lelystad, The Netherlands). After pre-culture, the medium was partly replaced or supplied with different additives. Pellets were all cultured at 37°C and 5% CO_2.

Histology and immunohistochemistry

Pellets were fixed in 200 µl 10% formalin (Sigma-Aldrich) with 0.1% eosin (Boom BV, Meppel, The Netherlands) for 1 week. Afterwards, pellets were embedded in 2.4% alginate (Sigma-Aldrich) and gelated by 3.7% formalin with 102 mM $CaCl_2$. Samples were dehydrated through EtOH 70% - 100% (Klinipath, Breda, The Netherlands) and 1 step xylene (Klinipath) for 1 hour. Pellets were incubated in paraffin (Leica, Amsterdam, The Netherlands) at 60°C for 2 hours before embedding in paraffin. The samples were cut into 5 µm slices using Microm microtome and captured on KP plus printer slides (Klinipath). Slides were fixed on a hot plate of 60°C for 1 hour. Before staining, slides were deparaffinised by two steps xylene and hydrated through EtOH 60% - 100%. Post staining, dehydration was performed through 70 -100% and 2 steps xylene. Cover slides were mounted with Depex (Merck, Whitehouse Station, New Jersey, United

States). Images were acquired using an Olympus BX51 microscope with DP73 digital camera (Olympus, Zoeterwoude, the Netherlands).

Safranin-O
Alginate was removed by 15 minutes incubation in a citrate buffer. Afterwards nuclei were stained with Weigert's Hematoxylin (Klinipath) for 5 minutes and washed in running water. Counterstaining with 0.4% aqueous Fast Green (Sigma-Aldrich) was performed for 4 minutes, after which slides were washed in 1% Acetic Acid (Boom BV). Present GAGs were stained with 0.125% aqueous safranin-O (Sigma-Aldrich) solution for 5 minutes. Short dehydration starting with 96% EtOH was needed to prevent safranin-O from washing of. Healthy cartilage explants were stained as positive control.

Sections stained with safranin-O staining were graded according to Bern scoring (0-9) for in vitro generated neocartilage, where a score of 9 shows histological a resemblance of hyaline cartilage (15). Pellets were scored for staining darkness, matrix formation, and cell morphology (Table 2).

Table 2 Scoring categories Bern score (15).

Scoring categories	*Score*
A. Uniformity and darkness* of Safranin O–fast green stain	
No stain	0
Weak staining of poorly formed matrix	1
Moderately even staining	2
Even dark stain	3
B. Distance between cells/amount of matrix accumulated	
High cell densities with no matrix in between (no spacing between cells)	0
High cell densities with little matrix in between (cells <1 cell-size apart)	1
Moderate cell density with matrix (cells approx. 1 cell-size apart)	2
Low cell density with moderate distance between cells (>1 cell) and an extensive matrix	3
C. Cell morphologies represented	
Condensed/necrotic/pycnotic bodies	0
Spindle/fibrous	1
Mixed spindle/fibrous with rounded chondrogenic morphology	2
Majority rounded/chondrogenic	3

*Section 3–4 μm thick.

Collagen II
Slides were blocked with 0.3% H_2O_2 solution for 10 minutes. Antigen retrieval steps contained Pronase (Roche, Basel, Switzerland) 1 mg/ml and Hyaluronidase (Sigma-Aldrich) 10 mg/ml both for 30 minutes at 37°C. The second blocking section was with PBS/BSA (Bovine Serum Albumin, Sigma-Aldrich) 2% for 30 minutes, after which incubation with the primary antibody Collagen II Mouse monoclonal antibody DSHB (1:1500 in PBS-BSA) (Santa-Cruz, Dallas, Texas, Unites States) and a normal mouse IgG1 (1:1800 in PBS-BSA) (Santa Cruz) as negative control took place overnight at 4 °C. Next day, samples were incubated with goat anti-mouse secondary antibody containing HRP (Dako, Heverlee, Belgium) for 30 minutes. DAB peroxidase substrate solution (Dako) was added for 5 minutes to obtain signal. Counterstaining was performed using Mayers Hematoxylin (Merck). As control healthy cartilage explants were used.

Determination of glycosaminoglycan and DNA contents in pellets and medium

Pellets were harvested and washed with 100 µl HBSS to remove GAGs present in remaining medium. Afterwards, pellets were dried for 60 minutes using a Savant speedvac. Pellets were digested in 200 µl papain digestion solution containing 250 µg/ml Papain (from papaya, Sigma-Aldrich) and 1.57 mg/ml Cysteine HCl (Sigma-Aldrich) in 2*Papain buffer (containing Na_2HPO_4 + EDTA.2H_2O, Merck) overnight at 60°C. For digestion of SF in aspirated medium, samples were diluted 1:1 with Hyaluronidase type II (Sigma-Aldrich) 0.1 mg/ml in 25mM Sodium Acetate (Merck) pH 6.5 and incubated for 30 minutes at 37°C.

For measuring sulphated GAG content in both pellets and medium, the DMMB assay was used as described by Farndale (16). Digested samples were diluted using PBS-EDTA, and 100 µl of the diluted sample was pipetted in duplo into a 96 wells plate with a flat and clear bottom (Greiner bio-one). Chondroitin sulphate (Sigma-Aldrich) was used for standardization in a dilution series with concentrations between 0 and 10 µg/ml. 200 µl of DMMB (Sigma-Aldrich) staining solution was added and extinction was measured at 525 and 595 nm using a plate reader. Aspirated mediums GAG content was corrected for GAG concentrations in added SF. Broad and High Sensitive assay of the Qubit® 2.0 Fluorometer were used for measuring DNA amounts in papain digested samples according to manufacturer's instructions.

RNA isolation, cDNA synthesis and expression mRNA

After harvest, pellets were immediately stored in liquid nitrogen and subsequently in -80°C. For RNA isolation the RNeasy Microkit (QIAGEN, Hilden, Germany) was used according to the manufacturer's protocol. RNA concentrations were measured with NanoDrop 2000c (Thermo-Fisher, Waltham, United States). For synthesis of cDNA the iScript cDNA Synthesis Kit (Bio-Rad) was used, with 50 or 100ng RNA input. The RT-PCR reactions were performed and measured using Bio-Rad CFX384 detection system (Bio-Rad).

All primers used were designed specifically for equine species using computer software (primer BLAST, http://www.ncbi.nlm.nih.gov/tools/primer-blast/), and were obtained from Eurogentec (Maastricht, The Netherlands). Marker genes of cartilage degeneration and regeneration were tested (Table 3). These included: Matrix Metalloproteinase-3 (MMP3), Matrix Metalloproteinase-13 (MMP13), A disintegrin and metalloproteinase with thrombospondin motif-5 (ADAMTS-5), Collagen type II A1 (Col2), Collagen III (Col3), Collagen I (Col1), and Cartilage oligomeric protein (COMP). The samples were tested in single reactions in a CFX 384 well plate (Bio-Rad). 4-fold dilution series were made from a 10 times diluted cDNA pool for the standard curve. To each well 4.0 µl 50 times diluted cDNA was added and 6.0 µl Master Mix containing the primers, and 2x iQ SYBR green SuperMix (Bio-Rad) dissolved in MQ. Also, a negative control with MQ was performed. To normalize the expression levels, reference genes were used. Several reference genes were tested according to GeNorm calculations in Excel (17). The best performing reference genes that were used for normalization, were Hypoxanthine phosphoribosyltransferase (HPRT) and Tyrosine 3-Monooxygenase/Tryptophan 5-Monooxygenase Activation Protein Zeta (YWHAZ) for experiment 1, and Ribosomal Protein L13 (RPL13) and Ribosomal Protein S19 (RPS19) for experiment 2. The PCR process consisted of an initial denaturation phase enduring 2 minutes at 95°C, and 40 cycles of a two-step reaction with 30 sec at 95°C for denaturation and primer annealing, and 30 sec at annealing temperature (Ta) for extension. Data were analysed with CFX manager and used to calculate relative expression according to the normalized relative quantity (NRQ) method (18) in Excel.

Table 3 Overview primer specifications.

Gene		Primer sequence	Amplification length (bp)	Annealing temperature (°C)
RPL13	FW 5'- 3'	GCGGAAGAACTCAAATTGG	112	63
	RV 5'- 3'	GCCTTGAAGTTCTTCTCCT		
RPS19	FW 5'- 3'	CACGATGCCTGGAGTTACTG	144	63
	RV 5'- 3'	GGAGCAAGCTCTTTATGTTTGG		
YWHAZ	FW 5'- 3'	CAAGCGGAGAGCAAAGTC	179	61
	RV 5'- 3'	AGACCCAATCTGATAGGATGT		
HPRT	FW 5'- 3'	AATTATGGACAGGACTGAA	121	58
	RV 5'- 3'	ATAATCCAGCAGGTCAGCAAAG		
MMP13	FW 5'- 3'	CAAGGGATCCAGTCTCTCTATGGT	90	55,5
	RV 5'- 3'	GGATAAGGAAGGGTCACATTTGTC		
Col2	FW 5'- 3'	GGCAATAGCAGGTTCACGTACA	79	55,5
	RV 5'- 3'	CGATAACAGTCTTGCCCCACTT		
Col3	FW 5'- 3'	GCTTCATCCCACTCTTATTCTG	N/A	67
	RV 5'- 3'	GGCTTCCAGACATCTCTATCC		
Col1	FW 5'- 3'	CGTGACCTCAAGATGTGCA	93	62
	RV 5'- 3'	AGAAGACCTTGATGGCGT		
ACAN	FW 5'- 3'	AAGACAGGGTCTCGCTGCCCAA	115	64
	RV 5'- 3'	ATGCCGTGCATCACCTCGCA		
MMP3	FW 5'- 3'	AAATAGCAGAAGACTTTCCAGG	96	65
	RV 5'- 3'	TCAAACTGTGAAGATCCACTG		
COMP	FW 5'- 3'	CCACGTGAATACGGTCACAG	104	65
	RV 5'- 3'	ACGTCTGCTCCATCTGCTTC		
ADAMTS-5	FW 5'- 3'	AGCCACGCCAGCATTGAGAACC	107	65
	RV 5'- 3'	AGTGTGGTGGCCGCGTTCTT		

Statistical analysis

Statistics were performed on data with sample sizes of n=3 or more. Software used was Rstudio (version 3.1.1 software). Normality was tested by using the kurtosis test and skewness test. Non-parametric data was transformed by log transformation or square root transformation into normally distributed data. Analyses were done by performing one-way ANOVA or two-way ANOVA, subsequently Tukeys post-hoc test was performed.

Results

Experiment 1

Passage 0 versus passage 1 chondrocytes performance in pellet culture

To determine whether culturing pellets was a feasible method to mimic an *in vivo* situation of the joint, P0 and P1 chondrocytes from middle carpal (MC) and metacarpophalangeal (MCP) joints were used to form pellets (Experiment 1). Histological sections of P0 chondrocyte pellets after safranin-O staining showed pellets that were very small and non-cohesive. Neither pellets formed out of P0 or P1 chondrocytes derived from the MCP joint showed staining for GAGs.

Additionally, MCP joint cells were less chondrogenically differentiated than P1 chondrocytes derived from the MC joint. Expression levels of mRNA markers reached undetectable levels for almost all samples in P0 chondrocyte pellets, as well as for pellets formed out of MCP joint cells (data not shown). Therefore, further analyses were performed on data gained from P1 chondrocyte pellets originating from the MC joints.

Culture medium enriched with healthy synovial fluid

In order to evaluate matrix production, immunohistochemistry for collagen II, safranin-O staining for GAG content and cartilage marker genes mRNA expression levels were assessed on MC joint derived P1 pellets (Experiment 1). All pellets cultured in chondrogenic medium stained for collagen II, with the darkest and most diffuse staining after TGF-β1 supplementation. Addition of 25% HSF, TGF-β1 and LPS to non-chondrogenic medium, resulted in focal staining spots, but these were less prominent than in pellets cultured in chondrogenic medium (Fig. 1).

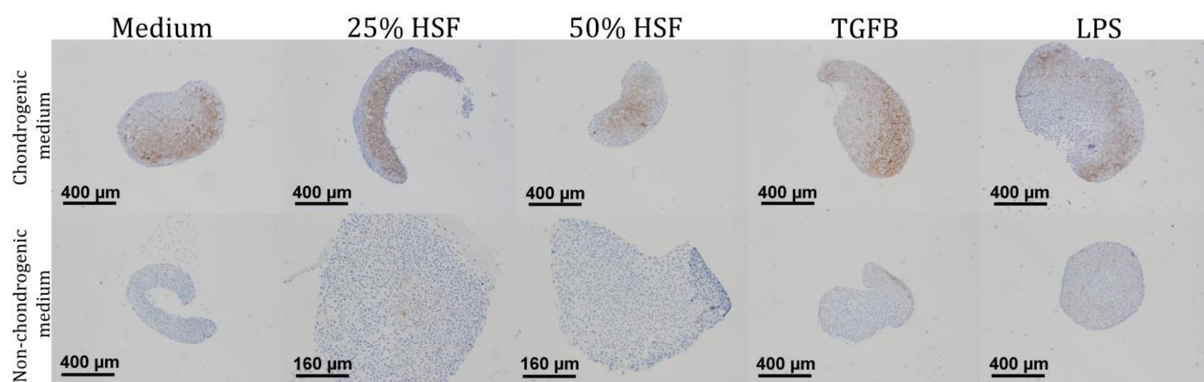

Figure 1 Representative collagen II staining in pellets cultured 1 week in chondrogenic medium or non-chondrogenic medium (control) supplemented with 25% HSF (healthy synovial fluid), 50% HSF, 10 ng/ml TGF-β1 (TGFB), or 10 ng/ml LPS. Brown staining indicates collagen II deposition. A 10x magnification was used for non-chondrogenic medium enriched with 25% HSF and 50% HSF, a 4x magnification for all other pictures.

Assessment of morphology and matrix production after safranin-O staining showed that all pellets contained round chondrogenic cells with matrix synthesized creating distance between cells. Lacunae around chondrocytes were specifically visible in pellets cultured in chondrogenic medium supplemented with 50% HSF or TGF-β1 (Fig. 2). Staining was darkest in TGF-β1 and LPS enriched medium. Addition of HSF to culture medium resulted in an increase in staining compared to plain medium. No staining was seen for pellets cultured in any of the non-chondrogenic medium conditions. In chondrogenic medium, only the addition of 25% HSF did not result in a higher Bern score compared to plain medium group (3.5±0.5 for plain medium versus 2.5±1.5 for 25% HSF). Medium enriched with 50% HSF resulted in a mean (±SD) Bern score of 5.0 (±1.0). Pellets cultured in non-chondrogenic medium demonstrated an increase in mean Bern score after addition of all conditions compared to plain medium, but did not reached a Bern score higher than 2 (Fig. 3A).

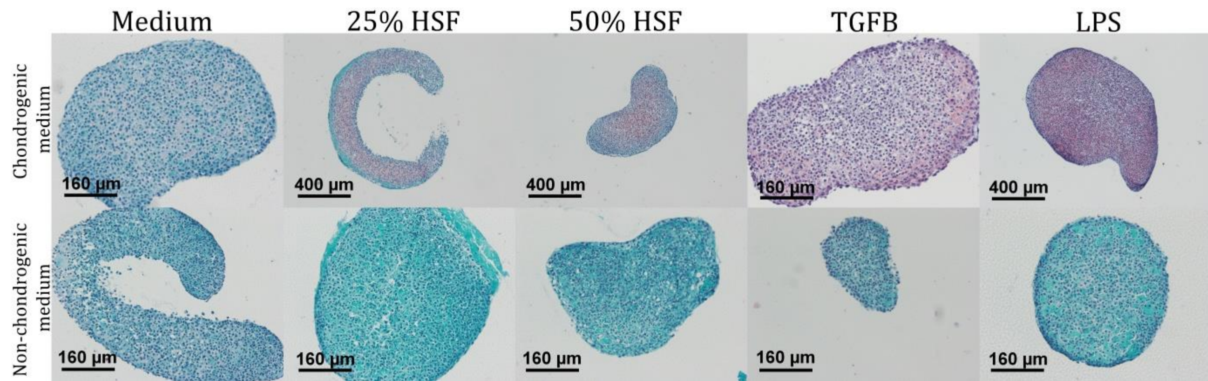

Figure 2 Representative safranin-O staining in pellets cultured 1 week in chondrogenic medium or non-chondrogenic medium (control) supplemented with 25% HSF (healthy synovial fluid), 50% HSF, 10 ng/ml TGF-β1 (TGFB), or 10 ng/ml LPS. Red staining in safranin-O indicates glycosaminoglycan (GAG) deposition. A 4x magnification was used for chondrogenic medium enriched with 25% HSF, 50% HSF and LPS, a 10x magnification for all other pictures.

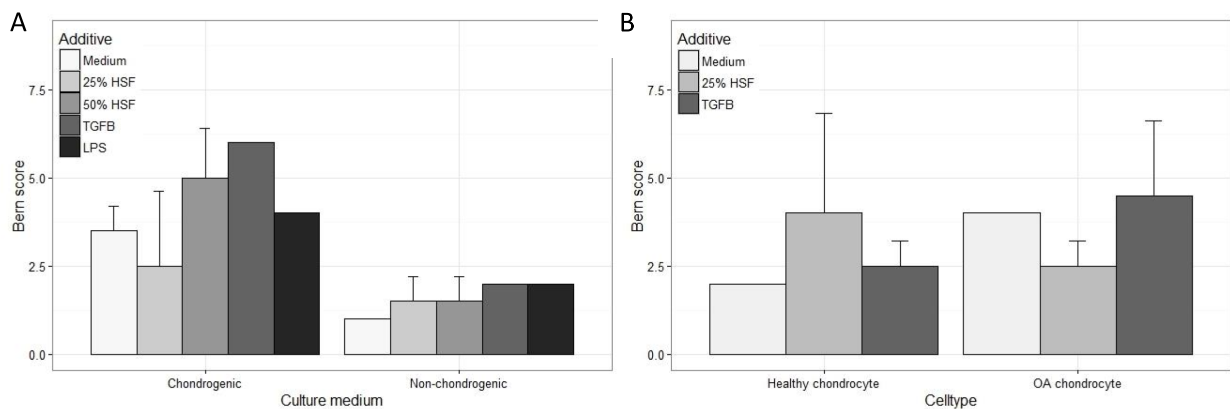

Figure 3 Bern scores of pellets stained with safranin-O (range 0-9). Bars represent mean + SD. **(A)** Pellets formed out of P1 middle carpal joint cells cultured in chondrogenic medium or non-chondrogenic medium (control) supplemented with 25% HSF (healthy synovial fluid), 50% HSF, 10 ng/ml TGF-β1 (TGFB), or 10 ng/ml LPS (n=2 pellets per condition, except for chondrogenic medium with LPS (n=1)). **(B)** Pellets formed out of healthy chondrocyte and chondrocytes derived from osteoarthritic joints (OA chondrocyte) cultured in chondrogenic medium (control) supplemented with 25% HSF, or 10 ng/ml TGF-β1 (n=2 pellets per condition).

