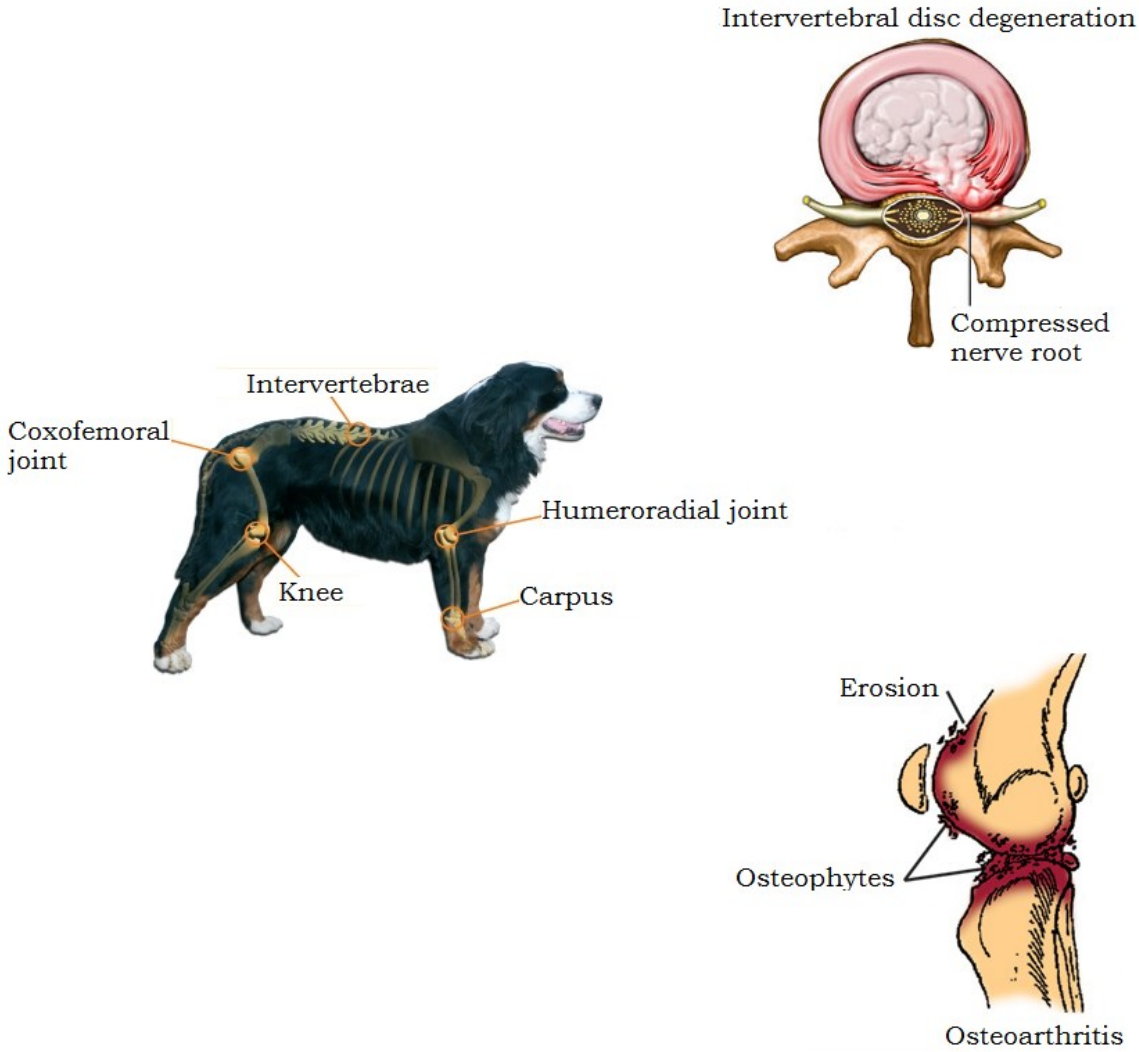
Minor Research: Regenerative treatments for OsteoArthritis and InterVertebral Disc degeneration



Minor Research
Veterinary medicine of Companion Animals
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Table of contents

Foreword

This report is part of the Minor Research and is a component of the Master of Medical Science of Companion Animals. I used the entire space of corrugation of the Master in order to do research. This was advised by my Supervisor, since I would like to work in research after I have obtained my Masters degree. My research period lasted for about 10 months (39 weeks).

Aim and scope: My research focused on regenerative medicine. The main focus of regenerative medicine is that with help from regenerative cells, such as pluripotent or multipotent stem cells or cells derived from the tissue that has to be regenerated, tissue can be regenerated which had been lost due to specific reasons (1,2). Regenerative medicine could therefore propose a curable treatment for diseases in which the body cannot regenerate on its own, the tissue that has been lost (3,4). Examples of such diseases are Osteoarthritis (OA) and Intervertebral disc degeneration (IVD degeneration) (1,5).

The experiments that I have performed are based on developing regenerative treatments for these diseases, which are highly diagnosed in canine as well as in human, and are still incurable (1,6-12). Moreover, Wiegant *et al*. and Bergknut *et al*. have shown that the dog can serve as an usable animal model for research towards OA and IVD degeneration in human (8,13). Therefore, if a regenerative treatment for becomes available for dogs, a translation could also be made towards human application.

The tissue specific elements that are lost in OA and IVD degeneration are tissue specific extracellular matrix components of cartilage and ECM resembling cartilage, respectively (1,6). In order to obtain these matrix components *in vitro*, I have used Bone-Marrow derived Stem Cells (BMSCs) and Chondrocyte-Like Cells (CLCs). BMSCs can differentiate into cartilage producing cells and CLCs are already differentiated cells in which they are able to produce ECM components (1,5). In order to achieve the differentiation and the production of ECM components, growth-factors Transforming Growth Factor Bèta1 (10 ng/mL; TGF-β1) and/or Bone Morphogenetic Protein-2 (100 and 250 ng/mL; BMP-2), were supplied to the culture.

Lay-out: The first chapter of this report explains the rationale behind the performed experiments. Therefore, more information about the diseases, regenerative cells and growth-factors is discussed. The second chapter contains the reports of the performed experiments written in the format of scientific articles. The third and last chapter contains a general discussion about the performed experiments regarding protocol optimization of chondrogenic differentiation of canine BMSCs. Finally, in the appendix information can be found about the scientific activities that I have followed.

Summary

This report focuses on regenerative medicine. The main goal of regenerative medicine is biological repair of degenerating cartilaginous tissue. Osteoarthritis and intervertebral disc degeneration (IVD degeneration) are diseases in which cartilaginous tissue degenerates. Both diseases are currently incurable and therefore researchers are trying to develop a regenerative treatment. In order to establish such treatment, regenerative cells are used, for instance mesenchymal stem cells or cells derived from the intervertebral disc tissue. In order to achieve the production of cartilaginous tissue by these cell types, growth factors: Transforming growth factor-β1 (TGF-β-1) and Bone morphogenetic protein-2 (BMP-2) are added to the cell-culture.

The first three experiments were based on optimizing chondrogenesis of canine BMSCs, while it was not known yet what the best technique is to obtain chondrogenises of canine BMSCs. Therefore, three different studies have been performed in which it was shown how important both expansion and the differentiation phases are of canine bone marrow derived mesenchymal stem cells (BMSCs). These studies showed that the best technique was to expand canine BMSCs with clonal, optimal density combined with fetal calf serum from which previous experiments showed that that serum did not have any negative influence on the chondrogenic potential of BMSCs. After expanding BMSCs, they were subjected to chondrogenesis and this could be most successfully achieved by adding TGF-β1 (10 ng/mL) combined with BMP-2 (250 ng/mL) to the culture media.

The last experiment was based on investigating the differences in response between chondrocyte-like-cells (CLCs) derived from chondrodystrophic (CD-) and non-chondrodystrophic (NCD-) dog breeds. IVD degeneration has a different pathophysiology within canine dog breeds which can be divided into CD- and NCD-dog breeds. In addition, there is also a difference in clinical presentation. This could indicate that there could also be differences in response in growth factors employed for regenerative treatment strategies when CLCs derived from the IVD tissue of these dog breeds are cultured *in vitro*. Consequently, the aim was to investigate the difference in response between chondrocyte-like-cells derived from CD- and NCD-dog breeds when cultured *in vitro* under the influence of TGF-β1 and BMP-2 in terms of matrix production. The results showed that micro-aggregates containing CLCs derived from CD- and NCD-dog breeds performed best when cultured with BMP-2 in the concentration of 250 ng/mL. Furthermore, micro-aggregates containing CLCs derived from CD-dog breeds showed higher GAG production in which they released more GAGs into the media when cultured with BMP-2 (250 ng/mL) compared to micro-aggregates containing CLCs derived from NCD-dog breeds. However, micro-aggregates containing CLCs derived from NCD-dog breeds were more efficient in depositing GAGs in their matrix.

Chapter 1: The rationale

1.1 Introduction

Cartilage has many important functions in the body and it is still very difficult to imitate.

In articular cartilage and extracellular matrix (ECM) resembling cartilage, two major problems were diagnosed in canine and also in humans: Osteoarthritis (OA) and Intervertebral disc (IVD) degeneration. OA is often the result of altered joint biomechanics, followed by excessive cartilage damage and subsequently an altered production of cartilage components, which cannot be healed by the body itself. IVD degeneration is the result of an altered cell population within the nucleus pulposus (NP), followed by an altered production of ECM resembling cartilage components, and subsequently loss of ECM integrity, making the tissue more prone to damage. This can also not be healed by the body itself. Therefore, both diseases are progressive and painful with no remedy yet (6,14-18).

Regenerative medicine aims for biologic repair of the degenerating cartilaginous tissues. With help from Bone-Marrow derived Mesenchymal Stem Cells (BMSCs) and Chondrocyte-like Cells (CLCs), tissue specific components of the ECM of the articular cartilage and IVD are employed *in vitro* as a first step for developing a regenerative treatment. These components are mostly collagen type II and proteoglycans and are both present in the ECM of the articular cartilage and of the IVD (1,4,19-25).

The regeneration of ECM components, can be obtained *in vitro* with the use of specific growth-factors which belong to the Transforming-Growth-Factor-Bèta-family; respectively Transforming-Growth-Factor-Bèta 1 (TGF-β1) and Bone-Morphogenetic-Protein-2 (BMP-2). These growth-factors take care of the transcription of Sox9, which is the master regulator of chondrogenic differentiation and essential for ECM specific gene expressions, such as collagen type 2α1 (COL2α1) and proteoglycans (2,26-28).

In this chapter OA and IVD degeneration will be discussed, followed by the role of growth-factors TGF-β1 and BMP-2 on the production of collagen type II and proteoglycans by BMSCs and CLCs.

1.2 OA and IVD degeneration

OA and IVD degeneration are two diseases which have high incidence rates in terms of diagnostics. Numerous dogs endure from the clinical consequences by showing symptoms of inter alia pain and lameness (1,6,7,9,11). In order to understand how these symptoms develop and what the significances of these diseases are, more information about the healthy body tissues and the degeneration processes are discussed in this paragraph. Also, the possible treatment options that are often performed by veterinarians are explained, followed by the prognosis for dogs diagnosed with OA or IVD degeneration.

1.2.1. *The healthy cartilaginous tissues*

<u>The articular cartilage</u>

OA is a disease of the articular joint. The articular joint consists of articular cartilage which is comprised of extracellular matrix (ECM) and chondrocytes. This ECM provides the extraordinary, compressive properties that characterize articular cartilage. It consists for 80% of water and 20% of solid matrix, which is comprised of collagen and proteoglycans (29,30). Approximately, 90% to 95% of the collagen fibres are comprised of collagen type II. The remaining part accounts for the collagen types: VI, IX, XI and XVI (12).

In addition, the solid part also contains proteoglycans in which aggrecan is most common found. This proteoglycan contains many negatively charged chains of glycosaminoglycans (GAGs), mainly keratin and chondroitin sulphate (chondroitin-6-sulphate). In the ECM, aggregates are formed consisting of aggrecan molecules bound by link protein to a hyaluronan molecule. These aggregates are negatively charged and due to osmosis they attract water and the ECM starts to swell. The collagen fibres counterbalance this process, resulting in a swelling pressure which exerts a force outwards to maintain the stiffness of the collagen network, as can be seen from figure 1. Under high tension, this rigidity results in lubrication of the cartilage and it causes synchronous movement of the GAG chains which causes water displacement. Hereby, the cartilage can resist further compression. Through these components, cartilage has viscoelastic properties, can resist sudden impact loading and is able to recover its normal shape after loading (30,31).

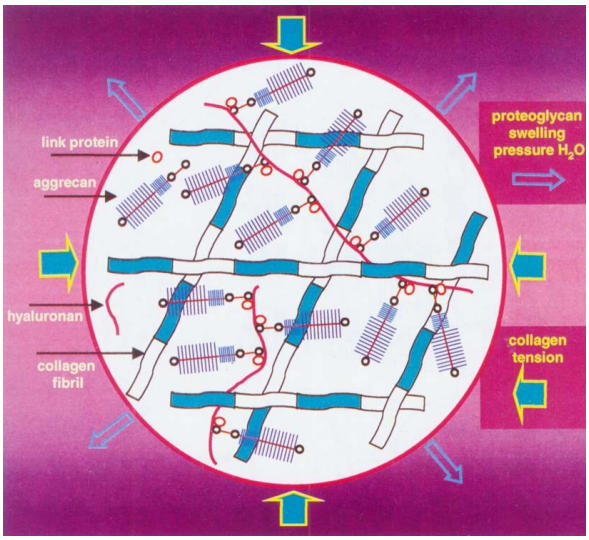

Figure 1: *Aggrecan-collagen II network in articular cartilage*. The formation of aggrecan aggregates combined with collagen fibres take care of the extraordinary and compressive properties that articular cartilage contains (31).

The ECM is produced by chondrocytes and in the healthy joint there is a dynamic balance between anabolism and catabolism (32). Adult chondrocytes have reduced proliferative and metabolic capacity. However, they are capable of repairing small defects associated with minimal loss of ECM components. Large defects associated with great loss, exceed their repair capacity, resulting in the progressive, painfully disease known as OA (29).

Articular cartilage is a non-vascular and non-innervated tissue. Chondrocytes receive their nutrition and cellular repair factors through diffusion from the synovial fluid (29). The synovial fluid also lubricates the synovial joint and is secreted by the synovial membrane (synovium). This membrane covers the joint and contains nerve-endings as well as synoviocytes which produce hyaluronate and synovia (6,30).

The synovium is surrounded by a joint capsule that consists of a tight fibrous connective tissue. It is passed through by nerves and veins, and it may also contain holes through which the synovium can protrude to form a cushion. The capsule adheres firmly to the articular bones in specialized “attachment zones”. These areas are important, because injuries occur often in or close to these zones as result of for instance wear and tear. Furthermore, accessory ligaments are also present which further supports and could also protect the capsule against loading, see figure 2 (33).

<u>The Intervertebral discs</u>

The IVDs have the function to interconnect the vertebral bodies from the second cervical vertebrae (C2) until the first sacral vertebrae (S1) (14,17). The IVD consists of, from the inside to the outside, the nucleus pulposus (NP), the annulus fibrosus (AF), the transition zone (TZ) and the cartilaginous endplates (EPs) (1,14,17) as can be seen in figure 2.

The NP contains as main cell type large notochordal cells (NC), which can be found in large clusters, consisting of 10 to 426 cells. These clusters offer mechanical integrity and facilitate long-term cell-survival *in vitro* (34,35). NCs are recognizable by their characteristic morphology: large cells (25-85 µm), containing many vacuoles in their cytoplasm (1,14,36,37). These vesicles play a role in the regulation of osmosis within the NP, containing a low-osmolality solution which is generated by ion pumps. These pumps are components of the vesicular membrane (38). The NCs produce an ECM that is rich in proteoglycan aggregates and collagen type II. Though, the proteoglycan content is in excess over the collagen content (24) and therefore the NP has a mucoid, transparent, bean-shaped composition. The most general proteoglycan that can be found is comparable to that in articular cartilage, namely aggrecan. All proteoglycan molecules contain side chains, comprised of GAGs, mainly chondroitin and keratin sulphate. These molecules are aggregated by link protein to hyaluronan, and are negatively charged, therefore creating a high osmotic gradient. Consequently, the NP is composed by over 80% of water (14,15).

The NP is surrounded by the AF, which consists of 60 % water and numerous concentric fibrous lamellae. These lamellae form an organized, dense network, consisting of aggregations of collagen fibres combined with elastic fibres and proteoglycans (1,14). These aggregations or cross-links prevent lamellar movement within the AF. The ventral part of the AF is thicker than the dorsal part (14,17), which is due to the fact that all vertebrae have ventrally their centre of gravity. This leads to a tensile stress that is absorbed by the NP and the ventral part of the AF (39). Within the AF there are two different kinds of cell populations. The outer layers contain a population of fibrocyte-like cells, producing mainly collagen type I. The inner layers contain a mixed population of fibrocytes and chondrocyte-like cells (CLCs) through which mainly collagen type II can be found (14,15).

Subsequently, the AF shows a transition zone at which it becomes less fibrous and more cartilaginous. This TZ is located near the centre of the IVD and forms the most inner part of the AF. It interconnects the AF with the NP and contains CLCs (14,15).

Furthermore, the outer layers of the AF contain limited blood supply and is also the only location in which nerve endings can be found (14,17), resulting in a non-vasculated and non-innervated NP tissue (1,14).

The fibres of the inner AF are firmly attached to the EPs and those from the outer AF are attached to the bony vertebral body epiphyses (14). The hyaline cartilaginous EPs form the upper and lower borders of the IVD and also contain CLCs as cell population (1,14,15). The EP is also highly hydrated, caused by the presence of proteoglycan aggregates interwoven in a collagen network comprised of mainly collagen type II (14). Nearby the EPs, there is a compactly formed vascular network from which small molecules (i.e. oxygen, glucose) can extend towards the cell populations present in the IVD tissue. This occurs through diffusion and/or osmosis (1,14,17). The composition of the EP is very important for the preservation of the integrity of the disc, because proteoglycans that are present in the ECM of the EP regulate the transport of the nutrition into and out of the discs (14).

Furthermore, the high water content of the IVD causes a high intradiscal pressure and the collagen content has as main function to direct tensile forces. Hereby, the IVD can

transfer the compressive forces between the vertebral bodies and can provide motion as well as steadiness to the spine (1,14).

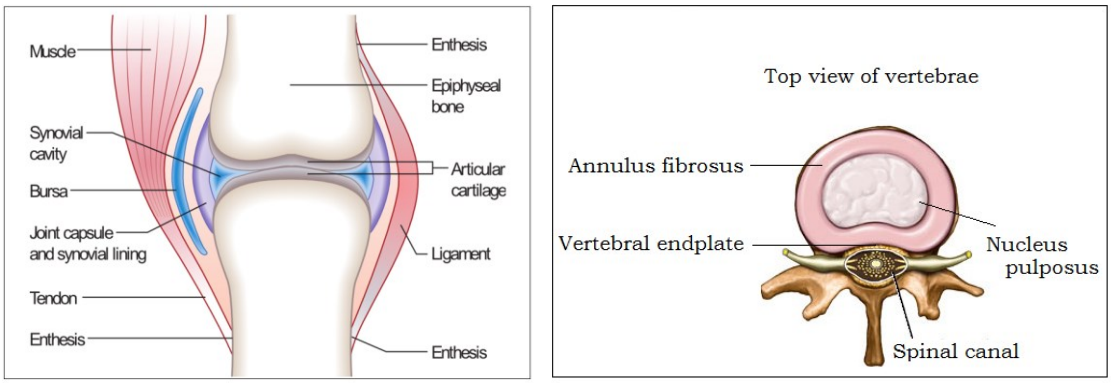

Figure 2: *Healthy structures of the articular joint and IVD* (116,119). Modified by MCME Müller.

1.2.2. The affected cartilaginous tissues (figure 3)

OA

As described above, articular cartilage is poorly vasculated and innervated. Together with the low cellular content, this tissue has a low regenerative capacity. Therefore, it is more prone to develop OA by cartilage catabolism, after the occurrence of excessive cartilage damage.

The chronic degenerative disease OA takes place in the articular joint (6). It appears often secondarily due to altered joint biomechanics which is regularly caused by risk factors. These risk factors can be divided into: systemic, such as genetics; biomechanical, such as joint instability; and environmental, such as nutrition (18,40-43). Therefore, the aetiology can often include multiple factors. However, the most important factor would most likely be trauma. This factor can appear as wear and tear or in the condition of severe injury (30).

The onset of the disease starts with injury of the cartilage in which, likewise, the chondrocytes are damaged. These cells are therefore triggered towards hypertrophy and cluster together; there is an increase in hydration of the ECM and an enhancement in matrix turn-over. These cells produce in this stadium not only common substances found in healthy cartilage (aggrecan and collagen type II), but also other substances like tenascin or collagen type I, III and X (6). Though, newly synthesized aggrecan molecules are also different in that way that chondroitin sulphate chains have decreased in height and the chondroitin - keratin sulphate ratio has shifted more in favour of chondroitin sulphate. Also the chondroitin sulphate is more present in the appearance of chondroitin-4-sulphate instead of chondroitin-6-sulphate (30).

Besides the increased anabolism, there is an even higher increased catabolism of ECM. The hypertrophied chondrocytes produce also higher concentrations of matrix metalloproteinases (MMPs) (6) which are enzymes that degrade cartilage matrix (29). This leads to a disparity between the forming and the degradation of cartilage substances, which is the main cause of the progressive degradation of articular cartilage (6). Consequently, the cartilage will contain less proteoglycans and combined with the altered production of aggrecan molecules, cartilage matrix will also decrease in water content, resulting in loss of stiffness of the collagen network. In that way, the tissue is more susceptible for developing mechanical disorders (30).

Interestingly, research has shown that these damaged chondrocytes have a decreased expression of the transforming growth factor-bèta receptor II and an increased expression of the receptors for tumor necrosis factor alpha (TNF-α) and interleukine-1 (IL-1) (6).

Due to cartilage loss that is released in the synovial joint, the synovium gets secondarily involved in the process of the disease. This membrane exhibits symptoms of a chronic inflammation (6) and this can cause a shift in the metabolism of the synoviocytes (44). Recapitulated, synoviocytes produce synovial fluid and hyaloronan which are essential for chondrocyte survival and for the formation of proteoglycan aggregates, which play a role in providing the cartilage mechanical strength (30,31).

The fibroblasts and synovial macrophages also present in the synovial tissue, take also part in this process. They produce among others catabolic factors, immunoglobulins, inflammatory cytokines (6), proteinases and oxygen-derived free radicals. These substances can be expelled into the synovia and can therefore reach the chondrocytes through diffusion, resulting in down regulation of proteoglycan and collagen production. Though, some substances, such as cytokines, can also cause the release of other proteinases, oxygen-derived free radicals and cytokines which further contributes to the degradation of the ECM (44).

Overall, a lot of factors are produced both by chondrocytes and synovium, which take care of the discrepancy between the levels of anabolism and catabolism.

In the beginning, there is stimulation of the production of cartilage substances through the release of anabolic factors. These factors are for instance, transforming growth-factor-Bèta (TGF-β), bone morphogenetic proteins (BMPs), insulin-like growth factor-1 (IGF-1) and fibroblast growth factors (FGFs) (29). The BMPs are part of the TGF-β family which will be discussed later on.

However, most factors are regulated by specific antagonists, just like the BMPs. The BMPs are partial regulated by *Noggin*, *Chordin, Gremlin* and *Follistatin*. Research has shown that no expression of *Noggin* is detectable in human OA chondrocytes. Although, compared to healthy chondrocytes, *Chordin* is low expressed and *Gremlin* and *Follistatin* are even higher expressed in human OA chondrocytes (45).

Besides stimulation, there is also an increase of these substances' degradation. There is an increased synthesis of MMPs and a diminish of "*Tissue Inhibitor Metalloproteinases*" (TIMP), which inhibit the activities of the MMPs. This is caused by the presence of inflammatory cytokines, for example IL-1β, interleukine-6 (IL-6) and TNF-α (29,46).

IL-1β increases the production of proteases, prostaglandin E_2 (PGE_2) and pro-inflammatory cytokines, such as IL-6. TNF-α also increases the production of IL-6. On molecular degree IL-6 amplificates the effects of IL-1 by inhibiting the synthesis of proteoglycans and by up regulating the production of MMPs. Furthermore, it also stimulates the recruitment of cellular defence into the synovial tissue and chondrocyte proliferation (46). The stimulation of the synthesis of pro-inflammatory cytokines, hence, amplifies itself due to the triggered inflammatory response.

Also, there is stimulation of pain receptors. The current inflammation causes damage of the cell membrane, resulting in production of inflammatory mediators, such as PGE_2. PGE_2 stimulates pain receptors, causing the animal to become aware of pain in his affected joint (47).

After a certain period of time, catabolism outweighs anabolism, establishing a progressive cartilage degradation. As a consequence of the continuous cartilage degradation, the subchondral bone will become uncovered and therefore will also become involved in this ongoing process. Consequently, it will develop osteosclerosis, combined with an increased quantity of osteoid and a low degree of mineralization. This raised the idea that a dysregulation in the bone remodelling process may be part of OA, which is confirmed by the fact that the current osteoblasts show a changed phenotype, in which the cells produce more IGFs and urokinase compared to healthy osteoblasts (6). Furthermore, the trabeculae of the spongy bone may be decreased and therefore the intratrabecular space increased in the deeper parts of the subchondral bone (44).

Finally, the release of cartilage components and membrane fractions (auto-antigens) as a consequence of cartilage injury can also trigger humoral immunity. These auto-immune responses can cause additional cartilage damage and therefore to additional release of auto-antigens. Therefore, the humoral inflammatory response as well as the cellular inflammatory response can cause a vicious circle which again amplifies itself, leading to a higher degree of cartilage injury (6,46).

IVD degeneration

The chronic degenerative disease IVD is a multifactorial process that takes place in the intervertebral disc. First of all, the disease will be discussed from a more general point of view. At the end more specific differences in pathology between two groups in which all dog breeds can be categorized, will be explained.

This categorization is based on the predisposition to chondrodystrophy, which is a skeletal disorder characterized by a disrupted enchondral ossification. Therefore, chondrodystrophic (CD-) dog breeds show disproportionally short extremities. Apart from this predisposition, IVD degeneration is more common in CD-dog breeds, although it is also diagnosed in non-chondrodystrophic (NCD-) dog breeds (1,48).

As mentioned, IVD degeneration is a multifactorial process and is marked by differences in the construction of cells and ECM that can be found in the NP, TZ, AF and EPs. IVD degeneration in canine is for a greater part still unexplored. However, this disease is comparable to the human variant and therefore the human pathophysiology will be discussed (14).

The degeneration of the IVD is associated with multiple factors among others, genetic predisposition, excess of mechanical load and trauma, resulting in changes in ECM content. The degeneration starts with cellular changes in which the NC cell population will be replaced by CLCs, resulting in a change in ECM composition (14). This results in a decreased proteoglycan content combined with breakdown of GAG molecules within the NP. Moreover, the composition of the proteoglycan molecules is also changed in which the chondroitin - keratin sulphate ratio has shifted more in favour of keratin. The decreased and altered proteoglycan content leads to a reduced water content. As a consequence, the size of the NP and the intradiscal pressure will also decrease. Furthermore, there is also an increased and altered collagen content: increased expression of collagen type III, V and VI can be found in the NP (14,15,49). In addition, organized collagen fibres have been replaced

by disorganized collagen fibres. This whole process of cellular changes in the NP and the production of disorganized collagen fibres is referred to as *chondrification* (50-54).

All of these changes within the NP cause an impaired mechanical function in which the NP is less able to equally distribute the compressive forces between the vertebral bodies. Consequently, the mechanical function is transferred to the surrounding AF (14,49).

The AF obtains the function of compressive load-bearing, which causes an alteration of its composition (14,49). This alteration results in matrix consisting of an increased collagen type II content (14) containing disorganized lamellar fibres and ingrowth of CLCs derived from the TZ. Hereby, the TZ increase in size, becomes more abnormal and therefore making it more difficult to distinguish the AF from the NP. Furthermore, there is also an increase in cross-links between these fibres. Consequently, the AF expanses in size, becomes more rigid and weaker. This may lead to the collapse of its structure, resulting in outwards bulging of the IVD (14). Though, it is also possible that the AF develops annular defects or tears through which the degenerated NP tissue can extrude (14,49). As a result of the AF being ventrally thicker than dorsally, the structural insufficiency and IVD rupture frequently take place dorsally (1,14). As a result, there is loss of tissue integrity of the NP and AF, leading to spinal instability (55).

The affected NP and also AF are not able anymore to manage with the load-bearing function, resulting in transferring the unevenly distributed forces onto the EP. In the early phases of the degeneration, the EPs become wider. In the later phases, the EPs become more irregular and due to unevenly loading, it may break at certain sections. These breaks usually take place in the centre of the EPs and therefore extruding of the NP can take place into the vertebral body. This is better known as a “Schmorls node”. This phase is associated with a decrease in collagen type II, proteoglycan and subsequently in water content. Eventually, in advanced stadia mineralisation of the EPs will occur and this will lead to capillary obstruction. As a consequence, diffusion of nutritional and waste solutes to and from the IVD tissue is inhibited and the disc height may decline (14).