Expression levels of collagen I and III increased progressively after addition of 25% HSF, 50% HSF in both chondrogenic and non-chondrogenic medium, however these differences were less prominent in non-chondrogenic medium. Addition of HSF did not result in an increase in collagen II mRNA levels, and only in a slight increase in COMP expression. On the other hand, expression of MMP3 and MMP13 showed a progressive increase in 25% and 50% HSF medium. Surprisingly, addition of LPS resulted in elevated expression levels of collagen II, collagen III and COMP compared to basal medium. Expression levels of MMP3 and MMP13 were found higher after LPS supplementation compared to medium, but lower compared to HSF addition (Fig. 4).

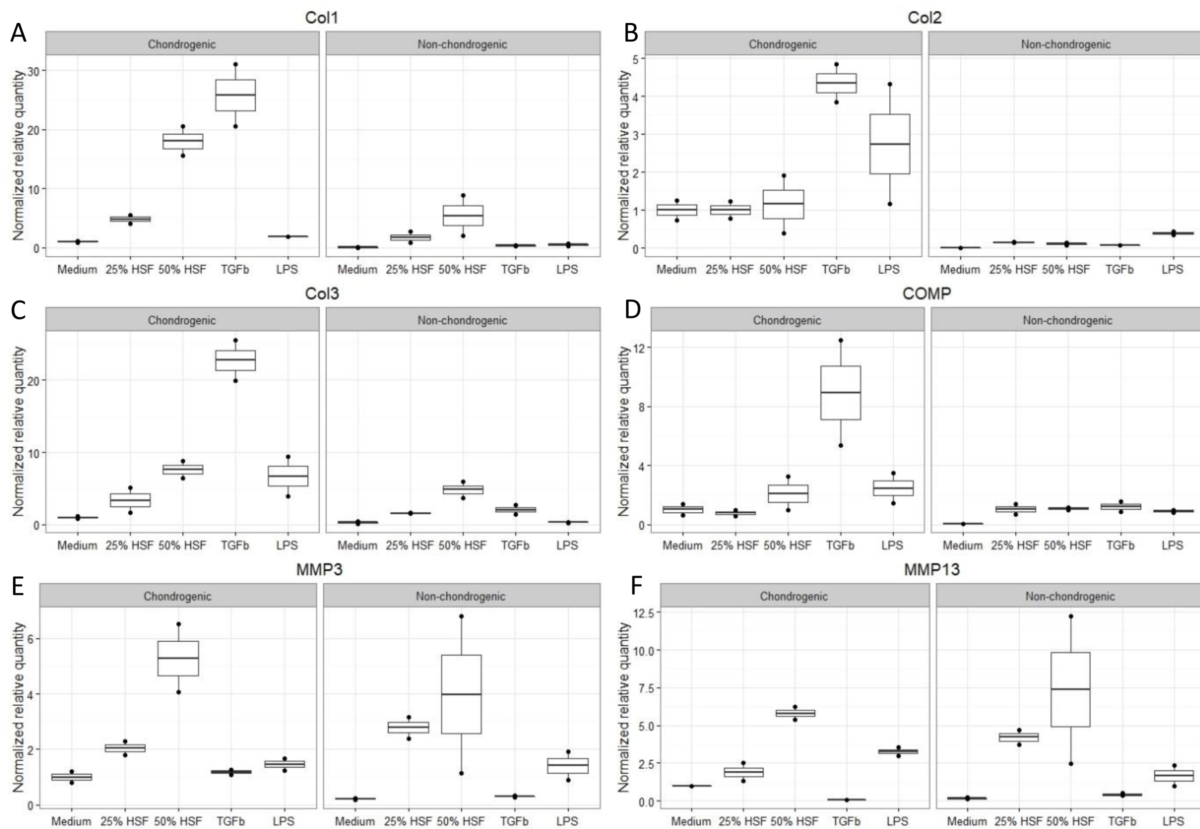

Figure 4 Normalized relative quantity of mRNA levels of **(A)** collagen I, **(B)** collagen II, **(C)** collagen III, **(D)** COMP, **(E)** MMP3, and **(F)** MMP13 in pellets (n=2 per condition) after 1 week of culture in chondrogenic medium or non-chondrogenic medium (control) supplemented with 25% HSF (healthy synovial fluid), 50% HSF, 10 ng/ml TGF-β1 (TGFB), or 10 ng/ml LPS.

These results show that addition of 25% HSF and 50% HSF improved matrix production and expression of both anabolic and catabolic marker genes, compared to medium. Likewise TGF-β1 and LPS addition induced matrix production and gene expression, which proves the role as positive control for TGF-β1 but contradicts the use of LPS as negative control.

Experiment 2

Healthy chondrocytes versus chondrocytes derived from osteoarthritic joints

Based on previous described results from experiment 1, in experiment 2 was chosen for culturing of P1 chondrocytes in chondrogenic medium enriched with 25% HSF. Under these culture circumstances the differences between pellets formed out of OA chondrocytes and healthy chondrocytes were examined.

In both healthy and OA chondrocytes, immunohistochemistry for collagen II existence in ECM showed staining after addition of TGF-β1, with a more evidently present and more diffuse staining in OA chondrocytes. The control group showed no staining for healthy chondrocytes, whereas the OA chondrocytes did display local staining. After adding 25% HSF in healthy or OA chondrocyte pellets, matrix was almost completely void of staining. Overall, collagen II appeared mainly synthesized in OA chondrocyte pellets (Fig. 5A).

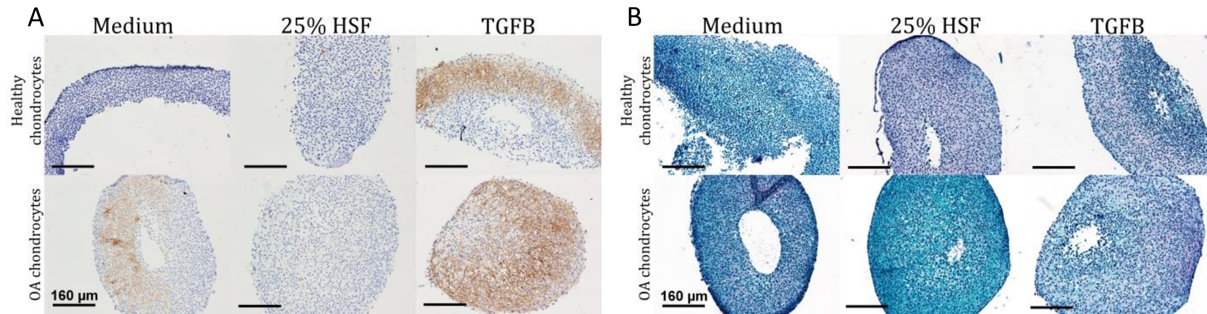

Figure 5 Representative **(A)** collagen II staining and **(B)** safranin-O staining in pellets (n=2) cultured 1 week in chondrogenic medium (control) supplemented with 25% HSF (healthy synovial fluid), or 10 ng/ml TGF-β1 (TGFB). Pellets were formed out of healthy chondrocytes and OA chondrocytes. Brown staining indicates collagen II deposition. Red staining in safranin-O indicates glycosaminoglycan (GAG) deposition. A 10x magnification was used.

Examining safranin-O stained sections presented an increase in GAG formation after adding 25% HSF or TGF-β1 in healthy chondrocyte pellets compared to control. While in OA pellets adding 25% HSF was not able to stimulate GAG formation. Staining was seen in OA pellets after adding TGF-β1 (Fig. 5B). Bern scoring confirmed the stimulating effect of 25% HSF and TGF-β1 in healthy chondrocyte pellets, and the decrease with HSF enriched medium in OA chondrocyte pellets (Fig. 3B).

Biochemical analyses of GAGs present in pellets showed lower mean GAG amounts in OA chondrocyte pellets compared to healthy chondrocyte pellets, except for the 25% HSF condition, which resulted in slightly lower GAG amounts in heathy pellets compared to plain medium (1.35 ± 1.05 versus 1.97 ± 0.10 µg/pellet, resp). However, a great variation is seen in healthy chondrocyte pellets after addition of 25% HSF (Table 4). Also, mean DNA amounts in OA chondrocytes were lower for every condition compared to healthy chondrocytes. This causes the relatively higher amounts of GAG normalized for DNA (GAG/DNA) in OA chondrocytes.

Addition of 25% HSF resulted in decreased levels of GAG/DNA in healthy chondrocytes, but showed increased levels of GAG/DNA in OA pellets. Two-way analysis of variance on GAGs released to the culture medium after 1 week of culture (4 days after medium change) showed no significant differences between cell types and no interaction between cell type and medium. However, post-hoc analysis showed a significant increase in GAG release for healthy chondrocytes after addition of 25% HSF compared to control (p=0.001). In OA chondrocytes addition of 25% HSF and TGF-β1 (both p<0.001) resulted in a significantly higher GAG release compared to medium. Both cell types had highest GAG release after addition of 25% HSF (Table 4).

Table 4 Differences in effects on matrix production of enriched chondrogenic medium on pellets formed out of healthy chondrocytes (healthy) and OA chondrocytes (OA).

Celltype	Additive	GAG (µg/pellet)	DNA (µg/pellet)	GAG/DNA (µg / µg)	GAG released to medium (µg /ml)	P-value
Healthy	Medium	1.97 ± 0.10	1.42 ± 0.20	1.41 ± 0.27	19.75 ± 1.76	-
	25% HSF	1.35 ± 1.05	1.32 ± 0.07	1.01 ± 0.74	34.12 ± 7.07*	0.001
	TGF-β1	2.20 ± 0.51	1.26 ± 0.11	1.73 ± 0.25	27.35 ± 11.14	0.284
OA	Medium	1.59 ± 0.08	0.87 ± 0.07	1.83 ± 0.04	15.48 ± 0.62	-
	25% HSF	1.94 ± 0.11	0.68 ± 0.06	2.86 ± 0.40	35.03 ± 7.44*	<0.001
	TGF-β1	1.60 ± 0.65	0.53 ± 0.48	4.20 ± 0.55	31.43 ± 2.49*	<0.001

GAG content, DNA content, and GAG/DNA (all n=2 per condition) measured after 1 week of culture, GAG release to medium measured (n=6 per condition) at 1 week of culture (4 days after medium change). Chondrogenic medium (control) was supplemented with 25% HSF (healthy synovial fluid), or 10 ng/ml TGF-β1. Values are presented as mean ±SD. * Significant difference compared to medium group of concurrent cell type.

Pellets formed out of OA chondrocytes showed higher mRNA expression levels for collagen I, collagen III and COMP compared to healthy chondrocytes under all conditions. A progressive increase in collagen I and -III expression was seen for HSF and TGF-β1 enriched medium in both cell types, whereas the addition of HSF did not stimulate COMP expression levels. Expression levels of collagen II were comparable for healthy chondrocytes and OA chondrocytes. Although, addition of 25% HSF increased expression levels in healthy chondrocytes, this effect was not seen in OA chondrocytes. Addition of TGF-β1 resulted in a clear increase in expression levels, particularly in one of the OA chondrocyte pellets (Fig. 6).

Expression levels of marker genes MMP3, -13, and ADAMTS5 were similar for healthy and OA chondrocytes cultured in medium or medium with TGF-β1, but showed a 2 fold (MMP3) to 80 fold (MMP13) increase in OA chondrocytes cultured in HSF enriched medium. This effect was not seen in healthy pellets (Fig. 6).

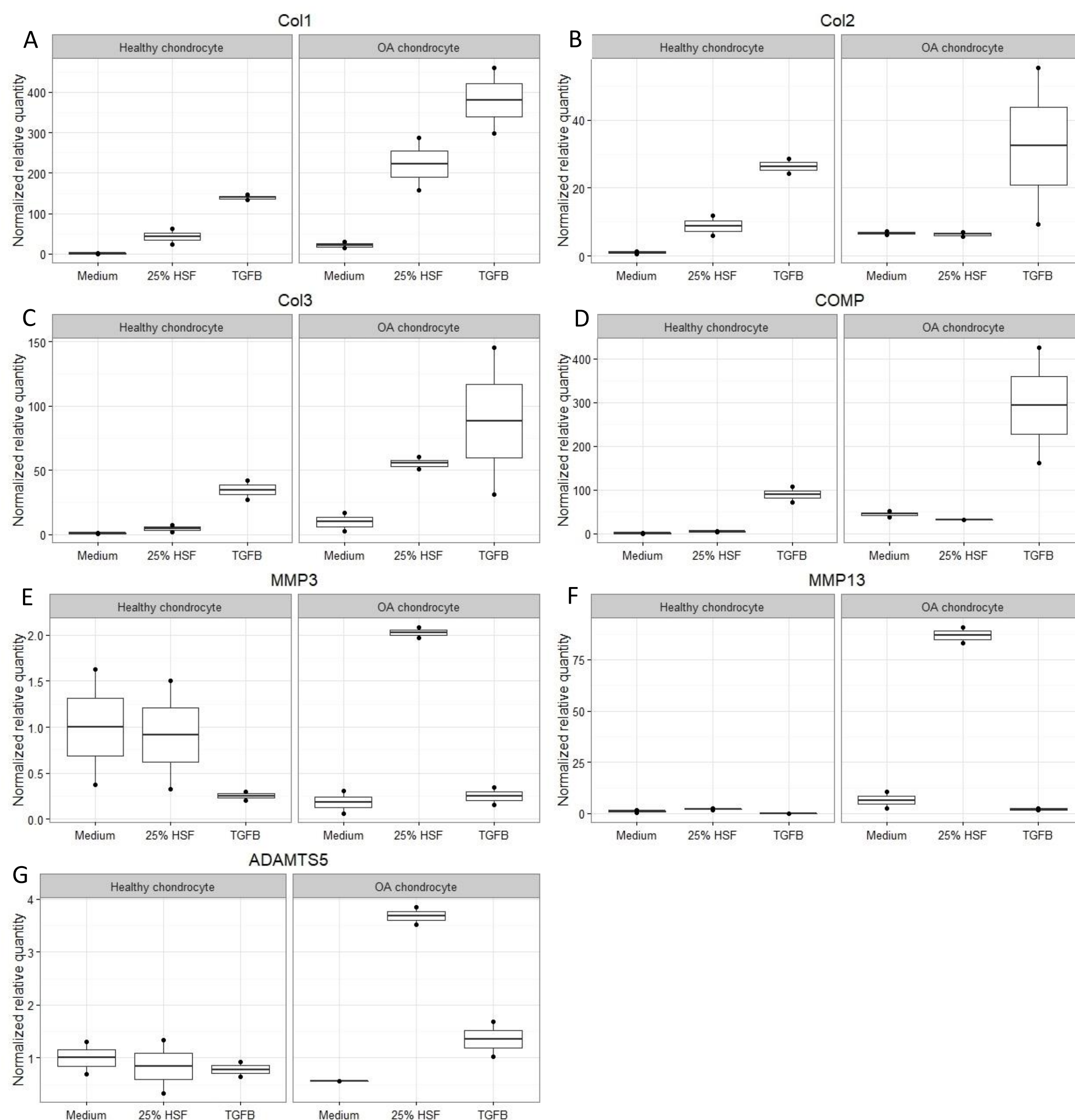

Figure 6 Normalized relative quantity of mRNA levels for **(A)** collagen I, **(B)** collagen II, **(C)** collagen III, **(D)** COMP, **(E)** MMP3, **(F)** MMP13, and **(G)** ADAMTS-5 in pellets (n=2 per condition) formed out of healthy chondrocytes and OA chondrocytes after 1 week of culture in chondrogenic medium (control) supplemented with 25% HSF (healthy synovial fluid), or 10 ng/ml TGF-β1 (TGFB). Boxplots show the measured points and the mean normalized relative quantity.

Discussion

The aim of this study was to optimize a 3D chondrocyte culture system, which allows us to investigate effects of SF on chondrogenesis and matrix production. To achieve this we investigated P0 and P1 chondrocytes in pellet culture, supplemented with SF from healthy joints. Additionally, differences between OA chondrocytes and healthy chondrocytes in pellet culture were explored. Our results demonstrated that P1 chondrocytes had higher gene expression levels of both cartilage marker genes and housekeeping genes, and formed histologically better pellets compared to chondrocytes immediately after harvest. Replacement of 25% and 50% of culture medium with SF resulted in improved GAG and collagen II production on histology compared to plain medium. Gene expression levels of both anabolic and catabolic cartilage marker genes increased progressively with a higher concentration of added SF. Pellets formed out of OA chondrocytes showed less safranin-O staining, but more collagen II staining compared to healthy chondrocytes under every condition.

Passage 1 chondrocytes perform better than passage 0 chondrocytes in pellet culture

Because of a limited amount of P0 chondrocytes, it is desirable to have the opportunity of expanding chondrocytes before pellet culture. A study on chondrocytes cultured in monolayer found that a higher passage number results in more dedifferentiated chondrocytes (2). Previous research on 3D cultured chondrocytes shows the possibility of culturing pellets up to P2 with sufficient pellet formation (6). Successful redifferentiation of chondrocyte in alginate beats is found possible up to 4 passages, although worsened each passage (19). Additionally, a study comparing chondrocytes from different passages seeded in scaffolds, found collagen II production was highest in P2 cells, but P1 cells contained a higher amount and more viable cells (10). Based on these findings, we hypothesized that P1 pellets were able to redifferentiate and form viable pellets. However, we expected that chondrocytes after passaging were more difficult to redifferentiate and effects of supplemented SF were less visible compared to primary chondrocytes.