Apart from the altered matrix synthesis and therefore the changed composition of the IVD there is also an altered metabolism during IVD degeneration. This metabolism has altered in favour of catabolism. Catabolic substances that are being produced consist mostly of MMPs and “*a desintegrin and metalloproteinase with trombospondin motifs*” (ADAMTS). These enzymes are involved in the degradation of respectively collagen and aggrecan (1,14,49). It has been shown from research that catabolic enzymes, such as MMP-13 and ADAMTS-5 are upregulated (14,15,49,56) and that especially upregulation of MMP-13 and ADAMTS-5 lead to severe degeneration (56). Furthermore, it has been reported that these catabolic enzymes are capable of enhancing the catabolism within the IVDs, though these proteins can be inhibited by “*tissue inhibitors of metalloproteinase*” (TIMPs) (14,49). However, the degeneration in the IVD may be provoked by loss of balance between catabolic enzymes and their inhibitors (15,49).

Furthermore, as a consequence of the occurred damage, pro-inflammatory cytokines are being released. The most commonly studied cytokines with respect to IVD degeneration are IL-1β, IL-6 and TNF-α (14,15,49). From research it appears that all of these mediators are higher produced in the degenerated IVD (15,49). It is also believed that they are responsible for the increased catabolism of the ECM and therefore accelerate IVD degeneration (14,15). IL-1β is capable of increasing the production of oxygen-derived free radicals, IL-6 and PGE2 within the IVD. These substances are able to inhibit proteoglycan synthesis (15,49).

As a result, there is a vicious circle ongoing of sustained injury combined with impaired regeneration, leading to chronic IVD degeneration (1,14,49).

Nevertheless, the IVD is a component of the functional spine and therefore IVD degeneration not only affects the disc, but also other spinal components, such as facet joints and vertebral bodies (57). Due to IVD degeneration, the structure is impaired in its load-bearing function and therefore facet joint loading is increased (58). This can cause secondary OA with for instance osteophyte formation. It can also lead to bone remodelling and spondylosis of the bodies of adjacent vertebrae (14).

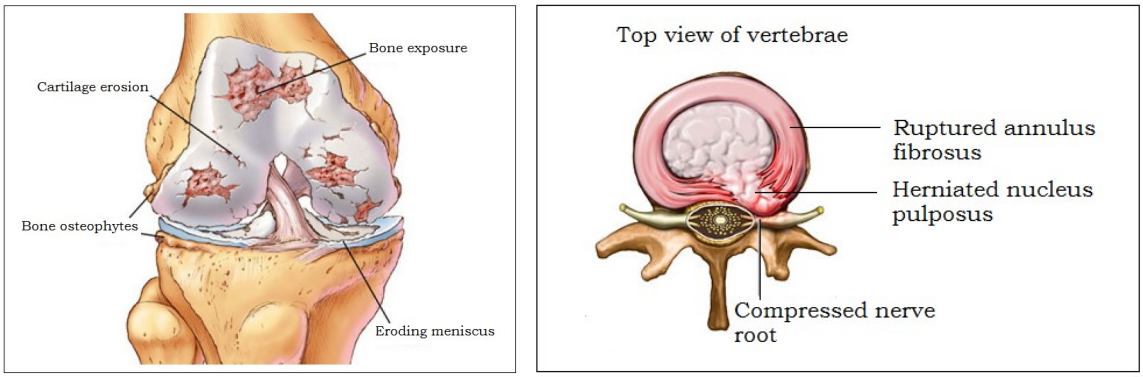

Figure 3: *Affected structures of the articular knee joint and IVD during OA and IVD degeneration* (116,120). Modified by MCME Müller.

As already mentioned all dog breeds can be divided into CD- and NCD-dog breeds, based on the predisposition to chondrodystrophy. Common CD-dog breeds are for instance the Dachshund, French Bulldog, Beagle, Cavelier King Charles Spaniel and Welsh Corgi. NCD-dog breeds that are mostly affected by IVD degeneration include the Dalmation, Dobermann, the German Shepherd, Labrador Retriever, Rottweiler and also mixed dog breeds (17,59,60). The degenerative process of the IVD is different within CD- and NCD-dogs. These different processes are in general referred to as respectively chondroid metaplasia and fibrous metaplasia (17).

Chondroid metaplasia or chondroid metamorphosis (17,48,50) is the result of loss of glycosaminoglycans and an increase in collagen content within the NP (17). Due to glycosaminoglycan loss, there is also a decrease in water content and therefore loss of intradiscal pressure. This leads to a decreased ability to cope with the load-bearing function of the NP (16,17). This shift of the gelatinous, semi-fluid NP into a drier NP can start as early as 2-3 months of age in CD-dogs and involves progressive replacement of NCs by CLCs surrounded mostly by dense ECM. This rapidly, progressive replacement is completed in most of the IVDs by the age of 1 year in CD-dogs and occurs along the whole vertebral column from C2 to S1 (17,48). Moreover, the CLCs are more prone to apoptosis and this increases with age (61).

As a result of the changed phenotype of the NP, dorsal rupture or calcification can proceed (16,17,48). After the degeneration of the NP is completed, degeneration of the AF can take place. This occurs often dorsally, resulting in dorsal rupture of the NP. This phenomenon can be observed from the age of 2 years (48). However, calcification of the NP occurs more frequently than dorsal rupture. In addition, this calcification process can start before chondroid metaplasia has completed within all IVDs from C2 to S1, and the

prevalence increases with age until the dog has reached the age of more than 27 months. Occasionally, calcification also occurs in the AF (48).

Fibrous metaplasia or fibroid metamorphosis is the degenerative process that is documented to occur predominantly in NCD-dogs (16,17,48,50). This process is age-related (17) and is reported to arise in NCD-dogs from the age of 5 years and older. By the age of 6 to 7 years approximately 50 to 70% of all NPs have undergone this metamorphosis (48). It includes a shift from a gelatinous NP to fibrous collagenisation of the NP. At the same time, there is also degeneration of the AF. This can occur along the whole spine, but not all IVDs will undergo this alteration (17,48). The transition to a fibrillar NP is caused by the change in the inherent cell population of the NP. In NCD-dogs NCs form the main cell population within the NP. However, age-related alterations in this population can occur. This is expressed as "slow maturation" and includes the appearance of collagen fibres derived from the TZ, into the NP. Subsequently, these fibres divide the NP into different islets of NCs. These NCs can undergo degenerative changes through which they obtain a more fibrocyte-like phenotype (48,50).

Although, recent studies have shown that the events that occur in the more developed phases of NP degeneration in NCD-dogs is comparable to those observed in CD-dogs (50,54,60). As well as in CD-dogs, replacement of NCs by CLCs can also take place in NCD-dogs in more advanced phases of the degenerative process (16,48). Although, calcification of the NP rarely happens in NCD-dogs (17,48), dorsal herniation of the NP can be more frequently observed (17,48).

Briefly worded, the degeneration process of both diseases has some common paths as shown in figure 4. There is an altered matrix production of ECM substances, making both structures more susceptible to injury. This injury can occur, because both tissues are not able anymore to carry out their load-bearing function. The appearance of tissue damage is accompanied by the release of certain pro-inflammatory cytokines. These cytokines are as well capable of altering matrix production in which proteoglycan synthesis is negatively influenced.

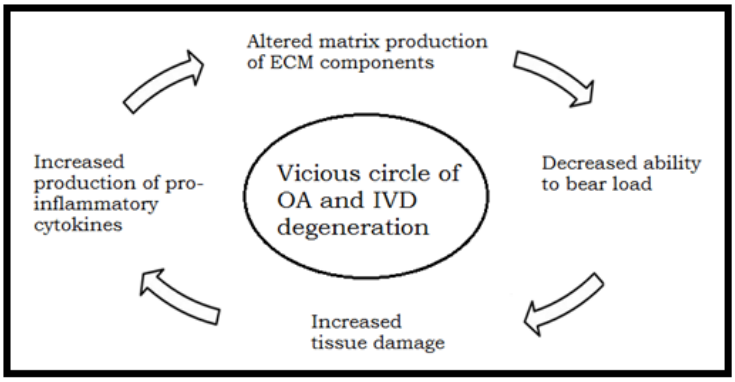

Figure 4: *Short overview of the pathophysiology of OA and IVD degeneration.*

1.2.3. Symptoms and diagnosis

The diagnosis of OA and IVD is based on a combination of patient information and history, affected body part(s), physical tests and radiographic results.

A suspicion of OA or IVD can arise after completing the anamnesis and physical examination. Radiographic results can confirm the diagnosis.

In the anamnesis it may come forward that the dog has a decreased appetite, has an aversion to move, limps a lot, suffers from back pain and shows symptoms of mild paraparesis to paraplegia (1,16,17,30). A history of former trauma that could have led to altered joint biomechanics is an important indication for OA. Furthermore, it is very important to ask the owner the right questions in order to receive all essential information (30).

The anamnesis is followed by a common inspection and then by a specific orthopaedic inspection. It starts with observation of the dog his gait. The gait can be abnormal in which the dog shows evident or not so clearly limping. Though, the dog can also show signs of an inability to walk due to weakening of his lower extremities (17,30).

Regarding the disease OA, it must be kept in mind that animals who suffers from OA will learn to compensate for their pain. Therefore, they can show another way to move (62). Furthermore, there is another symptom that is very characteristic for OA and that is that lameness can also be present in a form of morning stiffness. This can improve with activity, but it can also worsen after energetic activity (30).

Additionally, in both diseases in highly advanced stadia, joint effusion can be seen within or nearby the affected area (30). In addition, a reduced possibility to accomplish different kinds of movements by the affected body part can also be found. And as a consequence of limping or the inability to walk, muscle atrophy can also be observed (14,30). Moreover, manipulation of the affected body part (joint or part of the spine) can confirm that the animal is in pain and/or the presence of crepitation (1,14,30).

Finally, the diagnosis of OA and IVD can be confirmed by radiographic imaging. For this purpose, X-ray photography, Computer tomography (CT) and Magnetic resonance imaging (MRI) can be used.

Most practices are more likely to have an X-ray device, rather than a CT or a MRI device due to the high purchase price. The X-ray can be made immediately from the affected joint in the case of OA. In the case of IVD degeneration myelography was routinely used in the past to visualize the spinal cord in order to see if IVD degeneration and/or subsequently herniation were present (16,17). In the more advanced phases of IVD degeneration, osteoarthritic changes can occur and can also be seen on X-ray (14). These changes are comparable to the alterations that can be seen on an X-ray in the moderate and chronic stadia of OA, for instance: narrowed joint spaces, sclerosis, bone remodelling and osteophyte formation (30).

It is also possible to use CT as a diagnostic tool (16,17,63). It is capable of measuring composition and quality of the cartilage. This correlates with the important matrix substances which are affected in the early beginning of the degeneration phase. This takes place before tissue loss has occurred (63). CT has also the benefit of being very perceptive to alterations in radiographic density. Therefore, it allows calcified material to be more easily observed. Furthermore, it can give a clear description of the location of where the protruded or extruded IVD can be found in relation to the spinal cord (16,17). Though, it is not sensitive enough to detect early degeneration of non-mineralized IVD tissue, in contrary to MRI (16,17).

MRI offers greater anatomic detail of the cartilage and non-cartilage structures, including bone and nerves. Therefore MRI is very appropriate to evaluate the joint or IVD as a “whole

organ" (16,64). Moreover, MRI can also visualize different kinds of disc-associated injuries, such as disc protrusion, and allows early detection of IVD degeneration (16,17).

1.2.4. Current treatments

Even today there is still not a treatment that can cure this disease, so that most used therapies are based on symptomatic treatment. These therapies can be divided into non-surgical and surgical as further explained below.

Non-surgical therapies

Alteration of exercise patterns

For dogs with OA it is essential to exercise frequently (18). This exercise is based on the activity pattern of the animal, because the amount of exercise for a patient fluctuates with the individual and with the severity of the disease (65). However, for dogs with IVD degeneration it is important to restrict the exercise. It is better for the patient to move as little as possible to reduce the risk of continuous herniation of the NP, while the injured AF heals (17).

It is also possible to assign the dog with OA or IVD degeneration for physical therapy. The goals for these treatments are to preserve the mobility of the affected body part and muscle strength while decreasing the additional damage or pain (1,49,65).

Weight loss

Previous experiments has been shown that losing weight in dogs that are obesed and have OA, reduced clinical symptoms associated with OA (42,62,65,66). The loss of weight can be obtained by feeding the dog a special diet (42).

Neutraceuticals

This term is defined as "a food (or part of a food) that provides medical or health benefits, including the prevention and/or treatment of a disease" (67).

These are ω-3 fatty acids, glucosamine and chondroitin sulfate, green lipped muscle and anti-oxidants. However, the latter has only been researched in human OA (68).

A literature review about this topic showed that ω-3 fatty acids has the highest power of proof of efficacy (68).

Non-Steroidal Anti-Inflammatoy Drugs (NSAIDs)

As mentioned earlier, dogs with OA or IVD degeneration can be in severe pain. Therefore, NSAIDs can be prescribed. The following NSAIDs are licensed for chronic use according to Butch KuKanich *et al*. and these include: carprofen, deracoxib, etodolac, firocoxib, mavacoxib, meloxicam, phenylbutazon, robenacoxib and tepoxalin (1,17,49,69).

Corticosteroids

It is also possible to administrate corticosteroids as pain medication, indicated for cases that cannot be dealed with other treatments. It is especially prescribed in conditions in which the animal suffers terribly and in which NSAIDs cannot provide sufficient pain relief (1,6,17).

However, these drugs could have terrible side effects which are a great disadvantage of these medications (65).

Surgical therapies

Another way of treating dogs with OA or IVD degeneration is to perform surgery. The appropriate surgical therapy for the patient depends on his clinical status and it is beyond the scope of this thesis to describe it in detail.

All surgical treatments have as main aim to improve clinical signs associated with these diseases (1,17,49,65).

Joint distraction

This treatment can be applied in dogs with OA and has as main aim to release mechanical stress on articular cartilage. This is done by avoiding articular surfaces to contact during movement by placing an external metal frame. This frame holds the two bones from the affected joint slightly apart from each other (70).

Microfracture

In this treatment the subchondral bone is pierced to stimulate generation of new articular cartilage. It makes use of the idea that recovery could be stimulated by recruiting marrow elements. This treatment can also be used in dogs with OA (71).

Decompression

This comprises treatments that can be used to treat dogs with IVD degeneration. The main aim is to alleviate compression of neural structures that can be present due to herniation of the NP. Decompression treatments are for instance: nucleotomy, dorsal laminectomy or ventral slot procedure (1,17,49).

1.2.5. Prognosis

When dogs are diagnosed with OA or IVD their prognosis is not favourable. As mentioned above there isn`t a cure for both diseases but only symptomatic which is part of palliative maagement. This treatment can only reduce pain and slow down the progress of the disease.

Though, recently it has been suggested that mesenchymal stem cells (MSCs) and CLCs could be promising in developing a regenerative treatment for both diseases, thereby changing the prognosis of dogs diagnosed with OA and IVD in the future (1,49,72,73).

1.3 Regenerative medicine

Due to the impressive characteristics of extracellular matrix that can be found in the articular joint and IVD, it is difficult to develop a treatment that could cure these diseases. Due to the altered production of matrix substances and subsequently tissue damage, biomechanical properties change tremendously, making the tissue even more prone for further damage (1,6).

In order to develop a regenerative treatment that could stop these diseases from progressing, the diseases should be tackled in their initiation phase. In the case of OA this could be done by for instance correcting the altered production of matrix substances by damaged chondrocytes, implanting “new healthy” cartilage tissue containing healthy chondrocytes and/or mesenchymal stem cells (74,75). However, this approach is very challenging, because it is currently still very hard to diagnose this early phase in dogs. Especially, due to the fact that dog-owners only come when their dogs already show

symptoms and this often means that the disease is already advanced. In the case of IVD degeneration this could be done by correcting the altered cell population found in the NP.

In order to accomplish recovery, research mainly focuses at present on using specific cell types in combination with certain growth-factors. In the case of OA a lot of studies have been performed on using mesenchymal stem cells (MSC) as potential cell source in order to obtain "new healthy" cartilage tissue *in vitro* (20,76,77). This is partly due to the fact that if chondrocytes are used as potential cell source, cartilage has to be harvested from the animals. Thus, low cell numbers are obtained. Furthermore, the isolated chondrocytes also have a limited lifespan and proliferative capacity. Interestingly, MSCs can overcome these limitations and are therefore a better potential cell source to use for research (75,78). MSCs are multipotent stem cells and have the capacity to differentiate into chondrocytes, osteocytes and adipocytes under the influence of specific growth-factors, such as TGF-β1 and BMP-2 (76,79,80). With respect to IVD degeneration, the transition of NCs into CLCs should be reversed. However, little is known about the responses of CLCs cultured *in vitro*. For instance, it is not known if there is any difference in response to certain growth-factors that are able to upregulate ECM synthesis, between CLCs derived from CD- and NCD-dogs (54). Therefore, very first differences in response to these growth-factors between these two cell populations needs to be investigated *in vitro*. This should be done in order to obtain a better understanding of what the consequences are from the transition of NCs into CLCs regarding the metabolic activity of these cells and with respect to CLCs derived from CD- or NCD-dogs.

In the first paragraph more information about these cell types is discussed, followed by an explanation of the mechanisms of action regarding to TGF-β1 and BMP-2 in the second paragraph.

1.3.1 MSCs and CLCs

MSCs are multipotent stem cells and are originally derived from totipotent stem cells. Totipotent stem cells are undifferentiated cells that can be found in early embryonal tissues after fertilization. They have the ability to form all cells that are necessary to develop a viable organism, including cells that form the placenta or umbilical cord. During embryogenesis, further cell divisions of totipotent stem cells give rise to pluripotent stem cells, forming the inner cell mass. These cells have the ability to form all cell lineages derived from the three embryonic germ layers: mesoderm, ectoderm and endoderm. After these cells are devoted to one of these three germ layers, they are called multipotent stem cells (81,82), such as MSCs. MSCs are derived from pluripotent stem cells which are devoted to the mesodermal germ layer and can be isolated from different tissues, for instance: bone marrow, adipose, neural, hepatic, umbilical cord and also from hematopoietic tissues (82,83).

MSCs isolated from bone marrow, are called bone-marrow derived mesenchymal stem cells (BMSCs) and have a non-hematopoietic origin. These cells can differentiate into cell lineages derived from the mesodermal germ layer, such as bone, cartilage, adipose, fibrous connective tissue and muscle cells (figure 5) (82).

Nevertheless, stem cells have more abilities than only to give rise to certain cell lineages. They also have self-renewal capacity and in some tissues they replace injured or dying cells. However, they are as well able to secrete paracrine factors which can stimulate the restoration of damaged tissue (84).

MSCs are described based on three inherent properties which separate these cells from other cell types. These properties correspond with the requirements suggested by the International Society for Cytotherapy, and these include (79):

- They must adhere to plastic under standard culture conditions.
- They must express positively the following surface markers: CD105, CD73, CD90 and negatively: CD45, CD43, CD14 or CD11b, CD79α or CD19 and HLA class II (79,85). Though, the CD90 expression could be lower than expected. Human studies have shown that there is a strong expression of CD90, but there could be differences in the expression of CD90 between species. It is known that decreased expression of CD90 could be influenced by cell passage number. In early passage all cells exhibit a variable expression of CD90 and this decreases with increasing cell passage number (86).
- They are able to differentiate into cells derived from mesenchymal cell lineages, including osteocytes, adipocytes and chondrocytes (figure 6) (79,85).

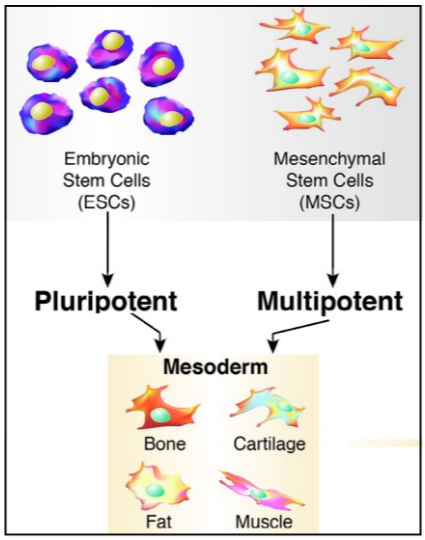

Figure 5: *Schematic example of pluripotent and multipotent stem cells.* Pluripotent stem cells devoted to mesodermal lineage are called multipotent mesenchymal stem cells. These cells have the ability to differentiate into osteocytes, chondrocytes, adipocytes and myocytes (81). Modified by MCME Müller.

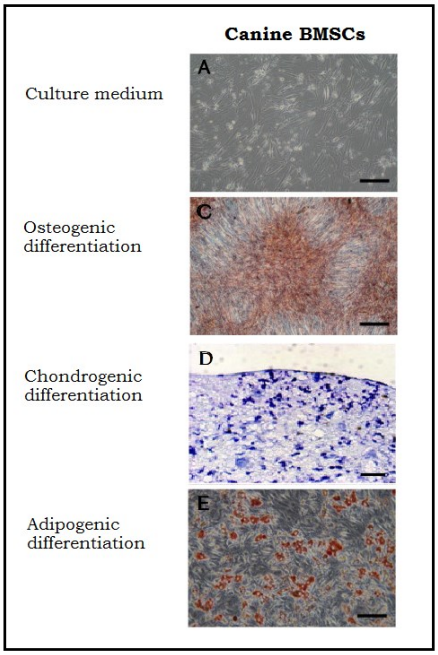

Figure 6: *Expansion and osteogenic, chondrogenic and adipogenic differentiation of canine BMSCs.* BMSCs cultured in expansion medium (**A**). Evident osteogenic differentiation by von Kossa staining (**C**); chondrogenic differentiation by Toloidine blue staining (**D**); and clear adipogenic differentiation by Oil Red O staining (**E**). Scale bars A-C-E 200 µm; D 20 µm.
Compiled figure from Hodgkiss-Geere *et al* and Takemitsu *et al* (23,86). Modified by MCME Müller.

The advantages of using MSCs in research are that they can be easily isolated in large quantities. Furthermore, they are simple to expand due to their proliferative capability in contrary to chondrocytes (5,75,78). Moreover, it appears that chondrocytes differentiated from MSCs can synthesize ECM components that are comparable to that of hyaline cartilage. For this phenotype, it is essential that these cells are cultured in a three-dimensional cell culture (pellet). The rationale behind it is that a pellet can provide the conditions that are similar to the ones that are found in the condensation phase during embryogenesis (87,88). Furthermore, a pellet consists of high cell density, providing better

settings for cell-to-cell interactions. These interactions are necessary in order to obtain the condensed condition which precedes chondrogenic differentiation (88-90).

BMSCs and adipose-derived MSCs (ADMSCs) are the two main MSC-sources that are used for cell therapy (5,87). This is because of the fact that they have good proliferation capability and they are easy to obtain, although, more MSCs could be obtained from adipose tissue rather than from bone-marrow (5,87). However, research showed that BMSCs undergo a more thoroughly chondrogenic differentiation in comparison to ADMSCs, because BMSCS present a more mature phenotype after the induction of chondrogenesis (87,91-93). Therefore, bone-marrow provides a better MSC-source to use in orthopaedic research.

As stated before, in the case of the IVD, during aging the NC-population becomes replaced by a cell-population that resembles articular chondrocytes, namely chondrocyte like cells (CLCs). CLCs are smaller than NCs (17-23 µm) (1,36,37) and are found solitary or in clusters, consisting of 4 to 6 cells in the NP (34). These cells show a different kind of stained actin skeleton (figure 7) and a decreased expression of gap junction protein. They also do not exhibit any signs of vesicles in comparison to NCs (34). In addition, CLCs are enclosed by lacunae that are not found within the NC-population (94).

The importance and mechanism leading to the development of these lacunae are still unclear. Though, there are some speculations regarding this. For instance, it could be a sign of the cells` influence on its pericellular environment (94).

The mechanism of NC-replacement by CLCs has still to be elucidated (54). However, research has shown that CLCs are also derived from notochordal lineage. T-box gene *brachyury* expression by CLCs is being largely investigated. This gene is necessary for NC differentiation and survival. Indeed, Risbud *et al*. showed that the CLCs express brachyury in the NP (36). The secretion of the protein *Sonic hedghe hog* (SHH) is being also under the scope, because it is necessary for normal disc development. Interestingly, Choi *et al*. showed that CLCs are derived from SHH-exhibiting NCs (95).

The shift in the residential cell-population of the NP is associated with early development of IVD degeneration (1,36). As mentioned earlier, dog breeds can be divided into CD- and NCD-dog breeds. In CD-dog breeds the replacement of the NC-population is completed when they have reached one year of age and in NCD- dog breeds this is finished when they have reached middle to old age (1,17,48).

As can be seen from figure 7, there also appear to be differences in morphology between CLCs derived from CD- and NCD-dogs. Consequently, it could be that these cells also respond differently *in vivo* in terms of metabolism.

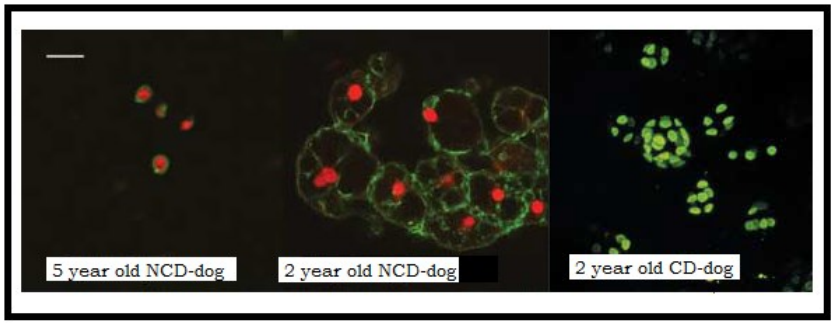

Figure 7: *Cytomorphology of CLCs and NCs.* CLCs derived from a 2 year old NCD- and CD-dog (resp. left and right) and NCs derived from a 2 year old NCD-dog (in the middle). Red staining = nuclei; green staining = actin; scale bar = 20 µm (53). Modified by MCME Müller.

1.3.2 Growth-factors TGF-β and BMP-2

In this project, two cell types are used: BMSCs and CLCs. BMSCs are multipotent stem cells and still need to differentiate before they are able to produce cartilage associated substances. The differentiation process for BMSCs occurs in two phases: condensation of stem cells followed by the actual differentiation process (2). CLCs are already differentiated and therefore they only need to be kept in their differentiated state in order to produce ECM components (1). In order to achieve this, growth factors are needed to be added to the culture. The growth-factors that are commonly used to achieve this purpose *in vitro* are transforming growth factor bèta (TGF-β) and bone morphogenetic protein (BMP) (2,49). For this project, TGF-β1 and BMP-2 are used. The following section will explain why there has been chosen to use these specific growth-factors. As well as how they induce differentiation in order to obtain cells that produce cartilage matrix associated substances and how they induce the synthesis of this matrix.

In the condensation process cell-to-cell contact is very important for mesenchymal stem cells in order to initiate differentiation. From research it is known that during this process ATP oscillations play a hugh role. These oscillations should induce the stem cells to secrete high amounts of adhesion molecules which take care of the enhancement of cell-to-cell contact and this would promote cellular aggregation of these cells (96). These oscillations are only induced by TGF-β1. BMP-2 is also present, however it appears that BMP-induced chondrogenesis in the early limb mesenchyme is reliant on preceding activation by the growth-factor TGF-β (97,98). Though, BMP-2 is able to encourage cellular condensation (2,97). Therefore, it could be assumed that TGF-β1 initiates the prechondrogenic condensation process in the early phase of chondrogenic differentiation and that BMP-2 is not sufficient to initiate this process, but can promote the cellular condensation in terms of growth in the ensued phases of chondrogenesis (97).

The chondrogenic differentiation process is initiated through specific growth factors. These factors are members of the transforming growth factor-Bèta (TGF-β) family, fibroblast growth factors (FGFs), insulin-like growth factors (IGFs) and the wingless-type (WNT) signalling pathway (2).

In the performed experiments, growth-factors that belong to the TGF-β family are used and are subtypes of TGF-β and BMP. TGF-β is subdivided into three subtypes: TGF-β1, -β2 and -β3. TGF-β1 is preferentially used in regenerative cartilage medicine (99). BMPs are a very large family and the most common used BMPs are BMP-2, BMP-4, and BMP-7. These BMPs are found during embryonic limb development and are able to induce chondrogenesis (100). Though, it appears that BMP-2 is most essential for skeletal development (101).

Both TGF-β1 and BMP-2 induce extracellular matrix production by specific signalling.

<u>TGF-β1 signalling</u>

Growth-factor TGF-β1 needs two receptors in order to transduce its signal into the cell. In healthy BMSCs and CLCs, these two receptors are principally known as type I (TGF-βRI) and type II (TGF-βRII). TGF-β1 binds to TGF-βRII which is an active kinase. This receptor then phosphorylates and activates TGF-βR1, which is the second step of the TGF-β signalling pathway. This receptor is also primarily known as activating receptor-like kinase-5 or ALK-5 (102). Though, TGF-β1 can also signal through another TGF-βR1: activating receptor-like kinase-1 (ALK-1). ALK-1 activation will result in phosporylation of the Smad 1-5-8 pathway,

which is commonly better known as the pathway activated by BMPs (103). Therefore, this pathway will be further described in the information about BMP-2 signalling.