In contrast with our hypothesis, we found that viable pellets were difficult to form out of P0 cells, and almost no gene expression of cartilage markers and housekeeping genes was present in P0 pellets. A study using equine P0 chondrocytes in pellet culture, was able to generate pellets comparable with hyaline cartilage (20). Although, a different pellet culture system was used; $5x10^5$ cells were seeded in polypropylene tubes for pelleting. Foetal calf serum (FCS) or human serum (HS) was added as growth factor to the medium. Higher cell numbers and different growth factors used in pellet culture might be beneficial for pellet formation. These differences in culture circumstances are a possible explanation for the failed pellet formation in our experiment. A more presumable explanation of our unexpected results is a technical failure in pellet formation, as we experienced problems with centrifugation to form the P0 pellets. To exclude this technical failure as a cause, another resembling pellet culture comparing P0 and P1 cells should be performed. Our results imply that chondrogenic potential is not completely lost after passaging chondrocytes once. Future research on the suitability of P2 cells is desired, because further passaging can provide a larger amount of cells. However, using P2 cells in pellet culture might impair chondrogenic potential of the chondrocytes.

The addition of SF obtained from healthy joints stimulates chondrogenesis

When assessing the differences in matrix production between supplementation of 25% SF and 50% SF to culture medium, we found higher Bern scores for pellets cultured in 50% HSF compared to pellets cultured in addition of 25% HSF. This indicates that culturing in higher amounts of SF improves GAG production. Consistent with our present findings, Lee et al. found improved matrix production, visible as increased GAG synthesis when HSF was added to culture

medium. Amount of GAGs increased simultaneously with higher percentage of SF up to 100% (9). Comparable results were found by van den Hoogen et al., who mentions increasing sulphate incorporation into GAGs in equine cartilage explants when cultured in HSF (8). A driving factor in this stimulation of chondrogenesis by SF might be the presence of nutritional components in SF (21).

When we increased the percentage of supplemented SF, gene expression levels raised for all cartilage marker genes. Unexpectedly, this effect was most prominent for collagen I and collagen III, and least for collagen II. Collagen I and collagen III are known as markers for chondrocyte dedifferentiation and fibrocartilage type repair tissue (22,23). Addition of TGF-β1 resulted in a similar gene upregulation as after SF supplementation. Therefore, upregulation of these collagen types is not necessarily correlated with dedifferentiated cartilage, since limited amounts of collagen III contribute to healthy hyaline cartilage matrix (24).

Remarkably, in experiment 2 different findings at histology were found compared to experiment 1. Addition of 25% HSF did not show an increase in staining intensity for collagen II, and only one out of two pellets stained positive for safranin-O. A possible explanation for these differences is the use of various chondrocyte donors and SF donors. The ability of chondrocytes to produce matrix components are different between donors, indicated by variable GAG amounts found in constructs containing human chondrocytes (25). In addition, SF composition is subject to large individual variations (26). To correct for this, multiple cell -, and SF donors were pooled. In future research, donors should be tested on performance before multiple donors are pooled.

Comparing our biochemical analyses, we found that GAG levels normalized for DNA (GAG/DNA) were not in line with our findings for GAG released to medium. Addition of TGF-β1 increased GAG/DNA levels, while after addition of 25% HSF in healthy pellets GAG/DNA levels decreased compared to medium control. In contrast, GAGs released to medium were significant higher after addition of 25% HSF. This finding indicates that GAG production is stimulated, but GAG deposition in ECM (partly) fails. Another possibility is that GAGs in ECM are degraded and released to medium. A comparable situation is seen in OA joints, where levels of GAGs in SF are increased after joint damage (27). For better evaluation of GAG synthesis, radiolabelled precursor [^{35}S]sulphate incorporation into GAGs could be used. This facilitates the ability to distinguish between newly synthesized GAG and GAGs already present (8). Another explanation for high GAG levels in medium might be the interference of GAGs present in SF added to the culture medium. We corrected GAG values released to medium for GAG amounts in added SF. To this end, samples were digested using hyaluronidase. After hyaluronidase digestion, tightly packed GAGs could be still present, which prevents DMMB molecules to fully bind GAGs. This irregular digestion might cause variation in multiple measurements of the same SF sample. Instead of using hyaluronidase, the use of papain digestion of SF might result in a more extensive digestion (28).

An intriguing accessory finding is that when pellets were cultured in non-chondrogenic medium instead of chondrogenic medium, effects of HSF on chondrogenesis were completely diminished. Even TGF-β1 addition was not able to stimulate chondrocytes to matrix production. The difference in composition between non-chondrogenic and chondrogenic medium is the addition of BSA and ITS to the latter, which are both known to stimulate chondrogenesis. For example, chondrogenic differentiation of human MSCs is found to be stimulated more by BSA compared to FCS (29), also insulin-transferrin-selenium ITS is found to have positive effects on chondrogenesis in 3D cultured bovine chondrocytes (30). Interestingly, Yang et al. reports that pellets formed out of chondrocytes expanded in medium without addition of FCS, showed no safranin-O staining. Even though human serum albumin (HSA), ITS and TGF-β2 were added to culture medium after pellets were formed (6). These findings imply that SF is not able to completely replace chondrogenic medium. However, culturing in 100% HSF resulted in 2 times

higher sulphate incorporation in GAGs compared to 20% HSF (8). Therefore, various concentrations of SF should be tested to determine whether higher percentages might enable replacement of chondrogenic medium.

Another essential point is that both TGF-β1 and LPS addition induced matrix production and gene expression of cartilage marker genes, which proves the role as positive control for TGF-β1 but contradicts the use of LPS as negative control. Other studies assessing chondrocyte monolayers where LPS was added to the culture medium, found increased levels of pro-inflammatory cytokines, which resulted in suppressive biosynthesis of ECM products (31,32). Further investigation into the effects of LPS on chondrocytes in pellet culture is needed when a pro-inflammatory state as negative control is desired.
Although TGF-β1 was found to stimulate chondrogenesis, staining for collagen II and safranin-O was not complete diffuse after 1 week of culture. In contrast, Heldens et al. reached diffuse and dark safranin-O staining upwards of 1 week of pellet culture. However, no credible comparison can be made, since human MSCs differentiated to chondrocytes were used (33). Yang et al. cultured human chondrocyte pellets in medium supplemented with comparable growth factors as used in our experiments, although insulin-like growth factor (IGF-1) was added. Histology was assessed after 4 weeks of culture, which resulted in diffuse safranin-O staining (6). This might indicate that for complete chondrogenic differentiation and matrix production of chondrocytes in pellet culture, a prolonged culture period is a requisite. Therefore, a prolonged pre-culture before adding different conditions might improve our current culture method. Future research into the use of pre-culture is desired to provide a more established pellet with a better resemblance to *in vivo* cartilage.

Chondrocytes derived from OA patients behave differently than healthy chondrocytes
New cartilage regenerating therapies often use autologous chondrocytes for implantation (1). Autologous chondrocytes from damaged joints might have impaired regeneration capacity, because metabolism is dysregulated when joints suffer severe OA (34). While some studies suggest that dedifferentiation in expansion causes loss of OA phenotype, others say that differences in phenotype stay visible (12–14). A study on differences between chondrocytes derived from different cartilage zones proves the ability of restoring original chondrocyte phenotype in culture. Small differences in phenotype seen in zonal specific chondrocytes, were restored after expansion (35).

We found more collagen II staining, but less safranin-O staining for GAGs in OA chondrocyte pellets compared with healthy chondrocyte pellets. Corroborant findings in GAG content were seen, with overall lower levels of GAG amount in OA cells. Interestingly, after addition of HSF, expression levels of matrix components collagen I and collagen III were highly upregulated in OA cells compared to both medium control and TGF-β1 addition. This effect was not seen in healthy chondrocyte pellets. Altered interaction between SF components and damaged chondrocytes could be the underlying cause for this finding. Addition of TGF-β1 resulted in comparable effects on gene expression between OA cells and healthy cells. Our findings indicate that characteristics of OA chondrocytes remain present after redifferentiation. These characteristics might be emphasized after addition of HSF, and are possibly (partly) masked by culturing in addition of TGF-β1. A limitation of the comparison between OA chondrocytes and healthy chondrocytes is that different cell donors were used. Osteoarthritic cells behaving differently from healthy cells in pellet culture, implies that usage of autologous chondrocytes from OA patients in regenerative therapies could give different outcomes compared to joints with healthy chondrocytes. This might contribute to a more difficult healing of cartilage defects in later stages of OA. More research is needed with similar donors for healthy cells and OA cells to confirm these findings.

Conclusion

Taken together, we found that pellet culture of passage 1 chondrocytes is a suitable way for researching effects of SF on chondrogenesis. We can conclude that addition of 25% SF to pellet culture is sufficient to find differences in chondrogenesis, provided that culture medium is enriched with chondrogenic growth factors. The addition of SF obtained from healthy joints is likely to stimulate chondrogenesis. Nevertheless, these positive effects were not visible in pellets formed out of chondrocytes from OA patients.

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Chapter 3

Effects of synovial fluid obtained from joints with different stages of joint damage on in vitro chondrogenesis

Abstract

BACKGROUND: Osteoarthritis (OA) is often accompanied with a disturbed joint homeostasis, which is reflected in synovial fluid (SF) composition. This disturbed joint homeostasis changes during OA development and might influence the effectiveness of cartilage regenerative therapies. The aim of this chapter was to investigate differences in effects of SF obtained from healthy joints, joints in a very acute stage of joint inflammation, and joints with more chronic osteoarthritic changes on cultured chondrocytes.

MATERIALS AND METHODS: Chondrocyte pellets were cultured for 1 week in chondrogenic medium (negative control) supplemented with 25% HSF (healthy SF), 25% LPSSF (SF obtained 24 hours after intra-articular LPS injection), 25% OASF (SF obtained from osteoarthritic joints), or 10 ng/ml TGF-β1 in experiment 2. In experiment 3 and 4, a pre-culture period of 2 weeks was tested prior to addition of the several conditions. Histological examination of collagen II and glycosaminoglycan (GAG) production was performed. In addition, GAG content, DNA content, GAG released to medium, and mRNA expression levels of cartilage markers were measured.

RESULTS: We found collagen II deposition in pellets cultured in addition of OASF, but not in pellets cultured in medium enriched with LPSSF or HSF. Safranin-O staining and GAG normalized for DNA (GAG/DNA) levels were higher in LPSSF and OASF groups, compared to the HSF group. Gene expression levels for collagen I and collagen III were higher after addition of LPSSF in culture, compared to OASF and HSF. On the other hand, collagen II and COMP were found higher after culture in medium enriched with OASF and HSF compared to LPSSF. Pre-culturing prior to addition of the conditions resulted in negative effects on histology and decreasing biochemical values after addition of every condition.

CONCLUSION: Both LPSSF and OASF stimulate matrix production in chondrocyte pellet culture more than HSF in terms of matrix production. However, addition of LPSSF resulted in more dedifferentiated chondrocytes. Pellets became unstable and degenerated after prolonged pre-culture before testing effects of SF.

Introduction

Osteoarthritis (OA) is a complex multifactorial degenerative joint disease, of which exact pathophysiology is not clarified yet. Patient follow-up studies found that joint trauma increases risk on developing OA over time (1). This early stage of cartilage damage arouses a disturbed joint homeostasis, with upregulation of inflammatory mediators and other anabolic and catabolic factors (2). Synovitis is often seen immediately after cartilage damage, where concentration of inflammatory mediators in synovial fluid (SF) peak early but decrease over time. Early treatment of these cartilage defects permits the ability to modify the course of OA development, since treating old defects results in worse cartilage repair (3). Nevertheless, early diagnosis of OA encounters some difficulties. Clinical signs may only occur when OA is further developed, and early changes in cartilage are not always visible with imaging techniques (4). Therefore, early treatment in OA is not always possible. Disturbed joint homeostasis is reflected in SF composition, and changes when OA progresses (5). When studying new regenerative therapies this changed joint environment in OA progression might influence the effectiveness of these therapies.

Several studies researched the influence of SF on *in vitro* chondrogenesis. An *in vitro* study using human cultured chondrocytes showed a significant decrease in proteoglycan contents and collagen type II amounts and distribution when SF from injured knees was added to culture medium (6). This indicates a negative effect of OA SF on chondrogenesis. In contrast, when SF obtained from healthy joints was added to cultured chondrocytes, similar effects as seen in *in vivo* chondrogenesis were found, characterized by high glycosaminoglycan (GAG) synthesis (7). However, effects of healthy synovial fluid on cultured chondrocyte and synovial fluid obtained from joints with different degrees of damage have never been compared within one study. Therefore, more insights are desired in differences on chondrocyte performance after addition of SF obtained from various stages in joint disease compared to healthy SF.

The aim of the experiments described in this chapter was to examine differences in effects of SF obtained from healthy joints, joints with an acute stage of joint inflammation and joints with severe osteoarthritic changes, on cultured chondrocytes. In order to achieve this, we used a previous established pellet culture method (Chapter 2), and we used a prolonged pre-culture period in order to test if a more developed pellet is required to pick up signs of matrix degradation. We hypothesized that 1) SF obtained from an acute stage of joint inflammation stimulates chondrogenesis and matrix production, while SF obtained from a joint with established OA will have inhibiting effects, and 2) that a prolonged pre-culture period provides a better established pellet, and that both signs of matrix production and degradation can be measured after this pre-culture.

Materials and methods

Experimental designs

All variables and fixed factors per experiment are summarized in Appendix I (Table 1). The current chapter describes experiment 2, 3 and 4.

Experiment 2

As described in Chapter 2. The current chapter reports effects of 25% healthy SF (HSF), 25% SF obtained 24 hours after intra-articular LPS injection (LPSSF) and 25% SF obtained from osteoarthritic joints (OASF) on healthy chondrocytes only.

Experiment 3 and 4
Healthy chondrocytes were obtained from three cell donors and were pooled (Table 1). Pellets were pre-cultured for 2 weeks in chondrogenic medium + 10 ng/ml human recombinant transforming growth factor β1 (TGF-β1, R&D Systems, Minneapolis, Minnesota, Unites States). A second group of pellets was pre-cultured in chondrogenic medium without TGF-β1. After pre-culture pellets were cultured in chondrogenic medium supplemented with 25% HSF or 25% OASF. Pellets cultured in addition of 10 ng/ml TGF-β1 functioned as positive control, while plain chondrogenic medium was used as negative control. Pellets were harvested at 0, 7, and 14 days after pre-culture (week 2, 3, and 4, resp.). Pellets were processed for histology (safranin-O), immunohistochemistry (collagen II), and assessed for GAG and DNA content in pellets. For each analysis n=3 pellets were used, except for GAGs released to medium n=6 samples were used.

Synovial fluid collection

Three types of SF donors were used; donors with healthy joints, joints with (chronic) osteoarthritic changes and joints with an acute stage of synovitis/arthritis. For each condition 2 or more donors (aged >2years) were pooled, and in each experiment different SF donors were used. Previously obtained and freezer-stored SF from metacarpophalangeal (MCP) and middle carpal (MP) joints from horses at a local abattoir and from patients euthanized at the clinic (with owner consent) was used as healthy SF (HSF) or osteoarthritic SF (OASF). Determination of OA diseased joints for OASF was based on macroscopic, microscopic and/or radiographic findings. Representing an acute stage of synovitis/arthritis, SF from horses 24 hours after injection with 3.5 ng lipopolysaccharide (LPS, E. coli O55:B5, Sigma-Aldrich, Zwijndrecht, The Netherlands) in 2 ml NaCl (LPSSF) was obtained from the tarsal joint (8). Punctures were performed using a 10 ml syringe with an 18 gauge needle. Joints were clipped and sterile prepared with 70% ethanol (EtOH) and chlorhexidine preparatory to puncture. Obtained SF was centrifuged for 5 minutes at 2520 g to remove cells and debris. The supernatant was stored at -80°C.

Table 1 Overview characteristics of the donors used for cell culture in each experiment.

Exp. nr.	Donor	Breed	Age	Sex	Joint	Cell type	Cell stage
2	Pony 1	Shetland pony	6	Gelding	MCP	Chondrocyte	P1
	Pony 4	Shetland pony	8	Mare	MCP	Chondrocyte	P1
	EQ17-001	Shetland pony	30	Unknown	MCP	OAC	P1
	EQ17-002	Shetland pony	20	Unknown	MCP	OAC	P1
3	EQ017-003	Shetland pony	6	Mare	MC	Chondrocyte	P1
	EQ017-009	Shetland pony	4	Mare	MC	Chondrocyte	P1
	EQ017-010	Shetland pony	3	Gelding	MC	Chondrocyte	P1
4	EQ017-003	Shetland pony	6	Mare	MC	Chondrocyte	P1
	EQ017-005	Shetland pony	6	Mare	MC	Chondrocyte	P1
	EQ017-006	Shetland pony	6	Mare	MC	Chondrocyte	P1

Cell stage represents the stage of the cells the pellets were formed with. Age is in years. OAC = chondrocytes from osteoarthritic joints. MCP = metacarpophalangeal joint, MC = middle carpal joint.

Cell isolation and expansion

Chondrocytes were harvested within 24 hours post mortem with a sterile scalpel knife, and cartilage pieces were kept in DMEM (high glucose, GlutaMAX, pyruvate) (31966, Gibco, Dublin, Ireland) + 1% p/s (penicillin/streptomycin, P11-010, Gibco) at 37°C. Within 24 hours after harvest, cartilage pieces were washed in sterile HBSS (Hanks Balanced Salt Solution, Gibco) + 1% p/s, and minced cartilage pieces were digested overnight on a roller plate at 37°C in 30 ml 0.15% collagenase II (Worthington, Lakewood, United States) solution in DMEM + 1% p/s. After digestion, cells were strained through a 70 µm cell strainer (Greiner bio-one, Alphen aan de Rijn, The Netherlands) to remove debris. Cells were stored in liquid nitrogen.

Cell culture experimental conditions
Cells were expanded using expansion medium in T175 flasks (Cellstar, Greiner bio-one) in a density of approximately 5000 cells/cm2. Expansion medium contained DMEM (high glucose, GlutaMAX, pyruvate) (31966, Gibco) + 1% p/s + 1.25 µg/ml Fungizone (Amphothericin, Gibco) + 10% FBS (Foetal Bovine Serum, Gibco,) + 0.5% 0.1mM ASAP (Ascorbic acid 2 – phosphate, Sigma-Aldrich) + 1 ng/ml βFGF (Basic fibroblast growth factor, AbD Serotec, Bio-Rad, Hercules, Californië, United States). When confluent, cells were trypsinized with 1x TripLE expres (Gibco) and counted with TC20 cell counter (Bio-Rad). Cells were resuspended in chondrogenic medium containing DMEM/F-12 (HEPES, no phenol red, L-glutamine, 11039, Gibco) + 1% ITS+ premix (Insulin Transferrin Selenium, Corning Life Sciences, Corning, United States) + 1% p/s + 1.25 µg/ml Fungizone + 5µl /ml 20mM ASAP + 1.5 mg/ml BSA (Bovine Serum Albumin, Sigma-Aldrich). Cells were plated out in an ultra-low cell attachment 96 wells plate with a round bottom (Costar 7007, Corning Life Sciences) in a cell density of $2x10^5$ cells per well and centrifuged for 5 minutes at 300 g to form pellets. Medium was refreshed each 3 or 4 days and stored in Micronic tubes (MICRONIC, Lelystad, The Netherlands). Pellets were all cultured at 37°C and 5% CO_2.