As showed in figure 8, binding of TGF-β1 to TGF-βRII, will lead to phosphorylation and activation of ALK-5. In turn, ALK-5 phosphorylates Smad 2 and Smad 3 and allowing them to bind to Smad 4. This complex is thereafter transferred to the nucleus where it can interact with a diversity of co-activators, co-repressors and transcription factors (102). In this manner, it activates the transcription of transcription factor Sox9 (104). Sox9 is the master regulator of chondrogenic differentiation and is essential for ECM specific gene expressions, such as collagen type 2α1 (COL2α1) and aggrecan (105,106). Hereafter, other members of the SOX-family are also transcribed: the Long-form of Sox5 (L-Sox5) and Sox6 (107).

Sox9 binds to the Smad-complex and is subsequently capable under influence of Smad 3, to recruit the transcriptional co-activator complex: CREB-binding protein/p300 (CBP/p300). Hereafter, Sox9 is able to associate with the COL2α1 enhancer which contains a Sox9-binding site (27,104). Though, binding of the complex L-Sox5/Sox6 is also necessary to secure binding of Sox9 to the COL2α1 enhancer (106,107). Therefore, the expression of COL2α1 is almost parallel to that of Sox9 (104). However, not only COL2α1, but also the gene aggrecan contains a Sox9 binding site. In order for Sox9 to be able to bind and to activate the aggrecan enhancer, binding of L-Sox5/Sox6 is also necessary (107,108).

Moreover, for the transcription of aggrecan not only the Smad-dependent activation of Sox9 is able to induce transcription. Beside Smad-pathway activation, TGF-β1 is also able to activate more different pathways, such as the mitogen-activated protein kinase (MAPK) pathway. The transduction via MAPK involves for instance extracellular signal-regulated protein kinase 1/2 (ERK 1/2) and p38 kinase. According to Li *et al.* ERK 1/2 and p38 kinase are at the same time active as Smad signalling and both are involved in regulating aggrecan expression. Although, the importance of the activated pathways varies in time. In early activation by TGF-β1 Smad signalling, ERK 1/2 and p38 kinase MAPK pathways play an important role in regulating Aggrecan expression. Although, after prolonged TGF-β1 treatment, aggrecan expression seems to be more dependent on p38 MAPK and ERK 1/2 activation (109,110).

BMP-2 signalling

Growth-factor BMP-2 also needs two receptors in order to transduce its signal into the cell. These receptors are known as type I receptors and comprise of ALK-2, ALK-3 (BMPRIa) and ALK-6 (BMPRIb) (111). BMP-2 preferentially binds to ALK-3 and ALK-6 and it has been shown that these receptors may transmit different signals (112,113). It appears that both receptors are capable of inducing chondrogenesis, although ALK-6 is more important than ALK-3 (26,113). The second receptor is known as type II receptors and comprise of BRII, ActRIIa and ActRIIb (111). After binding of BMP-2, receptor type II are being activated which in turn phosphorylates and activates receptor type I. BMP-2 can activate Smad pathway, but also p38-mitogen-activated protein kinase (p38-MAPK). The activation depends on whether the receptor complexes are preformed or not (112,114).

Binding of BMP-2 to preformed receptor complexes initiates Smad-signalling (112,114). Therefore, phosphorylated type I receptors phosphorylate and activate Smad 1-5 and 8. These activated Smads then bind to Smad 4, a Co-Smad, and will be transferred into the nucleus. There it can modulate gene transcription by interacting with transcription factors, co-activators and co-repressors (figure 8) (112). One of the genes that will be described in this manner is Sox9 (115). Though, binding of BMP-2 to non-preformed receptor complexes

results in activation of p38-MAPK which also results in Sox9 transcription (114,115). The activation of p38-MAPK results in increased binding of the transcription activator complex NF-Y/p300 to the promoter of the Sox9 gene (2,115). The effect of Sox9 regulating chondrogenesis and stimulating ECM specific gene expressions, such as COL2α1 and aggrecan has already been described above.

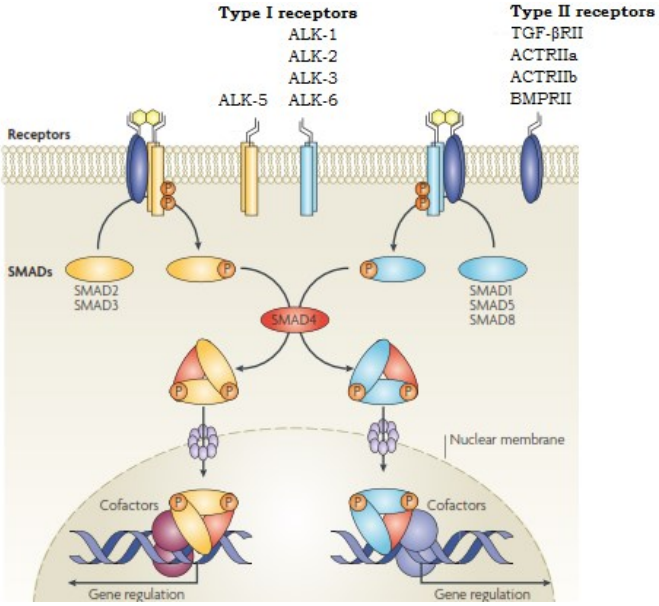

Figure 8: *TGF-β and BMP signalling through the canonical Smad-dependent pathway*. This figure shows how both growth-factors are able to activate different Smad-proteins which then bind to Smad 4. This leads to nuclear translocation of the formed complex which can modulate gene transcription. Original figure obtained from Schmierer *et al*. (121). Modified by Margot Müller.

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Chapter 2: Chondrogenic differentiation of Canine Bone-Marrow derived Mesenchymal Stem Cells and matrix production by Canine Nucleus Pulposus Cells

2.1 Introduction

In this chapter all scientific reports can be found about the performed experiments. The reports are put in order based on the used cell population; (a) Canine Bone Marrow derived Mesenchymal Stem Cells (BMSCs) followed by (b) Canine Chondrocyte-Like Cells (CLCs).

(a) <u>Canine BMSCs</u>

Rationale: BMSCs are a potent cell population to use in regenerative research towards osteoarthritis which is characterized by loss of cartilage components. These stem cells have the characteristics to be easily obtained from bone-marrow and to be easily expanded *in vitro* (1-3). Furthermore, they have the potency to be differentiated towards chondrocytes in which they are capable of producing cartilage extracellular matrix (ECM) components (4,5). Therefore, BMSCs are a potential cell source to obtain these components *in vitro*. These components combined with chondrocytes derived from differentiated BMSCs, could be used to develop a regenerative treatment for osteoarthritis. However, currently it is not known yet how to obtain the most successful differentiated canine BMSC-culture *in vitro* which has the highest production of these ECM components. Therefore, the first three experiments concentrated on protocol optimization for obtaining chondrogenically differentiated BMSCs.

Approach: In the first experiment it was investigated how long it is needed to add Transforming Growth Factor-Bèta 1 (TGF-β1) to the cell culture consisting of canine BMSCs, in order to successfully induce chondrogenesis. The results from this experiment led to a better understanding of the importance of the expansion phase of canine BMSCs, which precedes the chondrogenic differentiation phase in which TGF-β1 was added. This information led to the purpose of the second experiment: to investigate the influence of different kinds of fetal calf serum (FCS) received from different manufacturers: Hyclone (SV30160.03), Lonza (DE-14-801F) and Gibco High Performance (16000-044) combined with the influence of colony forming units, in the expansion phase. This phase was followed by the chondrogenic differentiation phase in which TGF-β1 was added according to the different media changing schedules corresponding to the first research. This experiment has provided notable results which showed that the FCS derived from the three different manufacturers combined with colony forming units, did not negatively influence the chondrogenic differentiation potential. Pellets from all conditions were able to differentiate into cartilage producing cells. However, still not all donors (one out of two) had responded. This led to the suggestion that some canine BMSC-donors may need an additional stimulus in order to achieve successful chondrogenesis of more donors. Therefore, another experiment was performed in which the (additive) effect of Bone Morphogenetic Protein-2 (BMP-2) on TGF-β1 was explored.

(b) <u>CLCs</u>

Rationale: During intervertebral disc degeneration (IVD degeneration), the inherent cell population of the nucleus pulposus is replaced by chondrocyte-like cells (CLCs). This replacement is associated with early development of IVD-degeneration (6,7). CLCs derived

from Chondrodystrophic (CD-) and Non-Chondrodystrophic (NCD-) dog breeds are capable of producing ECM components, resembling to those produced by chondrogenically differentiated BMSCs (8). However, given that the clinical presentation of intervertebral disc degeneration differs between these two groups (9,10), it could be that there are differences between CLCs derived from CD- and NCD-dog breeds when cultured in vitro under the influence of growth factors TGF-β1 and BMP-2.

Approach: In this experiment we investigated the response of CLCs derived from CD- and NCD-dog breeds *in vitro* when cultured under the influence of TGF-β1 and BMP-2, as well as if there are differences in response between these cell-populations derived from CD- and NCD-dog breeds.

2.1.1. References

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2.2 *Reports of the performed experiments*

PROTOCOL OPTIMIZATION OF CHONDROGENIC DIFFERENTIATION OF CANINE BONE-MARROW DERIVED MESENCHYMAL STEM CELLS:

The influence of different media changing schedules containing TGF-β1

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Abstract

Introduction: Osteoarthritis is diagnosed in about 20% of the adult dog population and is often a secondarily result of changed joint biomechanics. It is characterised by a vicious circle of cartilage damage, combined with an inadequate repair response, resulting in loss of extracellular matrix (ECM) components. In order to regain these components and to breakthrough this circle, bone marrow derived stem cells (BMSCs) could be used by chondrogenically differentiating them towards cartilage producing cells. It has already been shown that chondrogenesis can be achieved by supplementing chondrogenic medium with transforming growth-factor beta 1 (TGF-β1). Though, it is not known yet what the best media changing scheme is for supplementing TGF-β1 to the BMSC-pellet-culture in order to induce chondrogenesis most successfully. Therefore, the aim of this experiment was to define the optimal media changing scheme of supplementing TGF-β1 to the BMSC-pellet-culture.

Methods: BMSC-pellets were cultured with either solely chondrogenic media or supplemented with TGF- β1 (10 ng/mL) according to three different media changing schemes in order to induce chondrogenesis. Samples were subjected to biochemistry assays for the quantification of glycosaminoglycan (GAG) and DNA content, and to histology.

Results: Outcomes of GAG and DNA quantification and the extracted GAG per DNA content, did not show clear differences between the control group and TGF-β1 groups, which was supported by histology.

Conclusion: The results obtained from all groups after three weeks of culture suggested that no chondrogenic differentiation had occured.

Introduction

Osteoarthritis (OA) is a chronic progressive disease and is diagnosed in about 20% of the adult dog population (1-3). It is very painful and therefore the most common reason of pain and limping in dogs (2-4).

OA often results secondarily as a consequence of changed joint biomechanics which is regularly caused by a combination of risk factors (3,5-8). The disease starts with initial damage of articular cartilage, resulting in loss of extracellular matrix components, such as

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collagen type II and proteoglycans. This damage leads also to the release of cartilage degradation products into the articular joint and subsequently to the stimulation of immune responses (3,4,9). The cartilaginous tissue is poorly vascularised and contains low cell content (9,10). These cells, chondrocytes, produce in the onset of OA more cartilage matrix, but this is reversed in later stages in which they are significantly injured and as a result they lose their ability to regenerate matrix. This results in an altered production in which the cells will produce mostly matrix metalloproteinases (MMPs), through which they will contribute to matrix damage as well. Currently, there is no cure to breakthrough this circle and thus the disease will reach the chronic state (3,4,9).

In order to regain these matrix components, such as collagen type II and proteoglycans and to breakthrough this vicious circle, scientists are trying to develop a regenerative treatment by using canine bone marrow-derived mesenchymal stem cells (BMSCs), they are trying to cure the present cartilage defect (10-12). However, before this treatment can be applied *in vivo* researchers first have to investigate how to obtain this matrix *in vitro* from canine BMSCs. In order to obtain this matrix, it is possible to chondrogenically differentiate these stem cells. It has already been shown that this can be achieved by supplementing chondrogenic medium with transforming growth-factor beta 1 (TGF-β1) (13-15).

TGF-β1 is an important growth-factor and upon receptor binding, it activates the Smad-pathway in which Smad-2 and Smad-3 are activated. These proteins will activate and form a complex with Smad-4, a co-Smad. This complex is translocated into the nucleus where it will induce transcription of transcription factor Sox9 (16-18). This factor is the master regulator of chondrogenic differentiation and is essential for ECM specific gene expressions, such as collagen type 2α1 (COL2α1) and aggrecan (16,19,20).

However, it is not known yet what the best scheme is for supplementing TGF-β1 (10 ng/mL) to chondrogenic media in order to obtain a chondrogenically differentiated canine BMSC-culture. Therefore, our aim was to define the optimal media changing scheme to obtain the most successfully chondrogenically differentiated canine BMSC-culture after three weeks of pellet-culture. We focused on glycosaminoglycan (GAG) content which are part of proteoglycans; DNA content in order to determine proliferation rate and for normalizing GAG content for the amount of present cells; and finally, histology.

Materials and methods

Bio-Bank and cell expansion

Canine BMSCs were obtained from 6 donors from the Bio-Bank of the Faculty of Veterinary Medicine of Utrecht. Donor breeds were Beagle (3 donors), Labrador (1 donor) and mixed breed (2 donors). Their age ranged from 4 to 40 months (Table 1). BMSCs were thawed in passage 0 or passage 1 and expanded in expansion media, containing minimum essential medium α (α-MEM; Invitrogen, 22561-021), 10% fetal calf serum (FCS; Gibco High performance, 16000-044), 1% penicillin-streptomycin (PPA cell culture company, P11-010), 1 ng/mL fibroblast growth factor (AbD Serotec, PHP105), 0.1 mM ascorbic acid 2-phosphate (Sigma Aldrich, A-8960) and 0.2% fungizone (Invitrogen, 15290). Cells were seeded with a density of 2-3 million cells per 175 cm^2 flask (Greiner Bio-One, 660175). Cells were transferred to new flasks with a density of 1,5-5,0 million cells per 175 cm^2 flask, when subconfluency was reached. Therefore, BMSCs were washed with Hanks balanced salt solution (HBSS; Gibco by Life technologies, 14025-050) and detached by trypsin (Triple Ex; Gibco by Life technologies, A12177-01). Cells were counted by using a cell counting device

(Bio Rad TC 20, Catalog #145-0101). During expansion, medium was replaced twice per week and cells were cultured under normoxic conditions, 5% CO_2 at 37°C.
Passage 3 cells were prepared to form pellets consisting of 200,000 cells when subconfluency was reached. Cells were washed with HBSS and detached by trypsin. Hereafter, expansion medium was added to inactivate trypsin by the presence of FCS. Cells were centrifuged, followed by draining away the expansion media. Thereafter, chondrogenic media, containing Dulbecco's Modified Eagle's Medium, high glucose, GlutaMax™ pyruvate (DMEM; Invitrogen, 31966), 1% selenous acid (ITS+) premix (B&D, 354352), 1% penicillin-streptomycin (PPA cell culture company, P11-010), 0.04 mg/mL L-prolin (Sigma Aldrich, P-5607), 0.2% fungizone (Invitrogen, 15290), 0.0001 mM dexamethasone (Sigma Aldrich, D8893) and 0.1 mM ascorbic acid 2-phosphate (Sigma Aldrich, A-8960), was added. Cells were homogenized and counted by using a cell counting device. Subsequently, pellets were formed in which donor cells were unintentionally plated as pellets in a 96-wells-plate (Costar, 7007). Pellets were divided into two groups: control group receiving basic chondrogenic media three times per week, and three TGF-β1 groups. Pellets were cultured under normoxic conditions, 5% CO_2 at 37°C.

Donor number	Dog breed	Age
152	Beagle	39 months
154	Beagle	40 months
754	Beagle	32 months
652	Mixed-breed	16 months
520	Mixed-breed	15 months
445	Labrador	4.3 months

Table 1: Information about included BMSC donors.

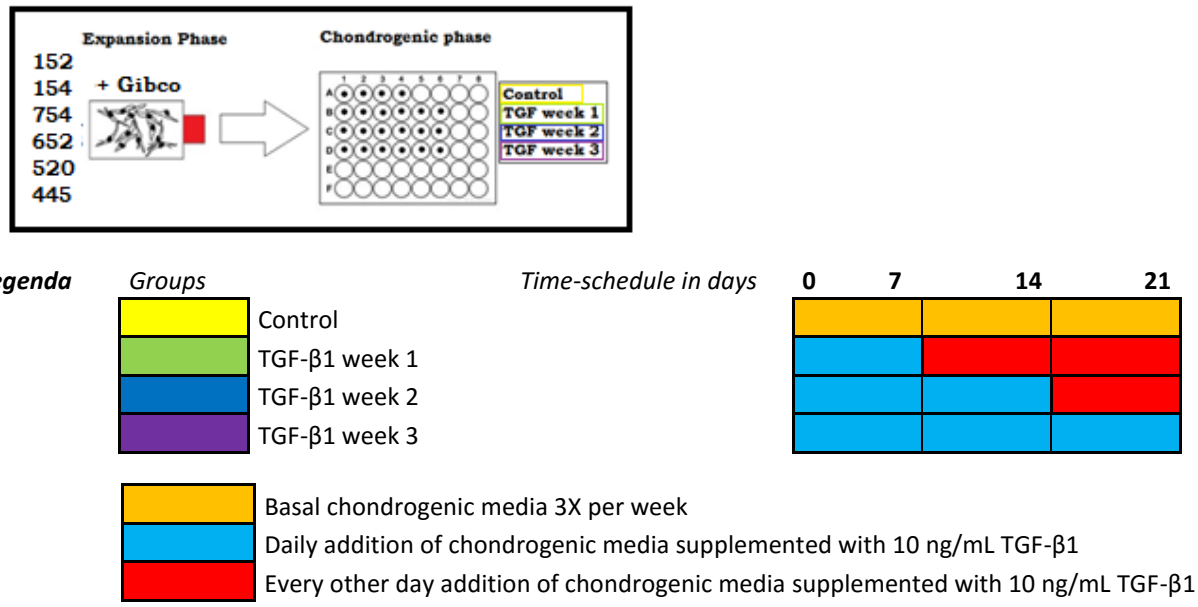

Figure 1: Experimental design. All donors were first expanded with FCS derived from the manufacturer Gibco. Thereafter, pellets were formed from all donors and were unintentionally plated in one of the four possible groups: control, TGF-β1 week 1, TGF-β1 week 2 or TGF-β1 week 3. All groups received chondrogenic media and the TGF-β1 groups also received TGF-β1 according to different time-schedules.

Microscopy

During the expansion and chondrogenic differentiation phase, cells were observed microscopically in order to get a good overview of the status of the cells. During the expansion phase, we observed the presence of colony forming units (CFUs). These units are a trait of mesenchymal stem cells which can be seen throughout this phase (21,22). During the differentiation phase, the appearance of all pellets and corresponding media was studied.

In-vitro chondrogenic differentiation

Chondrogenesis was established with chondrogenic media supplemented with 10 ng/mL TGF-β1 (R&D systems, 240-B-010) according to different time schedules.

During this experiment (Fig. 1) the *control* group received basic chondrogenic media and media was changed three times per week. The TGF-β1 groups received for three weeks chondrogenic media supplemented with TGF-β1, from which *TGF-β1 week 1* media was daily changed for one week and then every other day for two weeks; *TGF-β1 week 2* media was daily changed for two weeks and then every other day for one week and *TGF-β1 week 3* media was daily changed for three weeks.

Biochemistry

GAG quantification

The glycosaminoglycan (GAG) content was quantified as a first control to identify if pellets were chondrogenically differentiated after the three weeks of culture. This was performed with the dimethylmethylene blue (DMMB) assay in which the colour component associates with the repeating negative charges (sulphates) present on the GAGs. Hereafter, a change in absorption spectrum can be measured (23).

For this assay, pellets (N = 3 per group) were digested in a papain digestion solution, comprising of 250 µg/mL papain (Sigma Aldrich, P3125) in papain buffer (pH 6.0) containing 31.3 µg/mL mononatriumdiwaterfosfaat-2-hydraat, 3.26 µg/mL ethylenediaminetetraacetic acid (EDTA) and 1.57 mg/mL L-Cysteine hydrochloride monohydrate (Sigma Aldrich, C7880) and were incubated overnight at 60°C. Next day, pellets were shortly vortexed and put back in the incubator at 60°C for another half an hour. Hereafter, samples were prepared to be measured undiluted.

Firstly, a standard curve was made by pipetting 50 times diluted chondroitin sulphate (CS; Sigma Aldrich, C4384; stock: 0.5 mg/mL) and phosphate buffered saline - EDTA (PBS-EDTA) in a 96-wells-plate (Greiner Bio-one, 655191). The standard curve contained end concentrations ranging from 0 to 10.0 µg/mL CS. Then 100 µL of sample was pipetted into the wells-plate. Hereafter, 200 µL of DMMB-solution (pH 3.0) was added. The DMMB-solution contains per 250 mL: 4 mg DMMB powder (Sigma Aldrich, 341088-1G) dissolved in 1.25 mL 100% ethanol and incubated for 2-16 hours, 0.59 g natriumchloride and 0.76 g of glycine. The spectrophotometer was used to measure the absorption rates by using 540 and 595 nm wavelengths. Prepared samples were measured in duplo and averaged. Results were extrapolated from the standard curve and both were corrected for the blank. The blank corresponds with the first outcome of the standard curve (0 µg/mL CS). Finally, absolute GAG (µg) content for each pellet was calculated.

DNA quantification and GAG per DNA determination

The digested pellets were further used for DNA quantification. Samples were prepared with Qubit™ dsDNA BR Assay Kit (Invitrogen, Q32853) and results were measured by Qubit® 2.0 Fluorometer (Invitrogen, Q32866).

A calibration curve was made according to the manufacturer (Invitrogen). Hereafter, samples were measured 20 times diluted. Absolute DNA (µg) content for each pellet was calculated. Finally, absolute GAG per DNA (µg/µg) content was determined with help from the absolute GAG (µg) per pellet content.

Histochemical staining

Histochemical staining was performed as a second control to determine if BMSCs had successfully differentiated towards the chondrocytic lineage. On day 21 pellets for histology (N = 2 per group) were harvested and fixated overnight with buffered neutral formalin containing 1% eosin at room temperature (±19 degrees Celsius). Next day, pellets were embedded in 2.4% alginate (Sigma Aldrich, A2033), containing 2.4 g of alginate dissolved in 100 mL HBSS, and formalin with 102 mM $CaCl_2$, containing 1.5 g calciumchloride-2-hydrate, 90 mL Mili-Q and 10 mL formalin. Embedded pellets were transferred between two blue biopsy pads into white embedding cassettes. Cassettes were stored in 70% alcohol. Samples were dehydrated through graded alcohol and xylene steps. Thereafter, pellets were embedded in paraffin and cut into 4µm sections. Sections were stained with Mayer`s Hematoxylin (J.T.Baker, 3870; diluted 1:1 with Mili-Q), Safranin-O (Sigma Aldrich, 58884; 0.125 % Safranin-O, dissolved in Mili-Q) and Fast Green FCF (Sigma Aldrich, F7252; 0.4 %, solved in Mili-Q). Mayer`s hematoxylin stains the nucleus of the cell blueish. Safranin-O stains GAGs in cartilage matrix, orange to red and fast green is used as a background staining and stains the rest of the pellet blue-greenish (24).

Sections were first deparaffinised through xylene and graded alcohol steps and hydrated to distilled water. Then sections were placed in citrate buffer for 10 minutes on a rocker (38 oscillations/minute) to remove alginate. Thereafter, sections were put in Mayer`s Hematoxylin for 10 seconds, followed by washing with distilled water until a clear appearance of the water was seen. Henceforth, sections were stained ones with Fast Green for 4 minutes, twice with 1% Acetic acid (1 mL Glacial acetic acid in 99 mL Mili-Q) for respectively 3 and 2 minutes and once with Safranin-O for 7 minutes. Lastly, sections were dehydrated through graded alcohol (2X 96% and 100% for 3 minutes) and xylene (2X 5 minutes) steps and mounted. Thereafter, images were made with a digital microscopic camera (Leica, DFC425C).

Statistical analysis

No statistical analysis was performed, because no chondrogenic differentiation had occured.

Results

Microscopy

During the expansion phase no CFUs were observed and the pellets looked in good health throughout the chondrogenic differentiation phase. Moreover, there was a clear difference in size between the different groups in which pellets from the control group were the smallest.

GAG and DNA quantification and GAG per DNA determination

The highest measured GAG and DNA content (Fig. 2) was slightly above 2.0 µg per pellet. The highest GAG per DNA content (Fig. 2) was between 1.5 and 2.0 µg/µg and originated from a control sample.

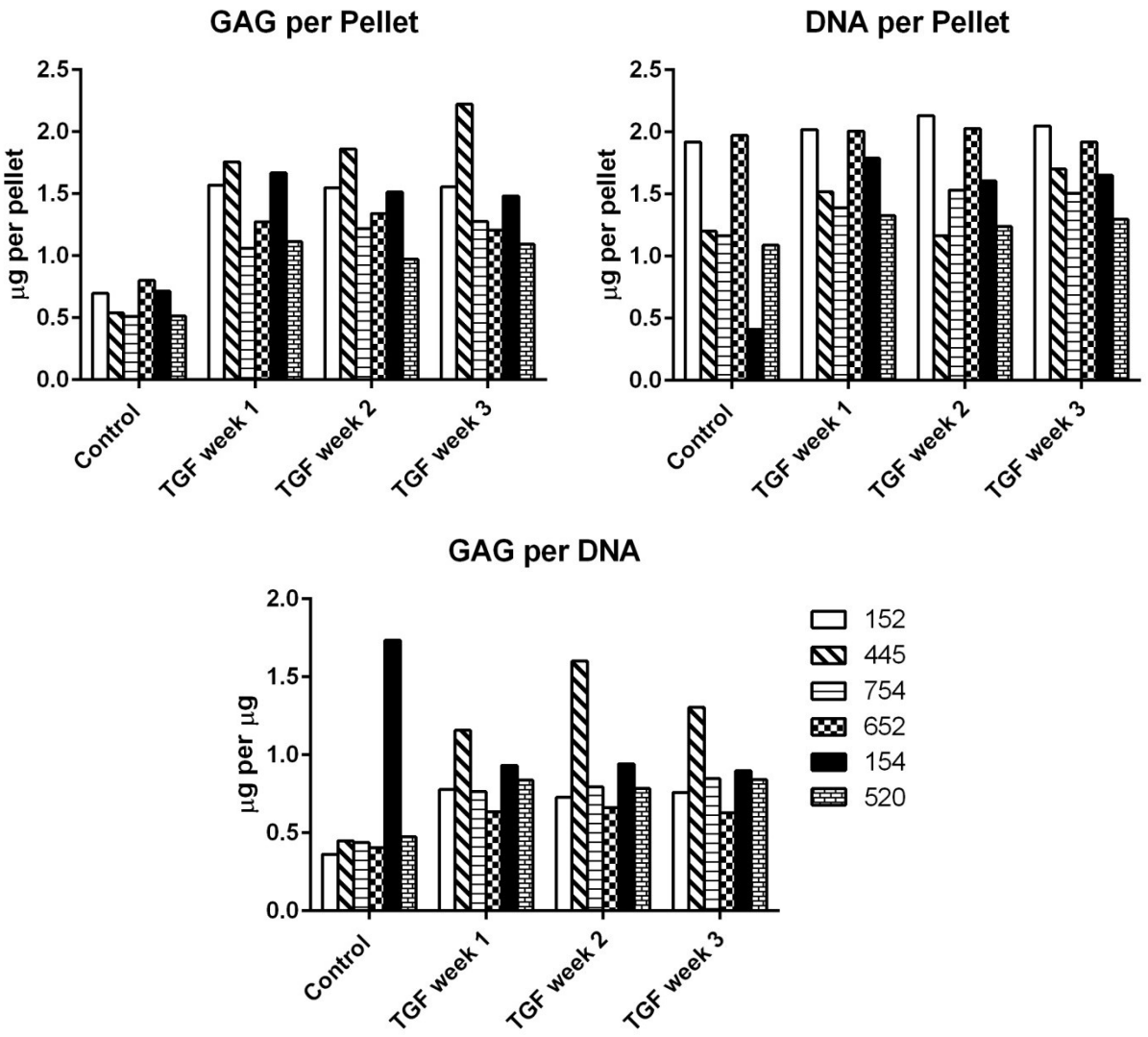

Figure 2: Results of the GAG- and DNA-assay performed on pellets derived from different donors and conditions on day 21. Absolute values are shown. Legend indicates the presentation of each donor in the graphs.

Histochemical staining

Mayer`s Hematoxylin and Fast green staining were present, while none of the donors stained positive for Safranin-O, that specifically stains GAG-rich matrix.

Discussion

The obtained absolute GAG per pellet and absolute GAG per DNA (Fig. 2) was very low after three weeks of culturing for what can be expected from chondrogenically differentiated BMSCs (*preliminary results from experiments performed by our research group*). There was also little difference between the results of the control and other conditions within the

same donor. Furthermore, histology also showed no positive signs of Safranin-O staining. Therefore, it can be concluded that no chondrogenic differentiation had been achieved with the current culture conditions.