Histology and immunohistochemistry
Pellets were fixed in 200 µl 10% formalin (Sigma-Aldrich) with 0.1% eosin (Boom BV, Meppel, The Netherlands) for 1 week. Afterwards pellets were embedded in 2.4% alginate (Sigma-Aldrich) and gelated by 3.7% formalin with 102 mM $CaCl_2$. Samples were dehydrated through EtOH 70% - 100% (Klinipath, Breda, The Netherlands) and 1 step xylene (Klinipath) for 1 hour. Pellets were incubated in paraffin (Leica, Amsterdam, The Netherlands) at 60°C for 2 hours before embedding in paraffin. The samples were cut into 5 µm slices using Microm microtome and captured on KP plus printer slides (Klinipath). Afterwards slides were fixed on a hot plate of 60°C for 1 hour. Before staining slides were deparaffinised by two steps xylene and hydrated through EtOH 60% - 100%. Post staining, dehydration was performed through 70 -100% and 2 steps xylene. Cover slides were mounted with Depex (Merck, Whitehouse Station, New Jersey, United States). Images were acquired using an Olympus BX51 microscope with DP73 digital camera (Olympus, Zoeterwoude, the Netherlands).

Safranin-O
Alginate was removed by 15 minutes incubation in a citrate buffer. Afterwards nuclei were stained with Weigert's Hematoxylin (Klinipath) for 5 minutes and washed in running water. Counterstaining with 0.4% aqueous Fast Green (Sigma-Aldrich) was performed for 4 minutes, after which slides were washed in 1% Acetic Acid (Boom BV). Present GAGs were stained with 0.125% aqueous safranin-O (Sigma-Aldrich) solution for 5 minutes. Short dehydration starting with 96% EtOH was needed to prevent safranin-O from washing of. Healthy cartilage explants were stained as positive control.

Sections stained with safranin-O staining in experiment 2 were graded according to Bern scoring (0-9) for in vitro generated neocartilage, where a score of 9 shows histological a resemblance of hyaline cartilage (9). Pellets were scored for staining darkness, matrix formation, and cell morphology (Table 2).

Table 2 Scoring categories Bern score (9).

Scoring categories	*Score*
A. Uniformity and darkness* of Safranin O–fast green stain	
No stain	0
Weak staining of poorly formed matrix	1
Moderately even staining	2
Even dark stain	3
B. Distance between cells/amount of matrix accumulated	
High cell densities with no matrix in between (no spacing between cells)	0
High cell densities with little matrix in between (cells <1 cell-size apart)	1
Moderate cell density with matrix (cells approx. 1 cell-size apart)	2
Low cell density with moderate distance between cells (>1 cell) and an extensive matrix	3
C. Cell morphologies represented	
Condensed/necrotic/pycnotic bodies	0
Spindle/fibrous	1
Mixed spindle/fibrous with rounded chondrogenic morphology	2
Majority rounded/chondrogenic	3

*Section 3–4 μm thick.

Collagen II

Slides were blocked with 0.3% H_2O_2 solution for 10 minutes. Antigen retrieval steps contained Pronase (Roche, Basel, Switzerland) 1 mg/ml and Hyaluronidase (Sigma-Aldrich) 10 mg/ml both for 30 minutes at 37°C. The second blocking section was with PBS/BSA (Bovine Serum Albumin, Sigma-Aldrich) 2% for 30 minutes, after which incubation with the primary antibody Collagen II Mouse monoclonal antibody DSHB (1:1500 in PBS-BSA) (Santa-Cruz, Dallas, Texas, Unites States) and a normal mouse IgG1 (1:1800 in PBS-BSA) (Santa Cruz) as negative control took place overnight at 4 °C. Next day, samples were incubated with goat anti-mouse secondary antibody containing HRP (Dako, Heverlee, Belgium) for 30 minutes. DAB peroxidase substrate solution (Dako) was added for 5 minutes to obtain signal. Counterstaining was performed using Mayers Hematoxylin (Merck). As control healthy cartilage explants were used.

Determination of glycosaminoglycan and DNA contents in pellets and medium

Pellets were harvested and washed with 100 µl HBSS to remove GAGs present in remaining medium. Afterwards, pellets were dried for 60 minutes using a Savant speedvac. Pellets were digested in 200 µl papain digestion solution containing 250 µg/ml Papain (from papaya, Sigma-Aldrich) and 1.57 mg/ml Cysteine HCl (Sigma-Aldrich) in 2*Papain buffer (containing Na_2HPO_4 + EDTA.2H_2O, Merck) overnight at 60°C. For digestion of SF in aspirated medium, samples were diluted 1:1 with Hyaluronidase type II (Sigma-Aldrich) 0.1 mg/ml in 25mM Sodium Acetate (Merck) pH 6.5 and incubated for 30 minutes at 37°C.

For measuring sulphated GAG content in both pellets and medium, the DMMB assay was used as described by Farndale (10). Digested samples were diluted using PBS-EDTA, and 100 µl of the diluted sample was pipetted in duplo into a 96 wells plate with a flat and clear bottom (Greiner bio-one). Chondroitin sulphate (Sigma-Aldrich) was used for standardization in a dilution series with concentrations between 0 and 10 µg/ml. 200 µl of DMMB (Sigma-Aldrich) staining solution was added and extinction was measured at 525 and 595 nm using a plate reader. Aspirated mediums GAG content was corrected for GAG concentrations in added SF. Broad and High

Sensitive assay of the Qubit® 2.0 Fluorometer were used for measuring DNA amounts in papain digested samples according to manufacturer's instructions.

RNA isolation, cDNA synthesis and expression mRNA

After harvest pellets were immediately stored in liquid nitrogen and subsequently in -80°C. For RNA isolation the RNeasy Microkit (QIAGEN, Hilden, Germany) was used according to the manufacturer's protocol. RNA concentrations were measured with NanoDrop 2000c (Thermo-Fisher, Waltham, United States). For synthesis of cDNA the iScript cDNA Synthesis Kit (Bio-Rad) was used, with 50 or 100ng RNA input. The RT-PCR reactions were performed and measured using Bio-Rad CFX384 detection system (Bio-Rad).

Table 3 Overview primer specifications.

Gene		Primer sequence	Amplification length (bp)	Annealing temperature (°C)
RPL13	FW 5'- 3'	GCGGAAGAACTCAAATTGG	112	63
	RV 5'- 3'	GCCTTGAAGTTCTTCTCCT		
RPS19	FW 5'- 3'	CACGATGCCTGGAGTTACTG	144	63
	RV 5'- 3'	GGAGCAAGCTCTTTATGTTTGG		
MMP13	FW 5'- 3'	CAAGGGATCCAGTCTCTCTATGGT	90	55,5
	RV 5'- 3'	GGATAAGGAAGGGTCACATTTGTC		
Col2	FW 5'- 3'	GGCAATAGCAGGTTCACGTACA	79	55,5
	RV 5'- 3'	CGATAACAGTCTTGCCCCACTT		
Col3	FW 5'- 3'	GCTTCATCCCACTCTTATTCTG	N/A	67
	RV 5'- 3'	GGCTTCCAGACATCTCTATCC		
Col1	FW 5'- 3'	CGTGACCTCAAGATGTGCA	93	62
	RV 5'- 3'	AGAAGACCTTGATGGCGT		
ACAN	FW 5'- 3'	AAGACAGGGTCTCGCTGCCCAA	115	64
	RV 5'- 3'	ATGCCGTGCATCACCTCGCA		
MMP3	FW 5'- 3'	AAATAGCAGAAGACTTTCCAGG	96	65
	RV 5'- 3'	TCAAACTGTGAAGATCCACTG		
COMP	FW 5'- 3'	CCACGTGAATACGGTCACAG	104	65
	RV 5'- 3'	ACGTCTGCTCCATCTGCTTC		
ADAMTS-5	FW 5'- 3'	AGCCACGCCAGCATTGAGAACC	107	65
	RV 5'- 3'	AGTGTGGTGGCCGCGTTCTT		

All primers used were designed specifically for equine species using computer software (primer BLAST, http://www.ncbi.nlm.nih.gov/tools/primer-blast/), and were obtained from Eurogentec (Maastricht, The Netherlands). Marker genes of cartilage degeneration and regeneration were tested (Table 3). These included: Matrix Metalloproteinase-3 (MMP3), Matrix Metalloproteinase-13 (MMP13), A disintegrin and metalloproteinase with thrombospondin motif-5 (ADAMTS-5), Collagen type II A1 (Col2), Collagen III (Col3), Collagen I (Col1), and Cartilage oligomeric protein (COMP). The samples were tested in single reactions in a CFX 384 well plate (Bio-Rad). 4-fold dilution series were made from a 10 times diluted cDNA pool for the standard curve. To each well 4.0 µl 50 times diluted cDNA was added and 6.0 µl Master Mix containing the primers, and 2x iQ SYBR green SuperMix (Bio-Rad) dissolved in MQ. Also, a negative control with MQ was performed. To normalize the expression levels, reference genes were used. Several reference genes were tested according to GeNorm calculations in Excel (11).

The best performing reference genes that were used for normalization, were Ribosomal Protein L13 (RPL13) and Ribosomal Protein S19 (RPS19). The PCR process consisted of an initial denaturation phase enduring 2 minutes at 95°C, and 40 cycles of a two-step reaction with 30 sec at 95°C for denaturation and primer annealing, and 30 sec at annealing temperature (Ta) for extension. Data were analysed with CFX manager and used to calculate relative expression according to the normalized relative quantity (NRQ) method (12) in Excel.

Statistical analysis
Statistics were performed on data with sample sizes of n=3 or more. Software used was Rstudio (version 3.1.1 software). Normality was tested by using the kurtosis test and skewness test. Non-parametric data was transformed by log transformation or square root transformation into normally distributed data. Analyses were done by performing one-way ANOVA or two-way ANOVA, subsequently Tukeys post-hoc test was performed.

Results

Experiment 2

Chondrocyte performance under influence of SF from joints with acute or chronic damage
To determine differences in synthetization of matrix components in pellet culture between pellets cultured under addition of LPSSF and OASF, healthy chondrocytes were cultured for 1 week under variable conditions (Experiment 2). After 1 week of culture, collagen II staining was present in pellets cultured in 25% OASF, and more evident and diffuse after addition of TGF-β1. Medium, 25% HSF, and 25% LPSSF did not show any collagen II staining (Fig. 1A).

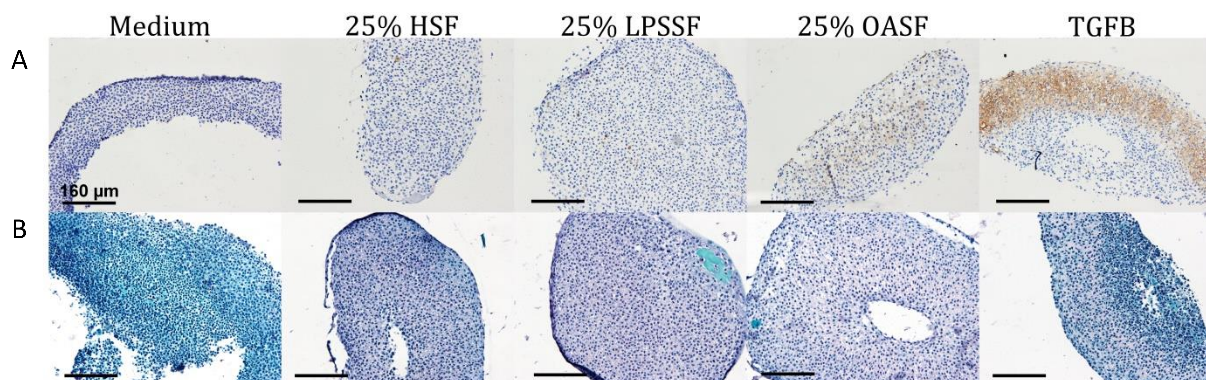

Figure 1 Representative **(A)** collagen II staining and **(B)** safranin-O staining in pellets formed out of healthy chondrocytes (n=2 per condition) cultured 1 week in medium (control) supplemented with 25% HSF (healthy synovial fluid), 25% LPSSF (synovial fluid after intra-articular LPS injection), 25% OASF (osteoarthritic synovial fluid), or 10 ng/ml TGF-β1 (TGFB). Brown staining indicates collagen II deposition. Red staining in safranin-O indicates glycosaminoglycan (GAG) deposition. A 10x magnification was used.

Morphological assessment of safranin-O stained sections showed round chondrogenic cells under all conditions. Matrix developed by the chondrocytes in pellets distanced cells from each other, and stained positive for GAGs in all conditions apart from the medium group. However, after addition of TGF-β1 exclusively low-degree staining was seen (Fig. 1B). Mean (±SD) Bern score was higher for all four conditions compared to chondrogenic medium, with a maximum score after addition of OASF (2.0±0.0 for plain medium versus 6.0±0.0 for 25% OASF). Addition of TGF-β1 resulted in a mean (±SD) Bern score of 2.5 (±0.5), which was the lowest of these four conditions (Fig. 2).

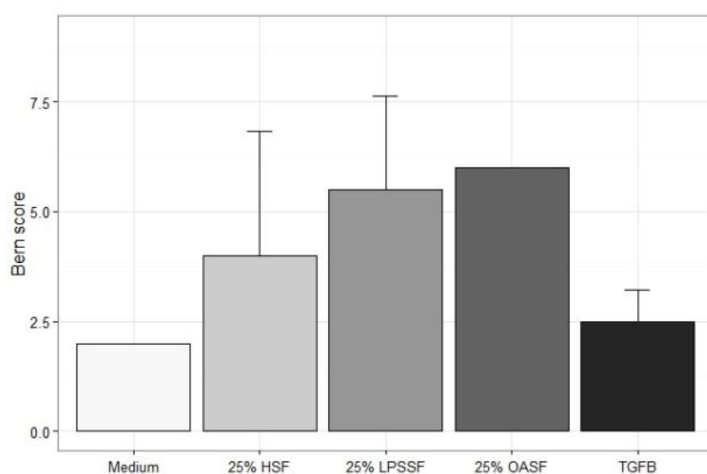

Figure 2 Bern score of pellets (n=2 per condition) formed out of healthy chondrocytes, cultured in medium (control) supplemented with 25% HSF (healthy synovial fluid), 25% LPSSF (synovial fluid after intra-articular LPS injection), 25% OASF (osteoarthritic synovial fluid), or 10 ng/ml TGF-β1 (TGFB). Bars represent mean + SD.

Quantitative analysis of matrix production showed that mean GAG amounts in pellets rose after addition of 25% OASF, 25% LPSSF, and TGF-β1 compared to medium and 25% HSF. Mean GAG content of pellets cultured in addition of HSF showed even a decrease relative to chondrogenic medium. No clear difference was seen between GAG contents in the 25% LPSSF and the 25% OASF group (2.23 µg/pellet and 2.27±0.23 µg/pellet, resp.). On the contrary, mean DNA content decreased after addition of all conditions relative to the medium and HSF group. Addition of 25% OASF and 25% LPSSF showed the lowest values of DNA content (1.01±0.10 µg/pellet and 0.84 µg/pellet, resp.). Pellet GAG amounts normalized for DNA content (GAG/DNA) was elevated in pellets cultured in addition of TGF-β1, 25% LPSSF, and 25% OASF compared to plain medium. Pellets cultured in addition of 25% LPSSF reached the highest levels of GAG/DNA content (2.65 µg/µg). In all groups the amount of GAGs released to culture medium were raised, with highest mean GAG release after addition of 25% LPSSF to culture medium (32.90 ± 13.67 µg /ml). However, one-way analysis of variance on GAGs released to the culture medium after 1 week of culture (4 days after medium change) showed no significant differences between supplemented medium (Table 4).

Table 4 Differences in effects on matrix production of enriched chondrogenic medium on pellets formed out of healthy chondrocytes.

Additive	GAG (µg/pellet)	DNA (µg/pellet)	GAG/DNA (µg / µg)	GAG released to medium (µg /ml)	P-value
Medium	1.97 ± 0.10	1.42 ± 0.20	1.41 ± 0.27	19.75 ± 1.76	-
25% HSF	1.35 ± 1.05	1.32 ± 0.07	1.01 ± 0.74	34.12 ± 7.07	0.104
25% LPSSF	2.23 ± N/A	0.84 ± N/A	2.65 ± N/A	32.90 ± 13.67	0.322
25% OASF	2.27 ± 0.23	1.08 ± 0.10	2.10 ± 0.02	28.29 ± 8.05	0.525
TGF-β1	2.20 ± 0.51	1.26 ± 0.11	1.73 ± 0.25	27.35 ± 11.14	0.715

GAG content, DNA content, and GAG/DNA (n=2 per condition, except for 25% LPSSF (n=1))) measured after 1 week of culture, GAG release to medium measured (n=6 per condition) at week 1 of culture (4 days after medium change). Chondrogenic medium (control) was supplemented with 25% HSF (healthy synovial fluid), 25% LPSSF (synovial fluid after intra-articular LPS injection), 25% OASF (osteoarthritic synovial fluid), or 10 ng/ml TGF-β1. Values are presented as mean ±SD.

Collagen I and collagen III expression levels increased in pellets cultured in addition of 25% HSF, 25% LPSSF, and 25% OASF compared to medium control. Highest expression levels were found in the 25% LPSSF group for both genes. Gene expression levels for collagen II and COMP also increased for all conditions relative to medium, but here addition of 25% OASF to culture medium resulted in higher expression levels than addition of 25% LPSSF. For all four genes pellets cultured in TGF-β1 resulted in the highest increase in gene expression (Fig. 3).

Expression levels of MMP3 decreased under every condition compared to medium. Lowest levels were found in pellets cultured in addition of 25% LPSSF and 25% OASF. In contrast, MMP13 mRNA expression was higher when 25% HSF, 25% LPSSF and 25% OASF was added to culture medium, compared to plain medium. Only little changes were found in mRNA expression of ADAMTS-5 (Fig. 3).

We experienced difficulties in handling medium supplemented with LPSSF in culture. Medium supplemented with LPSSF in culture tended to form cloths, resulting in high viscosity of culture medium. Therefore, this condition was excluded in following experiments.

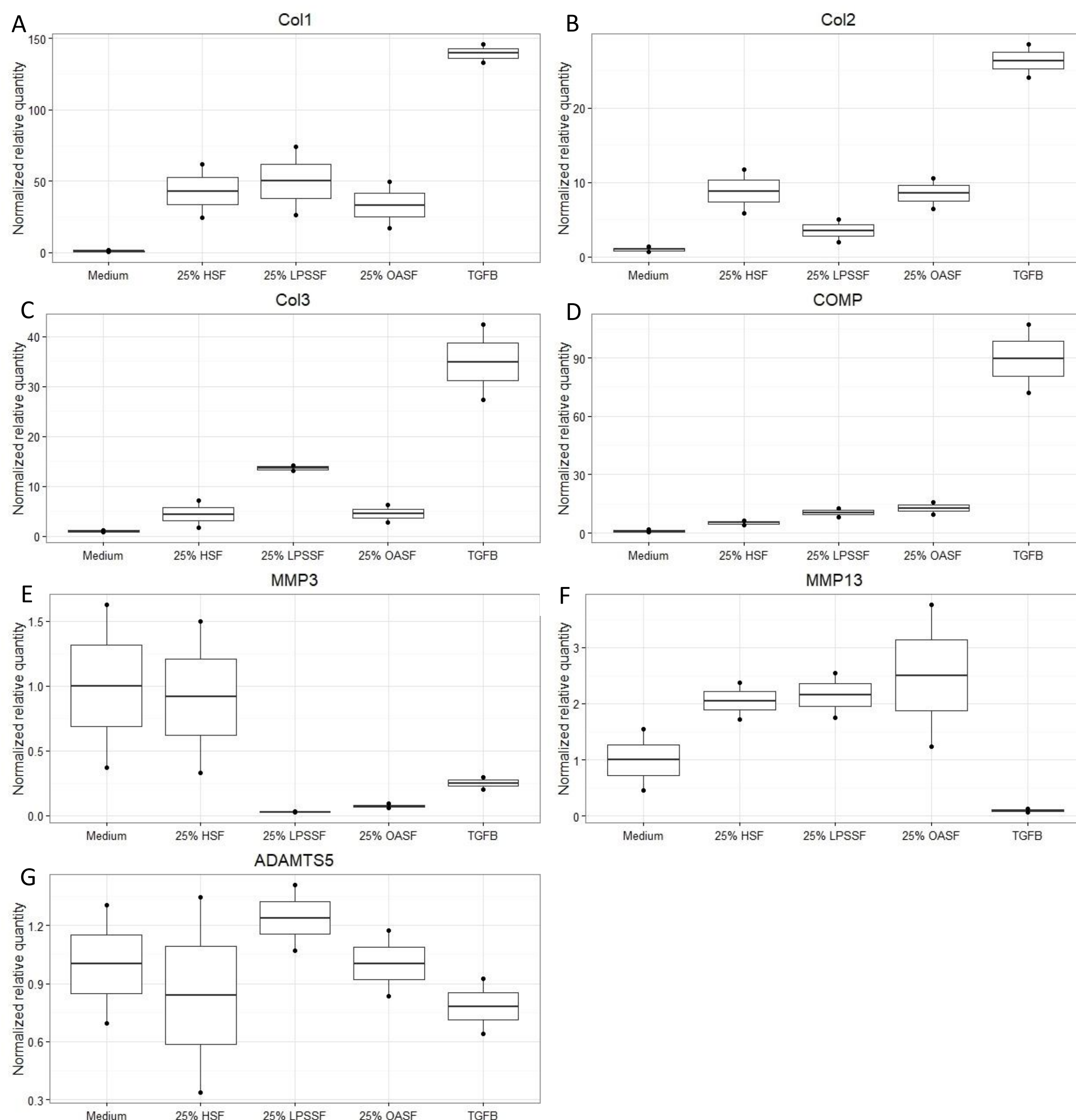

Figure 3 Normalized relative quantity of mRNA levels of **(A)** collagen I, **(B)** collagen II, **(C)** collagen III, **(D)** COMP, **(E)** MMP3, **(F)** MMP13, and **(G)** ADAMTS-5 in pellets (n=2 per condition) formed out of healthy chondrocytes after 1 week of culture medium (control) supplemented with 25% HSF (healthy synovial fluid), 25% LPSSF (synovial fluid after intra-articular LPS injection), 25% OASF (osteoarthritic synovial fluid), or 10 ng/ml TGF-β1 (TGFB).

Experiment 3 and 4

Influence of pre-culture on effects of SF enriched medium in pellet culture
In order to determine if effects of SF enriched medium on chondrocytes in pellet culture becomes more evident after a prolonged pre-culture period, histology and biochemical GAG and DNA analysis were performed on pellets cultured for 4 weeks (Experiment 3 and 4). Only results obtained from experiment 4 are described, since experiment 3 did not provide processable results due to poor performing chondrocyte donors.

At 2 weeks of pre-culture in chondrogenic medium without TGF-β1, showed absence of collagen II staining. However, in addition of 25% HSF, 25% OASF and TGF-β1 in week 3 collagen II production is visible as a few focal spots compared to chondrogenic medium alone. This effect appears to decrease after 4 weeks of culturing (Fig. 4A). At 2 weeks of pre-culture in chondrogenic medium supplemented with TGF-β1, a dark and diffuse staining for collagen II was present. Under all added conditions, staining darkness and load diminished at week 3 and 4. Staining darkness decreased when culturing in TGF-β1 was continued. Almost complete disappearance of collagen II staining was seen after 4 weeks culture in 25% OASF, while culturing in 25% HSF and TGF-β1 preserved staining the most (Fig. 4B).

Pellets pre-cultured in chondrogenic medium showed cells with a fibroblastic phenotype, and no staining for safranin-O was seen under any of the conditions (Fig. 4C). When pellets were pre-cultured in medium with TGF-β1, chondrocytes had a more round appearance, and more matrix was produced. However, merely at 2 weeks of pre-culturing in TGF-β1 light GAG staining darkness was seen (Fig. 4D).

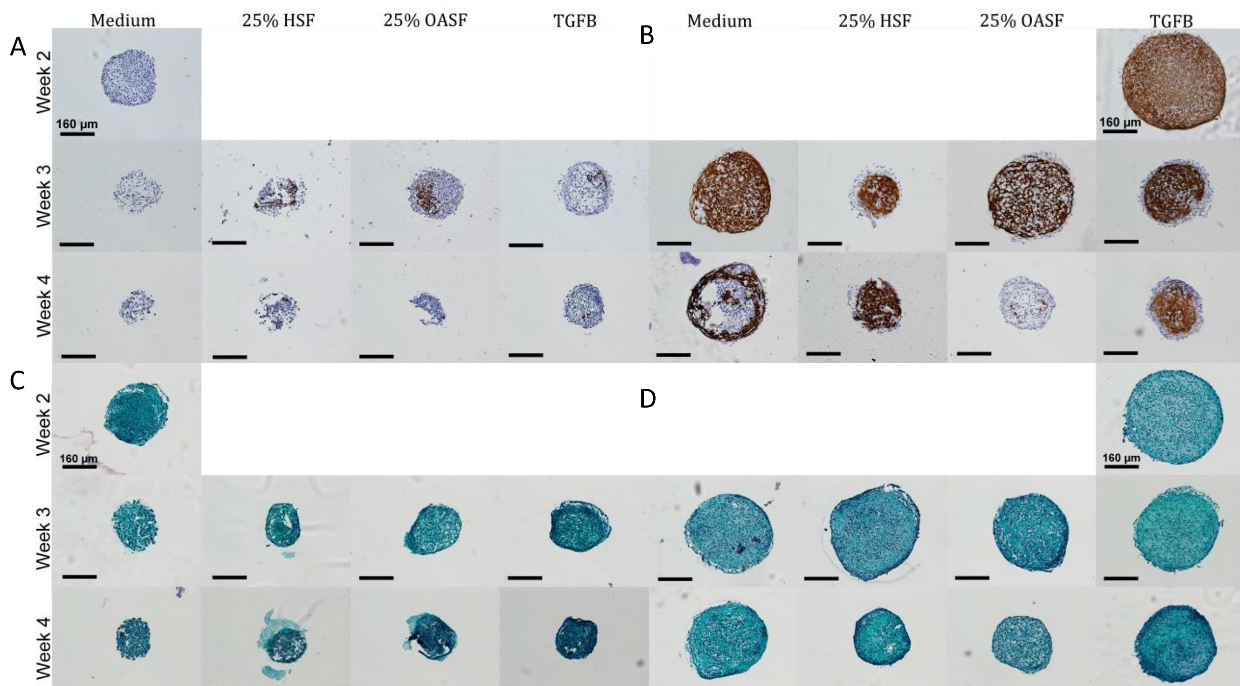

Figure 4 Representative **(A)(B)** collagen II staining and **(C)(D)** safranin-O staining of pellets pre-cultured for 2 week in **(A)(C)** chondrogenic medium, or **(B)(D)** chondrogenic medium with 10ng/ml TGF-β1 (TGFB). After pre-culture medium (control) was enriched with 25% HSF (healthy synovial fluid), 25% OASF, or 10 ng/ml TGF-β1 until 4 weeks of culture. Brown staining indicates collagen II deposition. Red staining in safranin-O indicates glycosaminoglycan (GAG) deposition. A 10x magnification was used.

Highest mean (±SD) GAG and DNA levels were measured in pellets precultured in TGF-β1 at 2 weeks (1.65±0.16 µg/pellet and 0.59±0.05 µg/pellet, resp.). Pre-culturing in chondrogenic medium without TGF-β1 supplementation showed GAG and DNA amounts that were consistently lower than pellets pre-cultured in TGF-β1. Under all conditions, both GAG and DNA

levels decreased at week 3 and 4 compared to pellets at week 2. At week 3, the 25% OASF group had significant higher GAG contents compared to 25% HSF for pellets pre-cultured in both non-chondrogenic medium (p<0.01) and chondrogenic medium (p<0.05). However, these significant differences disappeared after 4 weeks of culture (Fig. 5). Amounts of GAG and DNA present in pellet matrix was determined in n=3 pellets per time point per condition. However, at week 4 only 2 pellets cultured in medium and 2 pellets cultured in HSF enriched medium in the medium-pre-cultured group could be evaluated due to technical failure.

After GAG levels were normalized for DNA content (GAG/DNA), under no condition statistically significant differences were found. Pellets pre-cultured in TGF-β1 maintained the same pattern for GAG/DNA as seen in GAG amounts, while pellets pre-cultured in chondrogenic medium turned over their pattern as seen in GAG amounts. However, GAG levels of pellets pre-cultured in chondrogenic medium were divided by very low DNA contents (Fig. 5C).

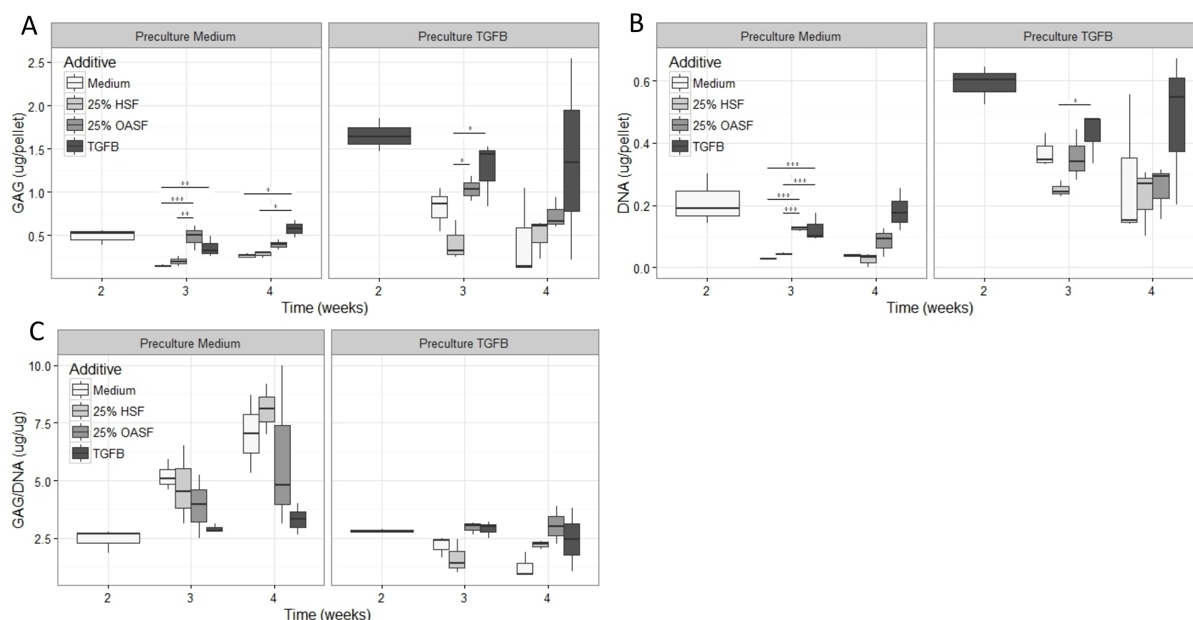

Figure 5 Pellets (n=3 per condition) **(A)** GAG content (μg/pellet), **(B)** DNA content (μg/pellet), and **(C)** GAG content corrected for DNA content (μg/μg) after 2, 3, and 4 weeks of culture are displayed. Pellets were cultured in medium (control) enriched with 25% HSF (healthy synovial fluid), 25% OASF (osteoarthritic synovial fluid), or 10 ng/ml TGF-β1 (TGFB). Pellets were pre-cultured for 2 weeks in chondrogenic medium, or chondrogenic medium with 10ng/ml TGF-β1. Significant differences *: p<0.05; **: p<0.01; ***: p<0.001.

Discussion

A proper culture design for culturing chondrocytes under influence of SF was determined in Chapter 2. This chapter shifts its focus toward *in vitro* chondrocyte performance under influence of SF obtained from healthy joints (HSF), joints with acute synovitis (LPSSF) and SF from osteoarthritic joints (OASF) (Experiment 2). Histological examination showed collagen II deposition after addition of OASF, but not after addition of LPSSF or HSF. On the contrary, Bern scoring for safranin-O staining showed resemblance between pellets cultured in addition of LPSSF and OASF, which were both higher compared to pellets cultured in HSF. Additionally, GAG/DNA amounts appeared to be higher in the OASF and LPSSF group compared to the HSF group. Gene expression levels of matrix components after LPSSF supplemented cultures were differently expressed compared to cultures supplemented with HSF or OASF. Additionally, to improve evaluation of potential degenerative effects of different SF conditions, a prolonged pellet culture period up to 4 weeks was tested (Experiment 4). A pre-culture period of 2 weeks

in medium with TGF-β1 resulted in a well-established pellet in terms of morphology and collagen II production, but only minor GAG formation was observed. Statistically significant higher GAG amounts were found at 3 weeks of culture in the OASF group compared to the HSF group. However, as of 3 weeks of culture, negative effects on histology and GAG and DNA content were observed after addition of every condition, including continued culturing in TGF-β1 compared to pellets at week 2.

Differences in chondrocyte performance after culture in SF obtained from joints with acute synovitis and osteoarthritic joints

Based on previous research we hypothesized that SF from joints with acute synovitis stimulates chondrogenesis, while SF obtained from joints with a more chronic stage of joint damage has inhibiting effects on chondrogenesis (3,6,13). In contrast, we found that SF obtained from joints with acute and chronic pathology both had stimulating effects on chondrogenesis in terms of collagen II and GAG production (Experiment 2). After pre-culture we observed higher GAG amounts after addition of OASF compared to HSF (Experiment 4). Previous research describes contradictory findings. A study of Yang et al. looked into effects of SF obtained from injured knee joints on cultured chondrocytes, and found an inhibiting effect on chondrogenesis (6). However, comparison was made only between medium supplemented with SF obtained from injured joints and culture medium supplemented with growth factors. No comparison was made with SF obtained from healthy joints. In accordance with Yang's study are the results from Heldens et al., where supernatant (culture medium) obtained from cultured synovium of OA patients was used in mesenchymal stem cell (MSC) culture to test chondrogenic differentiation (14). A negative effect was found on safranin-O staining and cartilage matrix gene expression. Similarly, no comparison with effects of HSF was made. Kiefer et al. compared viability of adipose derived stem cells (ADSc) after addition of both HSF and OASF, and found impaired cell viability for cells cultured in several dilutions of OASF (15). When cultured in HSF, cell viability was not affected. Nevertheless, no differentiated chondrocytes were tested in this research. Matrix production was not assessed in this study. In contrast with previously described research, a study assessing cartilage explants from OA patients in culture with OASF or medium reports no differences in GAG amounts between the groups (16). Only an increase in DNA amounts was found after addition of OASF. After evaluation of our results and results presented in previous research, we emphasize that a proper comparison between OASF and HSF is crucial to draw conclusion about the effects of OASF on chondrogenesis. In our experiments very low GAG levels were found, and GAG levels were decreasing for each condition after 2 weeks of pre-culture. This might question the relevance of the differences we found between conditions. Future research in comparing HSF and OASF on chondrogenesis with a larger sample size is desired to confirm our findings.

We established that both OASF and LPSSF supplementation in pellet culture had positive effects on matrix production. Nonetheless, differences in upregulated matrix components were found between pellets cultured in the addition of OASF and LPSSF. Collagen II deposition was solely seen under influence of OASF. Gene expression showed higher levels of collagen II mRNA in pellets cultured in OASF compared to LPSSF, while addition of LPSSF to culture medium induced an increase in mRNA expression of dedifferentiation markers collagen I and collagen III (17,18). Results were expected to be vice versa, with chondrocyte dedifferentiation after addition of OASF and matrix synthesis after addition of LPSSF. In contrast with our findings, Zhong et al. found a gradual decrease in collagen II mRNA expression in cartilage with different stages of OA (19). Collagen I mRNA expression was more likely to increase in OA cartilage. Another *in vivo* study showed that patients with acute synovitis had increased inflammation markers and raised collagen II amounts in SF, which suggests an improvement in collagen II production (20). However, high levels of matrix components in SF is also seen after matrix degeneration (21). This degeneration of collagen II in cartilage matrix is due to cleavage of collagen II provided by collagenases produced by chondrocytes (22). This indicates that high collagen II components found in SF are not always correlated with high collagen II synthetization. Additional research

with biochemical analyses for quantification of collagen II content will provide for more insights about collagen II production and break-down.