There were a lot of factors that can be of influence on the chondrogenic differentiation potential of BMSCs during cell culture. The culture period for obtaining chondrogenic differentiation of BMSCs consists of two different stadia: the expansion phase and the chondrogenic differentiation phase. The expansion phase was the most important phase, because if something goes wrong the chance of obtaining chondrogenic differentiation will be decreased (25,26). However, the same accounted for the chondrogenic phase in which suboptimal culture conditions can also lead to a decreased chance of obtaining cartilage producing cells (27,28).

During the expansion phase factors that may have influenced cell fate, were for instance, cell density and therefore the potential for BMSCs to form CFUs or another example, the substances that were present in FCS. Cell density during the expansion phase of stem cells appears to be an important factor on influencing cell fate. From research it appeared that cell density influences the forming of colony forming units (CFUs) (21,29,30). A high cell seeding density appeared to be detrimental for the forming of CFUs by human, rat and feline bone marrow-derived stem cells (21,30,31). The high seeding cell density also negatively influenced the potential of forming CFUs by the following cell passages (26). These CFUs were of importance, because specific morphological characterized cells derived from these colonies seems to have good capacity to differentiate into the different possible lineages of Bone marrow-derived mesenchymal stem cells: osteoblasts, adipocytes, myoblasts and chondrocytes (17,21,22,26,31-34). Before the onset of this experiment, BMSCs were expanded from passage 0 or passage 1 to passage 3. Normally, cells are expanded in a 75 cm^2 flask with a cell density of 0.4 million cells or in a 175 cm^2 flask with a cell density of 0.8-1.0 million cells (*Protocol "Expansion of Bone Marrow derived Mesenchymal Stem Cells" from our own research group*). In this experiment cells were expanded with a density of 2.0 to 3.0 million cells per 175 cm^2 flask. The cells were regularly microscopically observed and during the entire expansion phase no CFUs were observed. Another factor of influence was FCS. FCS was added to expansion medium because it contains many substances which were nutritious for stem cells. FCS was derived from calf fetuses. It was obtained during slaughter of pregnant cows in which blood was drained away from their still living fetuses. The obtained blood was processed into serum. This FCS contained many substances and not all substances were known (25,35). Furthermore, the FCS was sold in batches and every batch was derived from one calf. Therefore, every batch derived from another calf will contain different kinds of substances (25,35,36). During previous performed experiments in which chondrogenic differentiation of BMSCs had been obtained, different kinds of FCS were used, but not the one that had been used during the expansion phase of this experiment (*Preliminary results from experiments performed by our research group*).

Also during the chondrogenic differentiation phase were a lot of factors that may have influenced chondrogenesis, such as pellet size and culturing in hypoxia or normoxia (27,28). Zhang *et al*. 2010 showed that there is a difference in efficiency of establishing chondrogenesis of human BMSCs when a standard pellet culture system was used or a micromass culture system. Both systems were comprised of 250,000 cells, though there was a difference in preparing these systems. The pellet was made by putting 250,000 cells in 500

µL media, followed by centrifuging them and the micromass system was made by putting 250,000 cells suspended in a volume of 12.5 µL into the plate and letting them adhere to each other for 2 hours at 37 °C, followed by adding 500 µL of media. They showed that the micromass culture system was more efficient in chondrogenically differentiating BMSCs, though the pellets also showed evidence of chondrogenic differentiation. This could be due to that they were better able to offer an optimal 3D-structure which tolerates cell-to-cell contact and diffusion of nutrients (28). In this experiment pellets were formed prior to the chondrogenic differentiation phase consisting of 200,000 cells. Zhang *et al*. 2010 showed that the pellet culture system was also able to chondrogenically differentiate human BMSCs (28) and therefore it is likely that our pellet system was also capable of inducing chondrogenesis of canine BMSCs. Besides pellet size, culturing under normoxic or hypoxic conditions could also have influenced this phase. It has been shown that culturing BMSCs under hypoxic conditions during the chondrogenic differentiation phase, leads to an increase in cartilage specific gene expressions as well as cartilage specific extracellular matrix production (37-40). This condition resembled embryonal cartilage development in which hypoxia was also present during chondrogenic differentiation of MSCs (41). Though, Gawlitta *et al*. 2012 showed that when pellets consisting of human BMSCs derived from four different donors were cultured for four weeks under normoxic conditions, all donors were capable of differentiating towards the chondrocytic lineage in contrary to hypoxic conditions (only two donors). This could be explained by the existence of variation between donors. They also showed that culturing under normoxic conditions leads to an increase of hypertrophic differentiation of chondrogenically differentiated BMSCs. In order to assess when this took place during the culture period, measuring GAG-content released into the medium appeared to be a good parameter, since less GAGs were released when cells were hypertrophically differentiating (27). During this experiment, pellets were cultured under normoxic conditions. Though, as showed by Gawlitta *et al*. chondrogenesis could still be achieved under these conditions (27) and therefore this factor should not have had that great influence on the negative outcome of this experiment. In addition, BMSCs were cultured in a 3D-pellet-system from which it is thought that this system provides an environment comparable to that found during embryonic cartilage development (13).

In conclusion, the high cell density combined with no visible signs of the formation of CFUs and the different kind of FCS used in the expansion phase could have influenced the chondrogenic differentiation potential of BMSCs and could therefore have led to negative results.

Future research recommendation

Following the obtained results from this experiment and the discussion of what may have influenced this outcome; it is recommended to investigate the influence of FCS derived from different manufacturers combined with optimal, clonal cell density during the expansion phase, on chondrogenesis. Likewise, it is also recommended to investigate simultaneously the influence of different types of media changing schedules containing TGF-β 10 ng/mL on the chondrogenic differentiation potential of BMSCs. That is because these factors can be investigated in our lab, since it does not have the possibility to culture under hypoxic conditions.

List of abbreviations

Not all abbreviations are mentioned below, due to that some are considered as common knowledge.

α-MEM	Minimum essential medium α
BMSCs	Bone Marrow derived Mesenchymal Stem Cells
COL2α1	Collagen type 2α1
CS	Chondroitin Sulphate
DMEM	Dulbecco's Modified Eagle's Medium
DMMB	Dimethylmethylene blue-assay
ECM	Extracellular matrix
FCS	Fetal Calf Serum
GAG	Glycosaminoglycan
HBSS	Hanks Balanced Salt Solution
MMP	Matrix metalloproteinase
OA	Osteoarthritis
PBS	Phosphate buffered saline
TGF-β1	Transforming Growth Factor bèta-1

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PROTOCOL OPTIMIZATION OF CHONDROGENIC DIFFERENTIATION OF CANINE BONE-MARROW DERIVED MESENCHYMAL STEM CELLS:

The influence of Fetal Calf Serum, Colony Forming Units and different media changing schedules containing TGF-β1

Margot M.C.M.E. Müller[1], Michelle Teunissen[1], Alberto Miranda Bedate[1] and Marianna A. Tryfonidou[1]

Abstract

Introduction: Osteoarthritis (OA) is a disease which is most prevalent in the adult dog population by 20%. OA is frequently a secondarily result of altered joint biomechanics. It is characterised by loss of extracellular matrix (ECM) components. In order to regain these components, canine bone marrow derived stem cells (BMSCs) can be used. The culture of BMSCs starts by expanding them *in vitro*. During the expansion phase, fetal calf serum (FCS) is added to the media. This serum is an undefined medium and could therefore influence the cells prior to chondrogenesis. Furthermore, BMSCs can form colonies during the expansion phase from which it is known that cells derived from these colonies have better differentiation potential. After the expansion, pellets are formed containing BMSCs and are subjected to chondrogenesis by supplementing chondrogenic medium with transforming growth-factor beta 1 (TGF-β1) according to different media changing schemes.
This study investigated the influence of FCS derived from three manufacturers (HyClone, Lonza and Gibco), the observation of colony forming units (CFUs) and the influence of different media changing schemes of supplementing TGF-β1 to the BMSC-pellet-culture on the chondrogenic differentiation potential of canine BMSCs.

Methods: Two BMSC-donors were expanded with FCS derived from three different manufacturers: Hyclone, Lonza and Gibco. After the expansion phase, pellets were chondrogenically induced for three weeks with either solely chondrogenic media or supplemented with TGF- β1 (10 ng/mL) according to three different media changing schemes. Samples were subjected to biochemistry assays for the quantification of glycosaminoglycan (GAG) and DNA content, and to histology.

Results: Outcomes of GAG and DNA quantification and the extracted GAG per DNA content, showed differences between the control group and TGF-β1 groups for both donors. For one donor these results are supported by histology.

Conclusion: FCS derived from the manufacturers HyClone, Lonza and Gibco combined with the observation of CFUs in the expansion phase could have positively influenced the differentiation potential of the expanded BMSC-culture. Moreover, FCS derived from the manufacturer Gibco has good potential regarding proliferation and preservation of the multipotency of BMSCs. However, no conclusion could be made about what time-schedule is the best for supplementing TGF-β1 to the BMSC-culture due to the (very) short culture period.

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Introduction

Osteoarthritis (OA) is an incurable disease which is most prevalent in the adult dog population in which 20% of these dogs are diagnosed with the disease. It is commonly a secondarily result of altered joint biomechanics and is characterised by progressive cartilage damage which evokes a lot of pain. Therefore, this disease is the most common cause of pain and lameness in dogs (1-3).

Cartilage damage causes loss of extracellular matrix (ECM) components. In order to regain these components, canine bone marrow derived mesenchymal stem cells (BMSCs) can be used. BMSCs are multipotent stem cells and are able to differentiate into cartilage-producing cells under the influence of certain growth-factors, such as Transforming Growth-Factor (TGF)-β1 (4,5).

The culture of BMSCs starts by expanding them *in vitro*. For the expansion an optimal, clonal cell seeding density was used, because high and low seeding densities have a negative influence on the potential to form colonies (6-10). By seeding cells with an optimal, clonal density, an optimal environment for canine BMSCs can be provided in which they are able to form colonies. These colony forming units (CFUs) are characteristic for the presence of stem cells (8,11) in which the colony is formed by originally one stem cell that had started to proliferate. Cells derived from these colonies appeared to have good differentiation potential (7-9,11-15). In order to stimulate the proliferation of the stem cells among other things, fetal calf serum (FCS) was added. This serum is an undefined medium and could therefore influence the cells prior to chondrogenesis. FCS is sold in batches and every batch is derived from one calf. Consequently, there are batch-to-batch variations (16-18).

After the expansion, pellets are formed and subjected to chondrogenesis by supplementing chondrogenic medium with TGF-β1. TGF-β1 is an important growth-factor which is capable of inducing the transcription of transcription factor Sox9 (19-21). This factor is the most important regulator of chondrogenesis and is necessary for ECM specific gene expressions, such as collagen type 2α1 (COL2α1) and aggrecan (19,22,23).

In our previous research, chondrogenesis of canine BMSCs was not obtained after three weeks of culture (24). This could be explained by the fact that no colony forming units were microscopically observed in the expansion phase of culture due to the high cell seeding density. Furthermore, we could not exclude that FCS may have influenced the chondrogenic capacity of the BMSCs during the expansion phase (24). Therefore, the aim of this experiment was to investigate the influence of FCS derived from three different manufacturers (HyClone, Lonza and Gibco), by expanding canine BMSCs with an optimal, clonal cell seeding density. Furthermore, we also varied the changing schemes of supplementing TGF-β1 to the canine BMSC-pellet-culture during chondrogenic differentiation. We determined chondrogenic effects by focusing on glycosaminoglycan (GAG) content of the pellets, DNA content of the pellets in order to determine proliferation rate and finally, histology to study the cell phenotype and matrix deposition.

Materials and methods

Bio-Bank and cell expansion

Canine BMSCs were obtained from the Veterinary Bio-Bank. Two canine cell donors were used: one adult and one pup (table 1). BMSCs were thawed in passage 0 and were expanded in expansion media (Fig. 1), containing minimum essential medium α (α-MEM; Invitrogen,

22561-021), 1% penicillin-streptomycin (PPA cell culture company, P11-010), 1 ng/mL fibroblast growth factor (AbD Serotec, PHP105), 0.1 mM ascorbic acid 2-phosphate (Sigma Aldrich, A-8960), 0.2% Fungizone (Invitrogen, 15290). Each donor was cultured under three different expansion media with FCS as the only variable: 10% FCS of HyClone (*SV30160.03), or* Lonza (*DE-14-801F), or* Gibco High Performance (16000-044). Donor cells were expanded in 75 cm^2 cell culture flasks (Geiner Bio-One, 658175) with a cell density of 0.4 million cells per flask. When cells reached subconfluency (70-90%) they were transferred to 175 cm^2 cell culture flasks (Greiner Bio-One, 660175) with a seeding density of 0.8-1.0 million cells per flask. Therefore, cells were washed with Hanks balanced salt solution (HBSS; Gibco by Life technologies, 14025-050), detached with trypsin (Triple Ex; Gibco by Life technologies, A12177-01) and counted by using a cell counting device (Bio Rad TC20, Catalog #145-0101). During the expansion phase, medium was refreshed biweekly and cells were incubated under normoxic conditions with 5% CO_2 at 37°C.

Passage 2 BMSCs were prepared to form pellets consisting of 200,000 cells when subconfluency (70-90%) was reached. Cells were washed with HBSS and detached with trypsin. Hereafter, expansion medium was added that contained the corresponding FCS in order to inactivate trypsin. Cells with medium were centrifuged, followed by replacing expansion media with chondrogenic media, containing Dulbecco's Modified Eagle's Medium, high glucose, GlutaMax™ pyruvate (DMEM; Invitrogen, 31966), 1% selenous acid (ITS+) premix (B&D, 354352), 1% penicillin-streptomycin (PPA cell culture company, P11-010), 0.04 mg/mL L-prolin (Sigma Aldrich, P-5607), 0.2% fungizone (Invitrogen, 15290), 0.0001 mM dexamethasone (Sigma Aldrich, D8893) and 0.1 mM ascorbic acid 2-phosphate (Sigma Aldrich, A-8960). Cells were homogenised and counted by using a cell counting device. Subsequently, pellets were formed and unintentionally plated as pellets in a 96-wells-plate (Costar, 7007) in one of the four possible groups: control, TGF-β1 week 1, TGF-β1 week 2 or TGF-β1 week 3. Each group of donor cells expanded with FCS derived from HyClone, Lonza or Gibco, was divided into the four groups mentioned above (Fig. 1). Pellets were incubated under normoxic conditions with 5% CO_2 and at 37°C

Donor number	Dog breed	Age
154	Beagle	40 months
445	Labrador	4.3 months

Table 1: Information about included BMSC donors.

Microscopy

During the expansion phase, we observed the presence of CFUs. During the differentiation phase, we observed the phenotype of all pellets and the aspect of the corresponding media.

In-vitro chondrogenic differentiation

For the induction of chondrogenic differentiation, chondrogenic media was supplemented with TGF-β1 (R&D systems, 240-B-010).