While collagen II production was higher for the OASF group compared to the LPSSF group, our results showed that addition of LPSSF and OASF to culture medium resulted in a similar increase in GAG formation on histology and biochemical analyses as compared to plain medium. Once more, we expected higher GAG production after addition of LPSSF compared to OASF. This hypothesis was supported by previous research looking into SF from patients with acute and chronic joints disease, where it was found that SF from patients with acute joint damage had increased proteoglycan components. Elevated proteoglycan levels in SF arise either by high turn-over or high proteoglycan breakdown (13). An explanation for our unexpected results might be that gene expression and staining intensity was relatively low, which masked real differences between groups. This is also confirmed by GAG amounts released to medium, which showed no significant difference between addition of LPSSF and OASF.

An explanation for the observed cloth formation in LPSSF culture media is an increase in coagulation proteins like fibrinogen in SF, recruited by an acute inflammation response (23). Subsequently, aggregates of fibrin in joint cavities are found in inflamed joints. Imitation of joint inflammation by antigen-induced arthritis induced these fibrin aggregates (24). A possible solution for this problem is to pre-heat SF, resulting in denaturation of fibrinogen. Thereafter precipitated fibrin aggregates can be removed (25). A disadvantage of this method is that other proteins present in SF could also be affected, which may vanish the differences in SF composition. Future research assessing if differences in SF composition stay visible in culture after pre-heating is desired to elucidate the possibility of using pre-heated SF culture.

Prolonged pre-culturing could provide a better starting pellet for detecting effects on matrix degradation

In our previous studies (Experiment 1 and 2, Chapter 2) we found that after 1 week of pellet culture in medium supplemented with TGF-β1, matrix components were not completely formed. Staining of collagen II and GAGs was present, although not diffusely spread. In order to simulate an *in vivo* situation, a better differentiated pellet is desired. Additionally, manifestation of matrix degeneration would become better visible when matrix is fully produced. To accomplish this improved cartilage matrix, pellets were pre-cultured in TGF-β1 for 2 weeks (Experiment 4). This pre-culture resulted in a dark and diffusely stained pellet for collagen II, but only light safranin-O staining was visible. Pre-culturing in chondrogenic medium without TGF-β1 was performed to test if stimulation of chondrogenesis by added conditions was masked by TGF-β1. No (prolonged) stimulating effects, however, were found. In contrast, degeneration was determined by abolished collagen II staining and decreasing GAG amounts. Unexpectedly, continued culture in medium supplemented with TGF-β1 resulted in a decrease of matrix components. This decrease suggests that the pellets formed after 2 weeks of culture could not be maintained for a longer period. This, in combination with insufficient GAG production at any of the time points, could lead to misinterpretations of the results found after addition of SF-enriched medium. Increasing weight was found in equine chondrocyte pellets cultured for 4 weeks (26). Another study culturing equine chondrocytes observed a constant increase of GAG amounts in pellets up to 4 weeks of culture, with maximum levels of 100 μg/pellet (27). Differences in chondrogenic potential of cells obtained from different donors might be an explanation for our results (28). Based on this previous described research, we conclude that a stable pellet culture of at least 4 weeks should be achievable.

Conclusion

In conclusion, our results indicate that SF from both acute injured joints and OA joints contain components stimulating chondrocyte regeneration. Contradictive to our hypothesis, SF from an

acute joint inflammation seems to have a worse effect on chondrogenesis compared to SF obtained from OA joints, considering upregulation of dedifferentiation markers. This finding indicates that addressing inflammation in early stages of cartilage damage is important in preventing OA development. Additionally, SF from OA patients might not be a disruptive factor in regenerative therapies in later stages. A prolonged pre-culture of 2 weeks enables to observe matrix degradation. However, it is important that a stable pellet can be maintained up to 3 or 4 weeks before evaluating the effect of different culture conditions. Further research on individual donor differences should provide more information about improved pre-culturing. Eventually, SF obtained from more stages in OA should be tested to provide better insights in OA development.

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Chapter 4

Differences in chondrocyte behaviour between horse breeds and pony breeds

Abstract

BACKGROUND: Prevalence studies of osteoarthritis (OA) in horses state that pony breeds are less sensitive for developing OA compared to bigger horse breeds. A possible cause is differences in chondrocyte behaviour. If chondrocytes behave differently between breeds, this can affect the outcome of *in vitro* studies and perhaps be translated to different outcomes in *in vivo* models for OA. Our aim was to evaluate differences in chondrocyte morphology and performance in 2D and 3D culture between horse breeds and pony breeds.

MATERIALS AND METHODS: Chondrocytes were harvested from four Shetland pony donors (donor A-D) and three horse breed donors (donor E-G), all aged 2-13 years. Chondrocyte morphology was assessed after 7 days of expansion. Afterwards, pellets were cultured for 1, 2, and 3 weeks with or without supplementation of 10ng/ml TGF-β1. Pellet size was determined at each week. Additionally, immunohistochemistry for collagen I and collagen II was performed. Safranin-O staining was used to asses glycosaminoglycan (GAG) production, in addition to measurements for GAG and DNA content in pellets, and GAGs released to medium.

RESULTS: Chondrocyte morphology differed between breeds, since cells from pony breeds remained more chondrogenic in expansion. No breed differences were found assessing collagen I, collagen II and GAG staining. Additionally, GAG normalized for DNA (GAG/DNA) levels and GAGs released to medium showed no significant differences. Individual donor differences in GAG/DNA and GAGs released to medium were found significant as from week 2. Highest GAG/DNA levels were found for donor B at week 3 (14.63±2.23 μg/μg) and lowest levels for donor D (2.64±1.07 μg/μg).

CONCLUSION: Horse and pony breeds differ regarding chondrocyte morphology in expansion, although not regarding chondrocyte performance in pellet culture. Between chondrocyte donors, large individual differences in chondrogenic potential is present, therefore selecting donors prior to future experiments might prevent unreliable data.

Introduction

Prevalence studies of osteoarthritis (OA) in horses state that pony breeds are less sensitive for developing OA compared to bigger horse breeds (1). A study in geriatric horses found a smaller range of motion in horse joints compared to pony joints, which is known as a common finding in equine OA (2). Likewise, this breed difference is seen in dogs, where bigger breeds are more likely to develop OA compared to smaller dog breeds (3,4). In men weight plays a role in OA prevalence, since it is found that obesity is an important risk factor in OA development. This is due to metabolic factors, but also because of increased biomechanical forces on cartilage (5). An important finding in studying mammalian species with different body weights, is that cartilage thickness is relatively thinner in animals that have higher body weights (6). Therefore, a relative increased cartilage load in higher weighted breeds might play a role in OA development.

In terms of tissue regeneration, interesting differences in second-intention wound healing between horse breeds have been found (7). Pony breeds appeared to have a more pronounced and faster wound contraction resulting in better wound healing, compared to horse breeds. This wound contraction is preceded by a stronger initial inflammatory response in ponies (8). This finding might be extrapolated to repair processes of other tissues, including joint tissues. However, concrete causes elucidating the differences in OA prevalence in ponies and horses have not been studied yet. If chondrocytes behave differently between breeds, this can affect the outcome of *in vitro* experiments with equine chondrocytes as well as *in vivo* models for OA (and patients with OA).

Our previous experiments resulted in relatively low levels of glycosaminoglycan (GAG) amounts in pellet matrix, and a decrease in pellet stability was seen after more than 2 weeks of culture (Experiment 4, Chapter 3). Notably, chondrocytes were harvested from Shetland pony donors only. Another study investigating equine chondrocytes in pellet culture was able to reach higher GAG levels, and maintained more stable pellets (9). However, chondrocyte donor breed was not mentioned in this study. Therefore, more insights in differences between chondrocyte donor breeds in pellet culture are desired. As a first step towards elucidating potential differences in chondrocyte behaviour between equine breeds, we set out an *in vitro* experiment to compare performance of chondrocytes derived from ponies and horses. We evaluated chondrocyte differentiation, morphology and matrix production of pony and horse donors in 2D and 3D culture. Based on our previous experiments we hypothesized that chondrocyte behaviour is different between horses and ponies, where we expected that pony breed chondrocytes produce less matrix compared to horse breed chondrocytes.

Materials and methods

Experimental designs

All variables and fixed factors per experiment are summarized in Appendix I (Table 1). The current chapter describes experiment 5.

Experiment 5

Chondrocytes were derived from four pony breeds (Donors A-D) and three horse breeds (donors E-G). Cell donors were not pooled (Table 1). Pellets were cultured in chondrogenic medium or chondrogenic medium supplemented with 10 ng/ml human recombinant transforming growth factor β1 (TGF-β1, R&D Systems, Minneapolis, Minnesota, Unites States). Pellets cultured in chondrogenic medium without additives were used as negative control. Pellets were harvested after 7, 14, and 21 days (week 1, 2, and 3, resp.) of culture. Pellet size was evaluated at each time point before harvest. Sample size of n=2 pellets were used for histology

(safranin-O), immunohistochemistry (collagen I and collagen II), and n=3 pellets were used for GAG and DNA contents. Released GAG amounts to medium were measured in n=5 samples.

Table 1 Overview characteristics of the donors used for cell culture in each experiment.

Exp.	Donor	Breed	Age	Sex	Joint	Cell type	Cell stage
5	Pony 1 (A)	Shetland pony	6	Gelding	MC	Chondrocyte	P1
	Pony 4 (B)	Shetland pony	8	Mare	MC	Chondrocyte	P1
	EQ017-003 (C)	Shetland pony	6	Mare	MCP	Chondrocyte	P1
	EQ017-005 (D)	Shetland pony	6	Mare	MCP	Chondrocyte	P1
	EQ032 (E)	KWPN	13	Mare	MCP	Chondrocyte	P1
	EQ031 (F)	Oldenburger	2	Mare	MCP/MTP	Chondrocyte	P1
	EQ037 (G)	Unknown	6	Unknown	MCP	Chondrocyte	P1

Cell stage represents the stage of the cells the pellets were formed with. Age is in years. MCP = metacarpophalangeal joint, MTP = metatarsophalangeal joint, MC = middle carpal joint.

Cell isolation and expansion

Chondrocytes were harvested within 24 hours post mortem with a sterile scalpel knife, and cartilage pieces were kept in DMEM (high glucose, GlutaMAX, pyruvate) (31966, Gibco, Dublin, Ireland) + 1% p/s (penicillin/streptomycin, P11-010, Gibco) at 37°C. Within 24 hours after harvest, cartilage pieces were washed in sterile HBSS (Hanks Balanced Salt Solution, Gibco) + 1% p/s, and minced cartilage pieces were digested overnight on a roller plate at 37°C in 30 ml 0.15% collagenase II (Worthington, Lakewood, United States) solution in DMEM + 1% p/s. After digestion, cells were strained through a 70 µm cell strainer (Greiner bio-one, Alphen aan de Rijn, The Netherlands) to remove debris. Cells were stored in liquid nitrogen.

Cell culture experimental conditions

Cells were expanded using expansion medium in T175 flasks (Cellstar, Greiner bio-one) in a density of approximately 5000 cells/cm2. Expansion medium contained DMEM (high glucose, GlutaMAX, pyruvate) (31966, Gibco) + 1% p/s + 1.25 µg/ml Fungizone (Amphothericin, Gibco) + 10% FBS (Foetal Bovine Serum, Gibco,) + 0.5% 0.1mM ASAP (Ascorbic acid 2 – phosphate, Sigma-Aldrich) + 1 ng/ml βFGF (Basic fibroblast growth factor, AbD Serotec, Bio-Rad, Hercules, Californië, United States). When confluent, cells were trypsinized with 1x TripLE expres (Gibco) and counted with TC20 cell counter (Bio-Rad). Cells were resuspended in chondrogenic medium containing DMEM (31966, Gibco) + 1% ITS+ premix (Insulin Transferrin Selenium, Corning Life Sciences, Corning, United States) + 1% p/s + 1.25 µg/ml Fungizone + 5µl /ml 20mM ASAP + 1.5 mg/ml BSA (Bovine Serum Albumin, Sigma-Aldrich) + 0.04 mg/ml L-Proline (Sigma-Aldrich) supplemented with 10 ng/ml TGF-β1. Cells were plated out in an ultra-low cell attachment 96 wells plate with a round bottom (Costar 7007, Corning Life Sciences) in a cell density of $2x10^5$ cells per well and centrifuged for 5 minutes at 300 g to form pellets. Medium was refreshed each 3 or 4 days and stored in Micronic tubes (MICRONIC, Lelystad, The Netherlands). Pellets were all cultured at 37°C and 5% CO_2.

Histology and immunohistochemistry

Pellets were fixed in 200 µl 10% formalin (Sigma-Aldrich) with 0.1% eosin (Boom BV, Meppel, The Netherlands) for 1 week. Afterwards pellets were embedded in 2.4% alginate (Sigma-Aldrich) and gelated by 3.7% formalin with 102 mM $CaCl_2$. Samples were dehydrated through EtOH 70% - 100% (Klinipath, Breda, The Netherlands) and 1 step xylene (Klinipath) for 1 hour. Pellets were incubated in paraffin (Leica, Amsterdam, The Netherlands) at 60°C for 2 hours before embedding in paraffin. The samples were cut into 5 µm slices using Microm microtome and captured on KP plus printer slides (Klinipath). Afterwards slides were fixed on a hot plate of 60°C for 1 hour. Before staining slides were deparaffinised by two steps xylene and hydrated

through EtOH 60% - 100%. Post staining, dehydration was performed through 70 -100% and 2 steps xylene. Cover slides were mounted with Depex (Merck, Whitehouse Station, New Jersey, United States). Images were acquired using an Olympus BX51 microscope with DP73 digital camera (Olympus, Zoeterwoude, the Netherlands).

Safranin-O
Alginate was removed by 15 minutes incubation in a citrate buffer. Afterwards nuclei were stained with Weigert's Hematoxylin (Klinipath) for 5 minutes and washed in running water. Counterstaining with 0.4% aqueous Fast Green (Sigma-Aldrich) was performed for 4 minutes, after which slides were washed in 1% Acetic Acid (Boom BV). Present GAGs were stained with 0.125% aqueous safranin-O (Sigma-Aldrich) solution for 5 minutes. Short dehydration starting with 96% EtOH was needed to prevent safranin-O from washing of. Healthy cartilage explants were stained as positive control.

Collagen II
Slides were blocked with 0.3% H_2O_2 solution for 10 minutes. Antigen retrieval steps contained Pronase (Roche, Basel, Switzerland) 1 mg/ml and Hyaluronidase (Sigma-Aldrich) 10 mg/ml both for 30 minutes at 37°C. The second blocking section was with PBS/BSA (Bovine Serum Albumin, Sigma-Aldrich) 2% for 30 minutes, after which incubation with the primary antibody Collagen II Mouse monoclonal antibody DSHB (1:1500 in PBS-BSA) (Santa-Cruz, Dallas, Texas, Unites States) and a normal mouse IgG1 (1:1800 in PBS-BSA) (Santa Cruz) as negative control took place overnight at 4 °C. Next day, samples were incubated with goat anti-mouse secondary antibody containing HRP (Dako, Heverlee, Belgium) for 30 minutes. DAB peroxidase substrate solution (Dako) was added for 5 minutes to obtain signal. Counterstaining was performed using Mayers Hematoxylin (Merck). As control healthy cartilage explants were used.

Collagen I
Slides were initially blocked with peroxidase block (Dako) for 10 minutes. Antigen retrieval steps contained Pronase 1mg/ml and Hyaluronidase 10mg/ml both for 20 minutes at 37°C. In the second blocking section 1:10 diluted NGS (Normal Goat Serum, Sigma-Aldrich) in PBS was added for 15 minutes. Afterwards, primary antibody Collagen I Rabbit monoclonal (1:400 in PBS-BSA) (Abcam, Cambridge, United Kingdom) and a normal Rabbit IgG (1:8000 in PBS-BSA) (Dako, 20mg/ml) as negative control diluted in Bright diluent (Immunologic) was incubated for 1 hour at RT. Next, incubation with Brightvision Poly HRP Anti-Rabbit (Immunologic, Duiven, The Netherlands) for 30 minutes took place. Bright DAB peroxidase substrate solution (Immunologic) was added for 5 minutes to obtain signal. Counterstaining was performed using Mayers Hematoxylin. As control foal bone containing part of the growth plate were used. Additionally, a negative control containing no primary antibody was taken into account.

Determination of glycosaminoglycan and DNA contents in pellets and medium
Pellets were harvested and washed with 100 µl HBSS to remove GAGs present in remaining medium. Afterwards, pellets were dried for 60 minutes using a Savant speedvac. Pellets were digested in 200 µl papain digestion solution containing 250 µg/ml Papain (from papaya, Sigma-Aldrich) and 1.57 mg/ml Cysteine HCl (Sigma-Aldrich) in 2*Papain buffer (containing Na_2HPO_4 + $EDTA.2H_2O$, Merck) overnight at 60°C. For digestion of SF in aspirated medium, samples were diluted 1:1 with Hyaluronidase type II (Sigma-Aldrich) 0.1 mg/ml in 25mM Sodium Acetate (Merck) pH 6.5 and incubated for 30 minutes at 37°C.

For measuring sulphated GAG content in both pellets and medium, the DMMB assay was used as described by Farndale (10). Digested samples were diluted using PBS-EDTA, and 100 µl of the diluted sample was pipetted in duplo into a 96 wells plate with a flat and clear bottom (Greiner bio-one). Chondroitin sulphate (Sigma-Aldrich) was used for standardization in a dilution series with concentrations between 0 and 10 µg/ml. 200 µl of DMMB (Sigma-Aldrich) staining solution

was added and extinction was measured at 525 and 595 nm using a plate reader. Aspirated mediums GAG content was corrected for GAG concentrations in added SF. Broad and High Sensitive assay of the Qubit® 2.0 Fluorometer were used for measuring DNA amounts in papain digested samples according to manufacturer's instructions.

Statistical analysis
Statistics were performed on data with sample sizes of n=3 or more. Software used was Rstudio (version 3.1.1 software). Normality was tested by using the kurtosis test and skewness test. Non-parametric data was transformed by log transformation or square root transformation into normally distributed data. Analyses were done by performing one-way ANOVA or two-way ANOVA, subsequently Tukeys post-hoc test was performed.

Results

Experiment 5

Chondrocyte morphology and pellet size in horse and pony donors
For evaluating dedifferentiation of chondrocytes in 2D culture, chondrocyte morphology was assessed after 7 days of expansion prior to pellet formation. It was seen that chondrocytes from ponies showed a rounder phenotype (Fig. 1A). On the contrary, horse chondrocytes gained a wide stretched fibroblastic like phenotype (Fig. 1B). Chondrocytes from both horses and ponies were observed to maintain contact with neighbour cells by cluster formation.

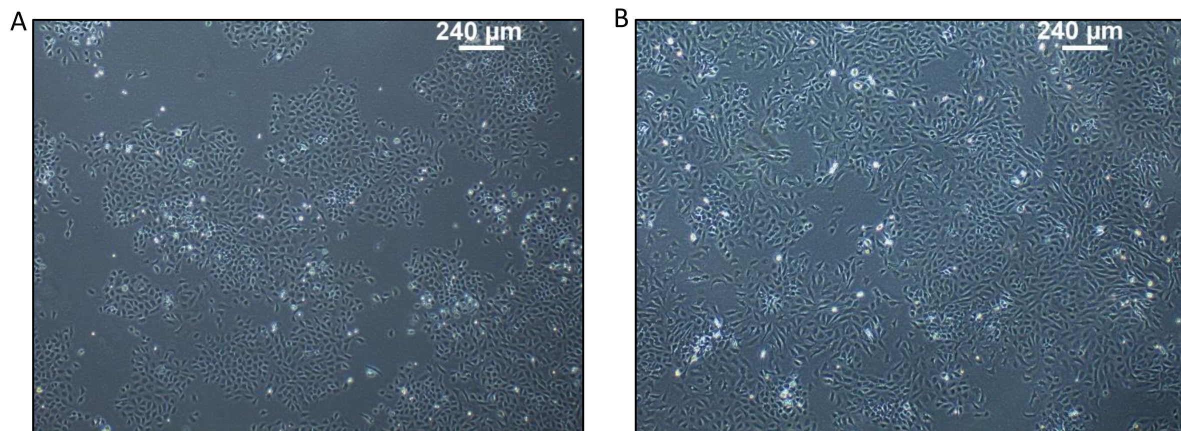

Figure 1 Visualisation of chondrocyte morphology after 7 days of expansion in culture medium. Representative chondrocyte morphology for **(A)** pony chondrocytes (donor A), and **(B)** horse chondrocytes (donor G). Bar represent 240 µm.

No clear differences in pellet growth between pony donors and horse donors were found. However, individual donor differences were observed (Fig. 2). Pellets from donor A, B, C, E, and F increased in size, while pellets from donor D and G decreased in size. At week 3, donor B provided the biggest pellet, followed by donor F Pellets cultured in chondrogenic medium without supplementation of TGF-β1 all decreased in size compared to week 1 of culture. At week 3, donor A and B had the largest size and donor C and D had the smallest size (data not shown).

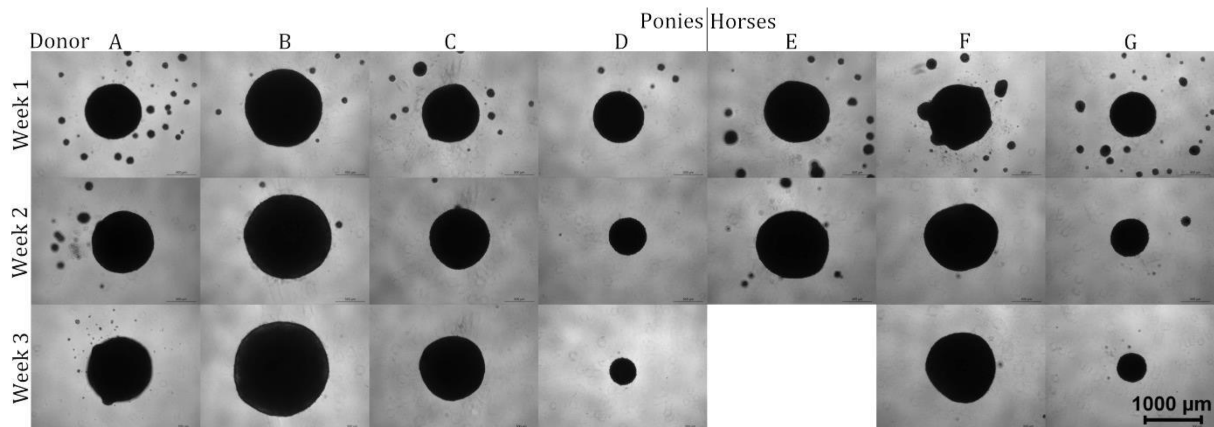

Figure 2 Representative pellet size at week 1, 2, and 3 of pellet culture. Donor A-D represent ponies, donor E-G represent horses. Pellets were cultured in chondrogenic medium supplemented with 10ng/ml TGF-β1. Bar represent 1000 µm.

Differences in matrix production between horse and pony donors

No clear differences were seen between horses and ponies concerning collagen I, collagen II, and safranin-O staining. However, individual donor differences were found. Pellets cultured in medium supplemented with TGF-β1 all showed little to moderate collagen II staining at week 1 (Fig. 3). Staining in pellets from donors A, B, C, E, and F showed a similar progressive staining intensity at week 2 and week 3 (Fig. 3). Staining darkness in donor D decreased at week 2 and 3. Donor G showed an increase at week 2 but this effect disappeared at week 3 (Fig. 3). Pellets cultured in chondrogenic medium showed less staining than pellets in TGF-β1. Moderate staining was seen for donor E at week 1 and week 2, donor F at week 1, and donor A at week 2. All other pellets were void of staining (Fig. 3).

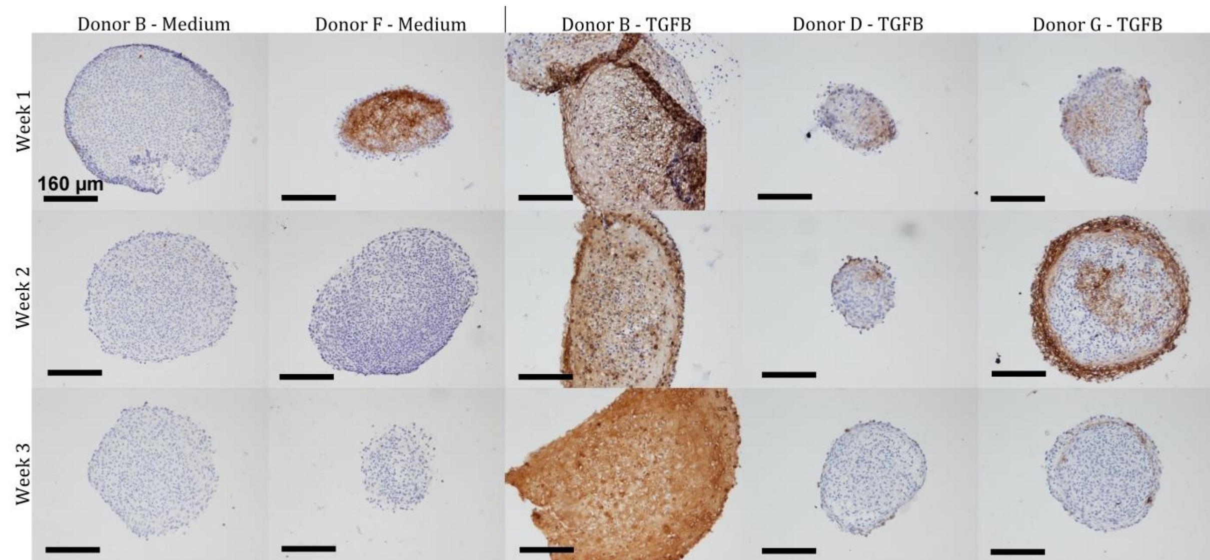

Figure 3 Representative staining for collagen II in pellets (n=2 per condition) after 1, 2, and 3 weeks of culture. All donors were cultured in chondrogenic medium (Medium), and chondrogenic medium supplemented with 10ng/ml TGF-β1 (TGFB). Donors A-D represent ponies, donors E-G represent horses. Brown staining indicates collagen II deposition. A 10x magnification was used.

Pellets cultured in medium supplemented with TGF-β1 showed no safranin-O staining at week 1. In week 2 pellets from donors A, B, C, E, F showed light staining, which became more prominent at week 3 for donors B and F. Other donors did not show staining at all at week 3. Donor D and G were completely void of staining at any time point. When pellets were cultured in chondrogenic medium only, no staining was seen at any of the time points (Fig. 4)

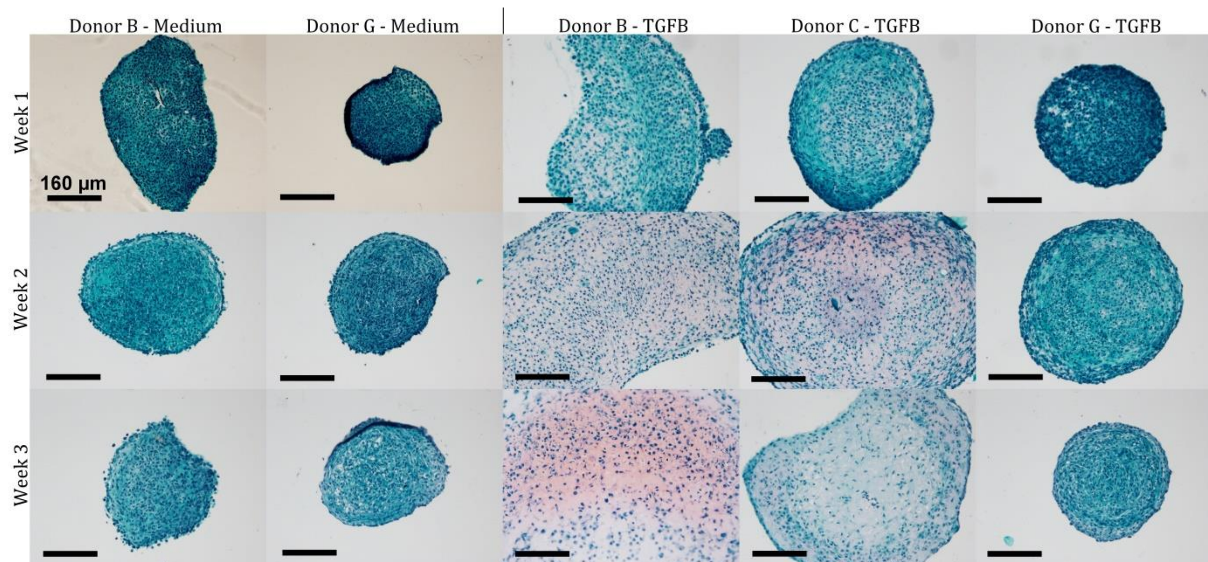

Figure 4 Representative safranin-O staining in pellets (n=2 per condition) after 1, 2, and 3 weeks of culture. All donors were cultured in chondrogenic medium (Medium), and chondrogenic medium supplemented with 10ng/ml TGF-β1 (TGFB). Donors A-D represent ponies, donors E-G represent horses. Red staining in safranin-O indicates glycosaminoglycan (GAG) deposition. A 10x magnification was used.

Pellets cultured in medium with TGF-β1 showed only light collagen I staining at week 1 for all pellets. An increase in staining darkness in week 2 and 3 was seen for donor B, C, D, and G, where donor G had the darkest staining. Pellets cultured in plain chondrogenic medium were comparable for each donor, where staining intensity increased each week (Fig. 5)

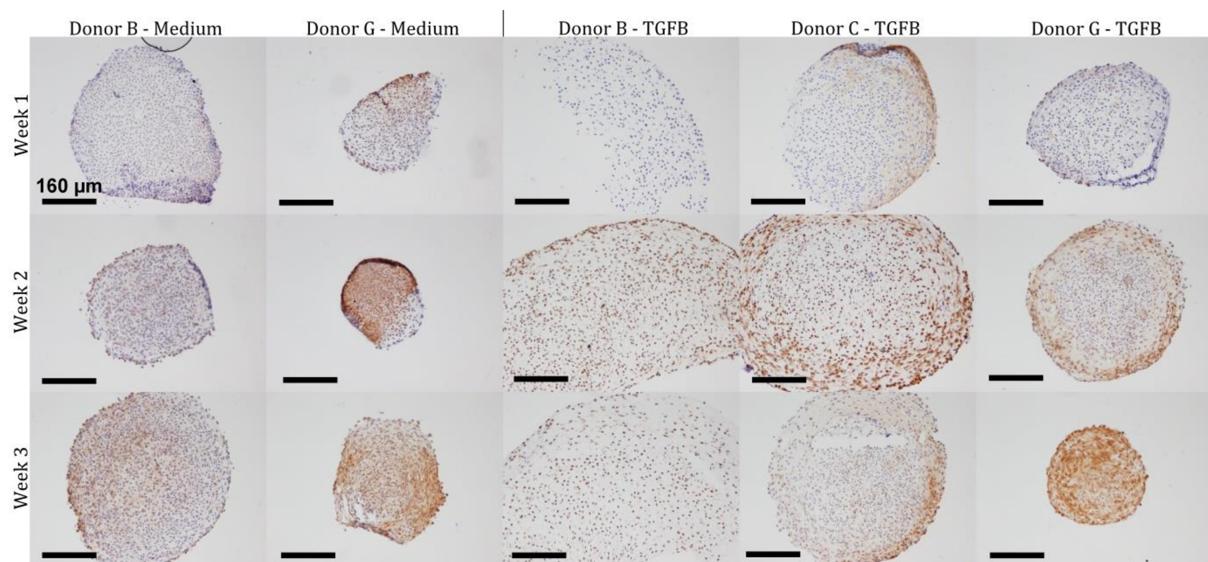

Figure 5 Representative staining for collagen I in pellets (n=2 per condition) after 1, 2, and 3 weeks of culture. All donors were cultured in chondrogenic medium (Medium), and chondrogenic medium supplemented with 10ng/ml TGF-β1 (TGFB). Donors A-D represent ponies, donors E-G represent horses. Brown staining indicates collagen I deposition. A 10x magnification was used.

For pellets cultured in chondrogenic medium with TGF-β1, one-way analysis of variance on GAG normalized for DNA (GAG/DNA) levels and GAGs released to the culture showed no statistical significant differences between horse breeds and pony breeds at any time point (Fig. 6).

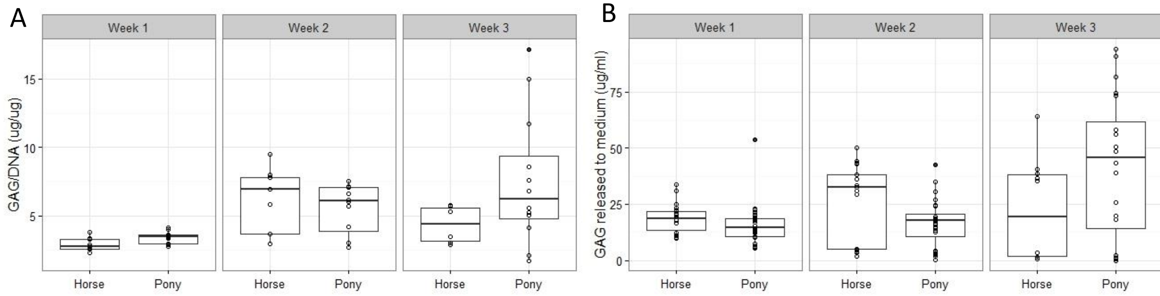

Figure 6 Pellets **(A)** GAG (n=3 per donor) content corrected for DNA (μg/μg) and **(B)** GAG released to medium (n=5 per donor) (4 days after medium change). Pellets were cultured for 1, 2, and 3 weeks in chondrogenic medium with 10ng/ml TGF-β1. Donors A-D represent ponies, donors E-G represent horses.

Significant differences between individual donors were found at week 2 and week 3. At week 2, donor A, B, C, E, and F had comparable mean (±SD) GAG/DNA levels (6.88±0.62 μg/μg, 6.47±0.58 μg/μg, 5.83±1.24 μg/μg, 7.21±0.97 μg/μg, and 8.07±1.08 μg/μg, resp.). Donor D and G had significantly lower mean (±SD) GAG/DNA levels (2.90±0.15 μg/μg and 3.43±0.33 μg/μg, resp., $p<0.05$). At week 3 differences between donors became more prominent; with highest mean (±SD) GAG/DNA levels for donor B (14.63±2.23 μg/μg) and lowest GAG/DNA levels for donor D and G (2.64±1.07 μg/μg and 3.13±0.24 μg/μg, resp.) (Fig. 7A). Amounts of GAGs released to medium showed statistical significant differences at week 2 and 3. Highest GAG release was reached at week 3 (donor B, 82.89±8.34 μg/ml). Pellets from donor D and G showed the lowest GAG release (0.81±0.89 μg/ml and 1.92±1.30 μg/ml, resp.) at week 3 (Fig. 7B).

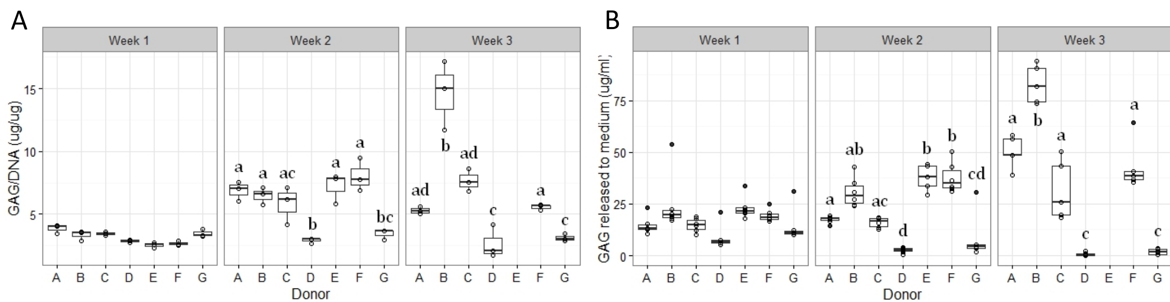

Figure 7 Pellets **(A)** GAG (n=3 per donor) content corrected for DNA content (μg/μg) and **(B)** GAG released to medium (n=5 per donor) 4 days after medium change. Pellets were cultured for 1, 2, and 3 weeks in chondrogenic medium with 10ng/ml TGF-β1. Donors A-D represent ponies, donors E-G represent horses. Significant differences of means between donor ($p<0.05$) within each week are indicated by letters. Analogues letters represent no significant differences.

Pellets cultured in chondrogenic medium only, started with DNA amounts comparable to pellets cultured in medium supplemented with TGF-β1 at week 1. However, DNA amounts of all donors decreased to level beneath 1.0 μg/pellet. Additionally, GAG amounts in pellets cultured in chondrogenic medium were all beneath 5.0 μg/pellet and decreased even further week 2 and week 3 (data not shown).

Discussion

The aim of this study was to determine whether chondrocyte performance differed between horse breeds and pony breeds. Our previous research resulted in relatively low levels of GAGs in pellet matrix, and a decrease in pellet stability after more than 2 weeks of culture (Chapter 3). Therefore, donor differences were evaluated for 3 weeks of pellet culture. Results from this experiment present that chondrocyte morphology stayed rounder in monolayer culture in

ponies compared to horses. Pellet size was not different between horse breeds and pony breeds, but individual donor differences were present. Some donors showed pellet growth, while others decreased in size after 1 week of culture. Histologically, no obvious differences were found between horse and pony breeds. Collagen II staining was similar for most of the donors, while safranin-O staining showed more donor differences. In addition, GAG/DNA levels and GAGs released to medium showed no differences between breeds. At 2 and 3 weeks of culture, pellets contained significantly different amounts of GAG/DNA and GAGs released to medium.

Chondrogenic morphology is likely to disappear after expansion of chondrocytes, resulting in dedifferentiated and hyperthrophic chondrocytes (11). Based on chondrocyte morphology, our data suggests that dedifferentiation occurs less in pony chondrocytes compared to horse chondrocytes. It is found that chondrocytes obtained from younger horses, were more likely to convert into a hyperthrophic cell type in culture (12). Donors chosen in our study were selected for the same age, however a relative young (2 years, donor F) and a relatively older horse (13 years, donor E) were included. This age difference might influence our findings. However, chondrocyte morphology was comparable between these two horses. When pony chondrocytes show less dedifferentiation in culture, this implies that pony donors are more suitable for studies where it is desirable for chondrocyte phenotype to be preserved. Further research in gene expression levels of dedifferentiation marker genes like collagen I and collagen X in 2D culture, might provide a clue about dedifferentiation levels in horses and ponies (13). Important is that cell donors should be chosen in the same age range, with exclusion of immature horses.

Differences between breeds concerning pellet size, histology and GAG content and release were not found. These findings might be explained by the limitations of this study concerning donor choice. The pony donors used were all in the same age range, but two different joints were used for chondrocyte harvest. In Chapter 2 (Experiment 1) we found that chondrocyte performance could differ between joints from the same donor. A study comparing chondrocyte behaviour in different species and joints, confirms these differences (14). Therefore, no exact comparison between donors can be made. Additionally, due to infection, one horse donor was removed from culture at week 3, whereby only 2 horse donors remained. Finally, background and breed from another horse donor was not completely known. It is found that Shetland ponies, which belong to Nordic breeds, have more genetic differences with warmbloods compared to other pony breeds. Also draught horses are genetically different from warmbloods, although they are still classified as horse breeds (15). This means that when the 'unknown' horse donor is classified as a draught breed, genetic differences might interfere with results. Therefore, further research with more donors representing horses and ponies is desired. Donors should be chosen based on similar age, joints and breed.

Interestingly, we found correlations between our histological and biochemical measurements regarding donor differences. Donors that obtained a bigger pellet size, had darker and more diffuse staining for collagen II and safranin-O, compared to donors that were smaller in size. Diminution of size and minor safranin-O staining was also in line with decreasing GAG and DNA levels. Additionally, collagen I staining, which reflects a more dedifferentiated type of cartilage, was more present in the smaller pellets with little GAG amounts. A study on redifferentiation potential of human chondrocytes in pellets cultured under different temperatures found similar results. Pellets that increased in size and weight had relatively higher mRNA expression of matrix components. This was confirmed by collagen II and safranin-O staining intensity, and GAG amounts (16). This suggests that a relative simple measurement like pellet size might function as a predictive factor for chondrocyte performance in matrix production, which might be helpful in selecting donors for future experiments.

Donor differences became significant as of 2 weeks of pellet culture, and matrix production was able to increase up to 3 weeks of culture. Some donors showed a decrease in matrix production after 1 week of culture. Comparing our results with previous research, a study of Ito et al.

cultured porcine chondrocytes in pellets to examine effects of several culture temperatures (16). At week 1 almost no staining was seen for collagen II and safranin-O, while complete staining was seen at 3 weeks of culture. At 3 weeks of culture GAG/DNA levels had a maximum of 11 µg/µg. A study with human chondrocyte pellets in varying oxygen percentages found dark and diffuse staining for safranin-O after 2 weeks of pellet culture, when cultured in 5% O_2 (17). Only little safranin-O staining was seen when cultured in 19% O_2. Another study where equine chondrocytes derived from several cartilage zones in pellet culture were assessed, complete safranin-O and collagen II staining was seen after 4 weeks of culture (18). Amounts of GAG in pellets raised in time up to 4 weeks of culture. Maximum values of GAG/DNA were 25 µg/µg at 4 weeks. This implies that pellet culture of equine chondrocytes is able to improve matrix production up to at least 4 weeks of culture, and that for complete matrix formation a minimum culture period of 3 weeks is needed. Additionally, our data suggests that for future research on differences between breeds or donors, a minimum culture period of 2 weeks is required.

Notably, our study did not find complete safranin-O staining at 3 weeks, while GAG/DNA levels reached up to 15 µg/µg. An explanation for this finding might be the use of other species and joints for chondrocyte harvest. It is known that chondrocytes derived from equine fetlock joints and shoulder joints increased cell number after addition of TGFβ. This effect was not seen in chondrocytes derived from the equine knee joint (14). In addition, Webber et. al. states that optimal culture conditions must be determined for each species individually (19). In our culture TGF-β1 and BSA was used. A study culturing equine chondrocytes supplemented culture media with TGF-β2 and HSA, which resulted in GAG levels up to 100 µg/pellet (9). Culturing in a hypoxic environment is also found to improve chondrocyte differentiation (17,20). Therefore, further research into the effect of several chondrogenesis stimulating growth factors in culture media and oxygen levels in culture might result in improved matrix production.

Conclusion

In conclusion, our results suggest that although monolayer culture indicated better maintenance of chondrocyte morphology in ponies compared to horses, no clear breed differences in chondrocyte behaviour exist in 3D pellet culture. Nevertheless, significant differences in chondrocyte performance were observed for individual donors. Additional research is desired using more donors per breed for more reliable results. Based on our findings, a minimum culture period of 2 weeks is required to test donor differences. When chondrocytes appear to differ between breeds, this might influence study outcomes to new therapies when only chondrocytes or *in vivo* models from a specific breed are used. Because of large individual differences found in the chondrogenic potential, donors should be specifically selected based on chondrocyte performance prior to future experiments in order to prevent unreliable data.

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Chapter 5

General discussion

Osteoarthritis (OA) has a great impact on the mobility of both animals and man (1,2). Due to limited regeneration capacity of cartilage, no satisfactory repair strategies have been found as yet. Studies on interactions between joint components in OA, introduced the definition 'whole joint disease' (3). A major role in these interactions is fulfilled by the synovial fluid (SF) present in the joint cavity. In OA development, joint homeostasis becomes disturbed and a reflection of this disturbed joint homeostasis can be found in SF (4). This altered joint environment might challenge the regenerative capacity of cartilage even more and might influence the effectiveness of new regenerative therapies. Therefore, this thesis focussed on investigating the influence of SF obtained from equine joints with different stages of osteoarthritic changes on the regenerative capacity of cartilage *in vitro*, by focusing on the vitality, matrix production and metabolic profile of chondrocytes in 3D cultures. Major results, limitations of our studies, and aspects for further research are discussed below.

The influence of synovial fluid on in vitro chondrogenesis

In healthy chondrocyte pellets cultured in medium enriched with healthy SF (HSF), we found a positive effect on chondrogenesis by increased staining for GAGs and collagen II, and increased cartilage marker genes (Experiment 1, Chapter 2). For evaluating the effects of SF obtained from different stages in OA development, HSF, SF from joints in an early stage of synovitis (LPSSF) and SF from joints with more chronic osteoarthritic changes (OASF) were used in culture.

Compared to the negative control (plain chondrogenic medium), HSF, OASF and LPSSF were all found to stimulate collagen II and GAG production on histology in healthy chondrocytes pellets. Although, pellets under influence of HSF induced less collagen II and safranin-O staining than pellets cultured in the addition of LPSSF and OASF. This finding was supported by biochemical analysis of GAG amounts per pellet, being lowest for pellets cultured with HSF. On the other hand, DNA levels were lower for LPSSF and OASF.

Gene expression levels of cartilage marker genes showed more differences between the addition of OASF and LPSSF in culture. Addition of both HSF and OASF resulted in increased collagen II mRNA expression compared to LPSSF and negative control. In contrast, LPSSF induced an increase in collagen I and collagen III mRNA expression compared to the HSF and OASF conditions. Expression levels of COMP, where highest under influence of OASF and lowest in HSF. Expression of inflammatory genes MMP3 and MMP13 were similar for all groups, and only MMP13 was slightly upregulated in the SF groups compared to both positive and negative control (Experiment 2, Chapter 3).

After 2 weeks of pre-culturing in TGF-β1, differences between pellets under influence of HSF and OASF remained visible. However, while a well-established pellet was formed in chondrogenic medium supplemented with TGF-β1 at 2 weeks, pellet degradation occurred under influence of all conditions at 3 and 4 weeks. Importantly, none of the conditions at any of the time points resulted in positive GAG staining on histology. We found that collagen II staining remained longer under influence of HSF than OASF. Biochemical assays showed that culturing in OASF reached significant higher GAG levels compared to HSF at week 3. However, mean (±SD) GAG amounts remained below 1.65±0.16 μg/pellet and were decreasing over time for all conditions (Experiment 4, Chapter 3).

Our findings are in line with previous research regarding the positive effects of HSF on chondrogenesis (5). On the contrary, addition of OASF has been reported to have negative effects on chondrogenesis, whereas our research shows stimulating effects (6). A major gap of knowledge in previous described research is the lack of comparison between effects of OASF and HSF within one study. When comparing the effects of OASF on chondrogenesis, with effects of medium enriched with growth factors, the latter is likely to be predominant over the biological amounts of stimulating factors present in SF.

Although HSF induced less collagen II and safranin-O staining and provided lower GAG amounts per pellet than LPSSF and OASF (Experiment 2, Chapter 3), DNA levels per pellet were higher, reaching similar levels as the positive control group. This might imply better pellet viability. Furthermore, high GAG production in an acute stage of synovitis is no direct indicator for the joint being in a reparative state, since also high levels of mRNA expression for collagen I and collagen III were found. This implies that in acute synovitis deregulation of chondrocyte phenotype may already occur. Based on the positive effects on chondrogenesis, our data indicates that SF in joints in a more chronic stage of OA might not be of overriding importance for cartilage regeneration. However, GAG formation in all of our experiments, but especially experiment 4 seemed remarkably low compared to earlier reported findings (7). With this in mind, the relevance and interpretation of differences between HSF and OASF (although significant) need to be considered carefully.

Pellet culture optimization

When optimizing pellet culture to test the effects of SF, we found P1 chondrocytes perform well in a 3D culture when cultured for 1 week in chondrogenic medium containing BSA and ITS supplemented with HSF or TGF-β1, although biochemically measured GAG levels remained low (Experiment 1, Chapter 2). Prolongation of culture period up to 4 weeks resulted in pellets with decreasing collagen II and GAG staining, and decreasing amounts of GAGs in pellets after 3 and 4 weeks of culture under all conditions (Experiment 4, Chapter 3). When individual chondrocyte donors were cultured for 3 weeks, some donors showed increasing GAG levels that were comparable with previously reported GAG levels. Pellets from other donors degenerated after 1 week and had reducing GAG levels. These individual donor differences were not found to be related to donor breed. Although in 2D culture, cell morphology for pony breed donors seemed to be better maintained (rounder shape) compared to horse breed donors (Experiment 5, Chapter 4). Interestingly, two out of the three donors pooled in experiment 4, were tested individually in experiment 5. Levels of GAG normalized for DNA appeared to be higher when cultured individually for both donors at week 2 compared to pooled donors. The effect of the third donor could not be taken into account (Experiment 4, Chapter 3 and Experiment 5, Chapter 4). A study on human articular chondrocytes confirmed these individual donor differences in GAG production (8). However, nothing is known about the negative effects of pooling chondrocyte donors. Our results imply that selection of donors is of great importance for maintaining a stable pellet during experiments, and that donor's breed might influence chondrocytes behaviour in culture. Additionally, our data suggests that when pooling donors, bad donors are determinative for how the pool performs. However, more research is desired into the potentially negative effects of pooling by comparing all donors from one pool individually.

Limitations of our studies

One of our major limitations is the relatively moderately performing chondrocyte pellets. This was characterized by very low GAG production in experiments described in Chapter 2 and 3, compared to Chapter 4 and previous research. Relevance of statistically significant differences found between pellets cultured in HSF and OASF in Chapter 3, are therefore debatable. Another limitation is our low sample size. Experiments performed in this project contained only n=2 or

n=3 pellets per condition. We found for instance GAG staining in only 1 out of 2 duplicates in experiment 2 (Chapter 2), which hampers interpreting results.

Additionally, a few donor-related limiting factors should be noted. Spread over all experiments, different chondrocyte donor pools were formed. Although pooling donors should overcome biased results due to individual donor differences, we experienced that in our experiments these individual donor differences remained important after pooling, since poor performing donors might be determinative for quality of the pool. Results obtained in different experiments are therefore less comparable. Furthermore, SF donors that delivered OASF were selected on macroscopic, microscopic and/or radiologic (cartilage) changes and pooled afterwards. Therefore, medium enriched with OASF may include various disease stages and a variety in SF composition. The fact that different pools were used for experiments 2 and 4, may explain different results found for OASF-enriched medium

Future research

Two main factors are important in future research on the effects of disturbed joint homeostasis in OA on regenerative capacity of chondrocytes in culture. First, improvement of several parts of the culture system we used is needed for more reliable results. Pursuing on our findings, inclusion and exclusion criteria regarding chondrocyte performance in matrix production should be composed. Based on these criteria individual donors should be tested before experiments are initiated. Assessing changes in pellet size could be an easy and fast parameter to determine whether pellets are able to produce sufficient matrix components. A revision on the need of a pre-culture period before testing several conditions should be executed, when suitable cell donors are found. Besides, further research on the differences in chondrocyte behaviour between different breeds could elucidate potential breed differences in OA pathology, which is relevant for both *in vitro* and *in vivo* experiments. Second, when testing SF obtained from joints in various stages of OA, these stages should be better determined. During a recently performed study at our faculty on Shetland ponies subjected to a cartilage groove model (9), SF samples were obtained at different stages of cartilage damage. The use of these, or comparable, samples could contribute to more uniformly experimental conditions and can eventually be correlated to diagnostic markers. For complete evaluation healthy SF should always be included.

Conclusion

In this thesis, effects of disturbed joint homeostasis on *in vitro* cartilage regeneration were investigated. In this respect, the effects of SF obtained from joints in an early stage of synovitis and joints with more chronic osteoarthritic changes were compared to the effects of healthy SF on chondrocyte pellet culture. We can conclude that addition of HSF, OASF and LPSSF all have positive effects on the matrix production of chondrocytes *in vitro* compared to chondrogenic medium. However, under influence of OASF and LPSSF matrix production was stimulated in a higher degree compared to HSF. On the other hand, SF from an acutely inflamed joint induces higher expression of cartilage dedifferentiation markers. These findings suggest that SF in both joints with an acute synovitis and osteoarthritic joints provoke matrix synthetization, and that in acute synovitis deregulation of chondrocyte phenotype may already occur. Finally, clues were found with respect to the importance and selection of well-performing individual donors. A major limitation in this project is that results were obtained assessing pellets with relatively low matrix production, due to poor performing cell donors. Therefore, the relevance and interpretation of differences between HSF, OASF and LPSSF that were found need to be considered carefully.

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Appendix I

Overview experimental designs

Table 1 Overview variables and fixed factors in experimental designs.

Exp.	Donors	Cell type	Joint	Cell stage	General medium	Additives	Pre-culture medium	Pre-culture + additives
1	Pony 1 Pony 4	Healthy chondrocytes	MCP joint MC joint	P0 P1	Chondrogenic Non-chondrogenic	General medium 25% HSF 50% HSF LPS 10 ng/ml TGF-β1 10 ng/ml	Chondrogenic	1 + 7 days
2	Pony 1 Pony 4 EQ17-001 EQ17-002	Healthy chondrocytes OA chondrocytes	MCP joint	P1	Chondrogenic	General medium 25% HSF 25% OASF 25% LPSSF TGF-β1 10 ng/ml	Chondrogenic	1 + 7 days
3	EQ17-002 EQ017-009 EQ017-010	Healthy chondrocytes	MC joint	P1	Chondrogenic	General medium 25% HSF 25% OASF TGF-β1 10 ng/ml	Chondrogenic TGF-β1 10 ng/ml	14 + 0 days 14 + 7 days 14 + 14 days
4	EQ017-003 EQ017-005 EQ017-006	Healthy chondrocytes	MC joint	P1	Chondrogenic	General medium 25% HSF 25% OASF TGF-β1 10ng/ml	Chondrogenic TGF-β1 10 ng/ml	14 + 0 days 14 + 7 days 14 + 14 days
5	Pony 1 Pony 4 EQ017-003 EQ017-005 EQ032 EQ031 EQ037	Healthy chondrocytes	MCP joint MC joint	P1	Chondrogenic	General medium TGF-β1 10 ng/ml	N/A	0 + 7 days 0 + 14 days 0 + 21 days

MCP = metacarpophalangeal, MC = middle carpal, HSF = healthy synovial fluid, OASF = synovial fluid obtained from osteoarthritic joint, LPSSF = synovial fluid obtained from joints 24 hours after LPS injection.

Appendix II

Courses

- Modern Methods in Data Analysis (MSc Epidemiology)
- Writing a Scientific Paper - Online (PhD Course Centre)
- Training on geNorm and global mean normalization - Online (qBase+)