Four groups were used in this experiment with a culture period of three weeks. The control group received basic chondrogenic media and media was changed three times per week (Mondays, Wednesdays and Fridays). The TGF-β1 groups received for three weeks chondrogenic media supplemented with TGF-β1 in the concentration of 10 ng/mL, from which *TGF-β1 week 1* media was daily changed for one week and then every other day for two weeks; *TGF-β1 week 2* media was daily changed for two weeks and then every other

day for one week and *TGF-β1 week 3* media was daily changed during the entire culture period.

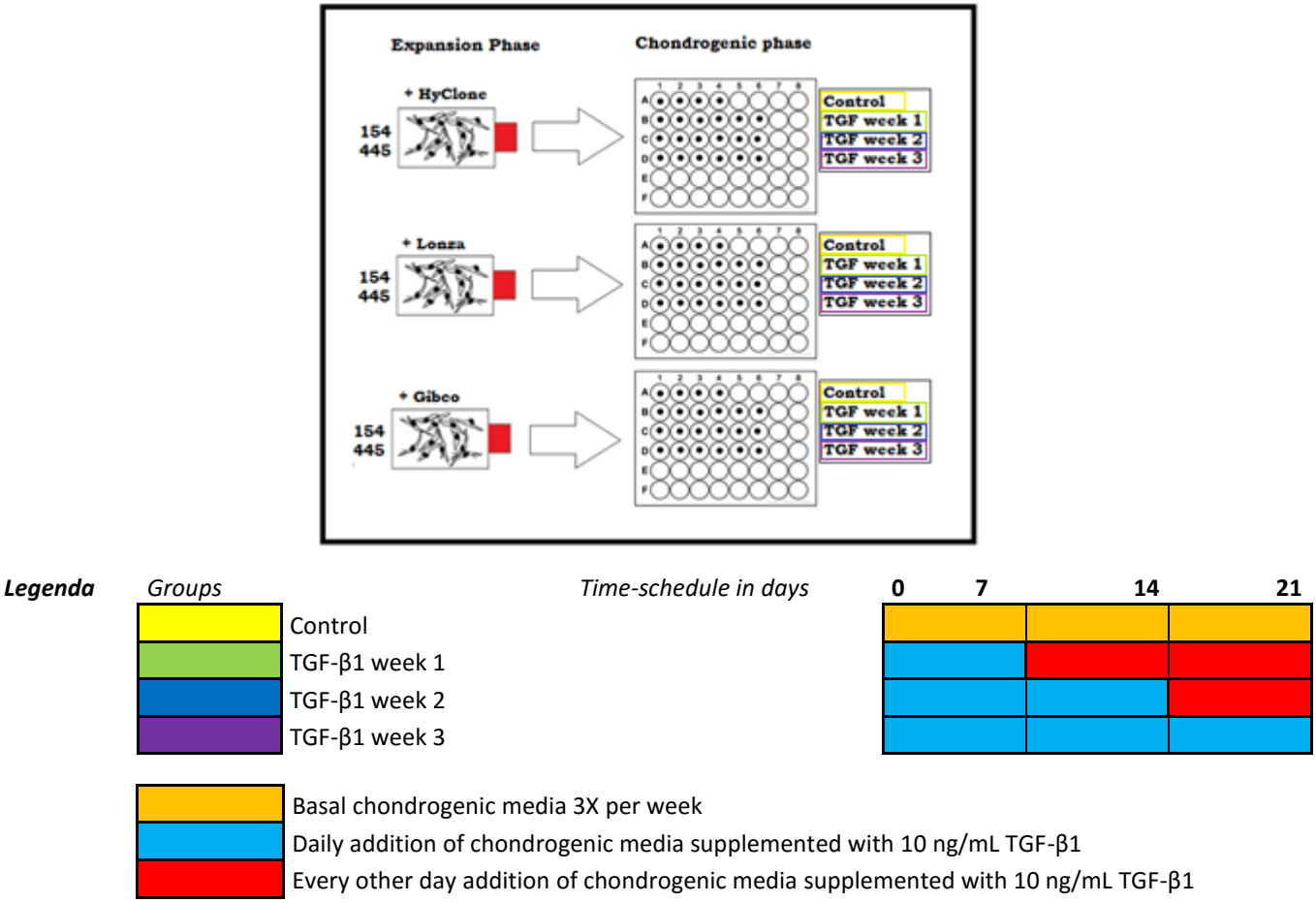

Figure 1: Schematic presentation of the experimental design. Passage 0 canine BMSCs derived from two donors (154, 445) were expanded with FCS derived from one of the three different manufacturers: HyClone, Lonza or Gibco. When subconfluency was reached of passage 2 cells, pellets were formed and plated in one of the four possible groups: control, TGF-β1 week 1, TGF-β1 week 2 or TGF-β1 week 3. All groups received chondrogenic media and the TGF-β1 groups also received TGF-β1 according to different time-schedules.

Biochemistry

GAG quantification

The dimethylmethylene blue (DMMB)-assay was used to quantify the amount of glycosaminoglycans (GAGs), which are produced by chondrogenically differentiated stem cells. The DMMB dye binds to the repeating negative charges on the GAGs which are caused by sulphates. After this association, a shift in the absorption spectrum can be measured (25) at wavelengths of 540 and 595 nm.

The DMMB-assay was performed as previously described (24) and was performed on pellets (N =3 per group) after the three weeks of culture. Briefly, pellets were washed with HBSS, were digested in a papain digestion solution and incubated overnight at 60 °C. Next day samples were vortexed and put back in the incubator at 60 °C for another hour. A standard curve was made using 50 times diluted chondroitin sulphate (CS, Sigma Aldrich, C4384; stock: 0.5 mg/mL) and phosphate buffered saline (PBS). The standard was made in

duplo according to the manufacturer (Sigma Aldrich) and ranged from 0 to 10.0 µg/mL CS. Hereafter, samples were measured undiluted in duplo in which 100 µL of sample was pipetted in a 96-wells-plate with flat bottom (Greiner Bio-one 655191), followed by the supplementation of 200 µL of DMMB-solution pH 3.0 (Sigma Aldrich). Absorption levels were measured by a spectrophotometer at 540 and 595 nm wavelengths. Results were extrapolated from the standard curve and both were corrected for the blank. The blank corresponds with the first outcome of the standard curve (0 µg/mL CS). Finally, absolute GAG (µg) content for each pellet was calculated.

DNA quantification and GAG per DNA determination

Papain digested pellets were used to measure DNA content. For this, samples were processed with Qubit™ dsDNA BR Assay Kit (Invitrogen, Q32853) and results were measured by the Qubit® 2.0 Fluorometer (Invitrogen, Q32866). Samples were prepared to be measured 20 times diluted and a calibration curve was obtained according to the manufacturer (Invitrogen). The obtained results were normalized for pellet content and absolute DNA (µg) per pellet was calculated, followed by calculating absolute GAG per DNA (µg/µg) content.

Histochemical staining

In order to determine if chondrogenesis was successful, pellets (N = 2 per group) were stained histochemically with Fast green, Safranin-O staining. Mayer`s Hematoxylin stains the nucleus of the cell blue. Safranin- O will stain GAGs in ECM orange-red. And fast green is used as background staining and will stain the rest of the pellet blue-greenish (26). The preparation and staining of pellets was performed as previously described (24). Briefly, pellets were overnight fixated with buffered neutral formalin with 1% eosin and thereafter embedded in 2.4% alginate (Sigma Aldrich, A2033), and formalin containing 102 mM $CaCl_2$. Embedded pellets were stored in white embedding cassettes in 70% alcohol. Samples were dehydrated through graded alcohol and xylene steps. Subsequently, pellets were embedded in paraffin and cut into 4µm sections. Sections were deparaffinised, hydrated to distilled water and disposed of alginate. Subsequently, sections were stained with Mayer`s Hematoxylin for 10 seconds (J.T.Baker, 3870), 0.4% Fast Green FCF (Sigma Aldrich, F7252) for 4 minutes and 0.125% Safranin-O (Sigma Aldrich, 58884) for 7 minutes. Sections were dehydrated and mounted. Images of stained sections were taken with a digital microscopic camera (Leica, DFC425C).

Statistical analysis

No statistical analysis was performed, because during the chondrogenic differentiation phase a lot of loose cells were microscopically observed and this was explained as a result of that on day 10th of culture a bacterial infection was confirmed. Consequently, the experiment was stopped and data were analysed in order to obtain information about what happened during these 10 days.

Results

Microscopy

During the expansion CFUs were observed in all cell culture flasks, regardless of the expansion condition. During the initial chondrogenic differentiation phase rounded pellets were formed. However, short after the beginning of this phase a lot of loose cells were

observed and a bacterial infection was confirmed by analysis of the culture media with the aid of cytospins.

GAG and DNA quantification and GAG per DNA determination

The TGF-β1 growth-factor groups produced more amounts of GAG per pellet under the influence of TGF-β1 in comparison to the control group for both donors (Fig 2.). BMSCs of donor 154 expanded with Lonza FCS, contained relatively more DNA compared to the pellet comprised of cells of the same donor expanded with HyClone or Gibco FCS (Fig. 2). Though, this pattern was not shown by donor 445. GAG per DNA content was consistently higher in the TGF-β1 treated groups compared with the control groups. Donor 154 revealed better chondrogenic potential when expanded with Lonza or Gibco FCS, while donor 445 seemed to perform better when expanded with HyClone FCS.

Histochemical staining

Regardless of the FCS employed during the expansion phase, all TGF-β1 treated pellets of donor 154 stained positive for GAG (Fig. 4). Interestingly, there was an evident difference in intensity of staining; pellets that had been expanded in Gibco FCS stained more intense than Lonza and HyClone. Sections derived from donor 445 pellets stained negative for Safranin-O.

Discussion

Regardless of the FCS used in the expansion phase, both donors showed chondrogenic differentiation potential based on GAG content of the pellet corrected for DNA (Fig. 2). In order to confirm this, sections of pellets were stained with Safranin-O that is specific for the detection of GAGs (26,27). In line with the GAG/DNA findings, pellets from donor 154 treated with TGF-β1 stained positively for GAGs, indicating that chondrogenic differentiation was achieved with GAG-rich matrix deposition. Moreover, there was a difference in red-staining intensity, pellets expanded with Lonza and Gibco FCS were more intense stained than those expanded with HyClone FCS. The same trend was also observed for GAG per DNA content. Furthermore, both donors showed formation of CFUs. This altogether indicated that FCS derived from manufacturers HyClone, Lonza and Gibco combined with the formed colonies in the expansion phase could have positively influenced the differentiation potential of the expanded canine BMSC-culture. FCS derived from the manufacturer Gibco showed good potential regarding proliferation and preservation of the multipotency of BMSCs (Fig. 2) and can therefore be still used by our research team. However, no conclusion was made about what time-schedule was the best for supplementing TGF-β1 to the BMSC-culture due to the (very) short culture period.

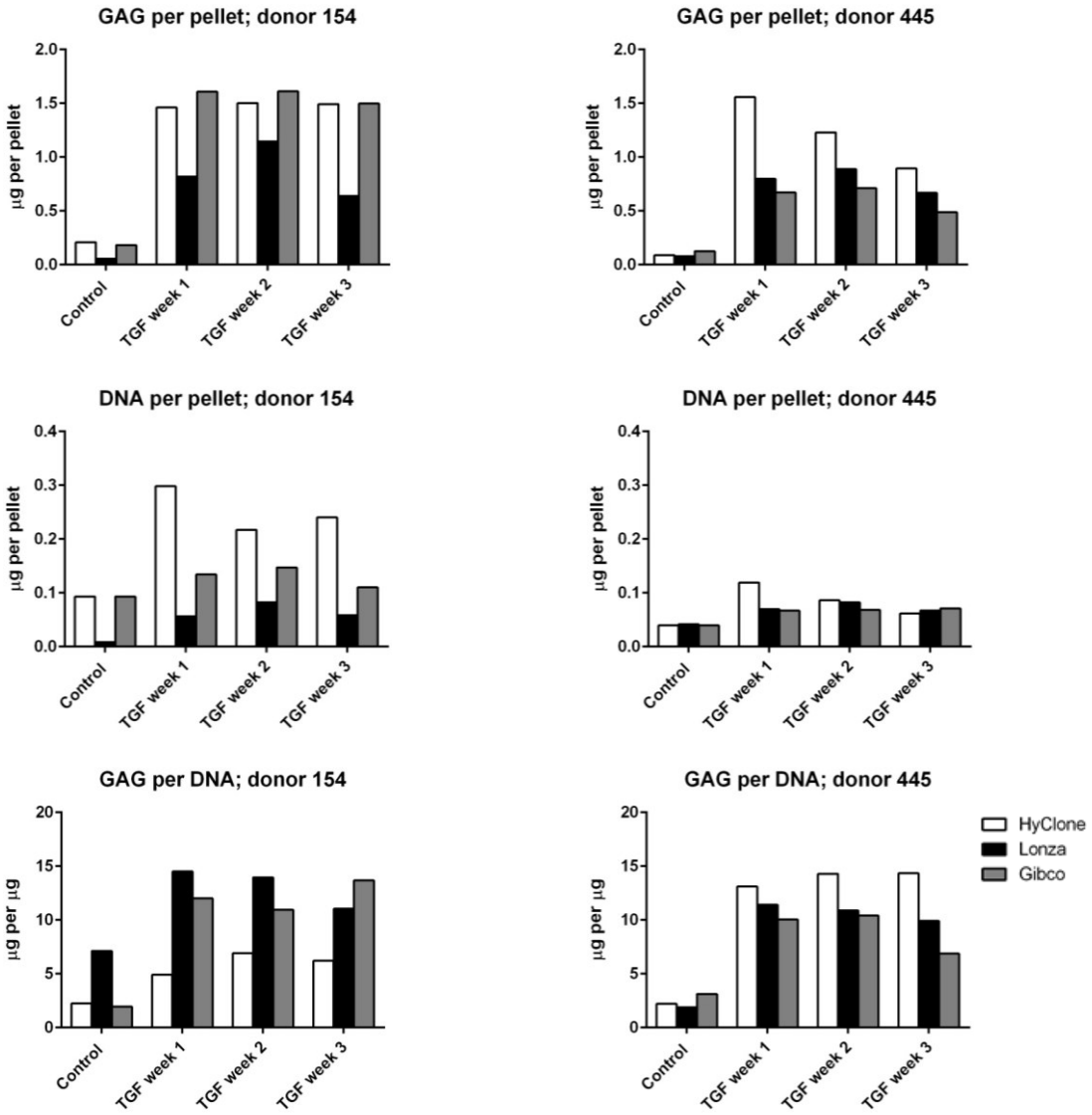

Figure 2: Quantification of the GAG- and DNA- content performed on pellets from different donors and conditions and the corresponding calculated GAG per DNA after ten days of culture. Absolute values are shown.

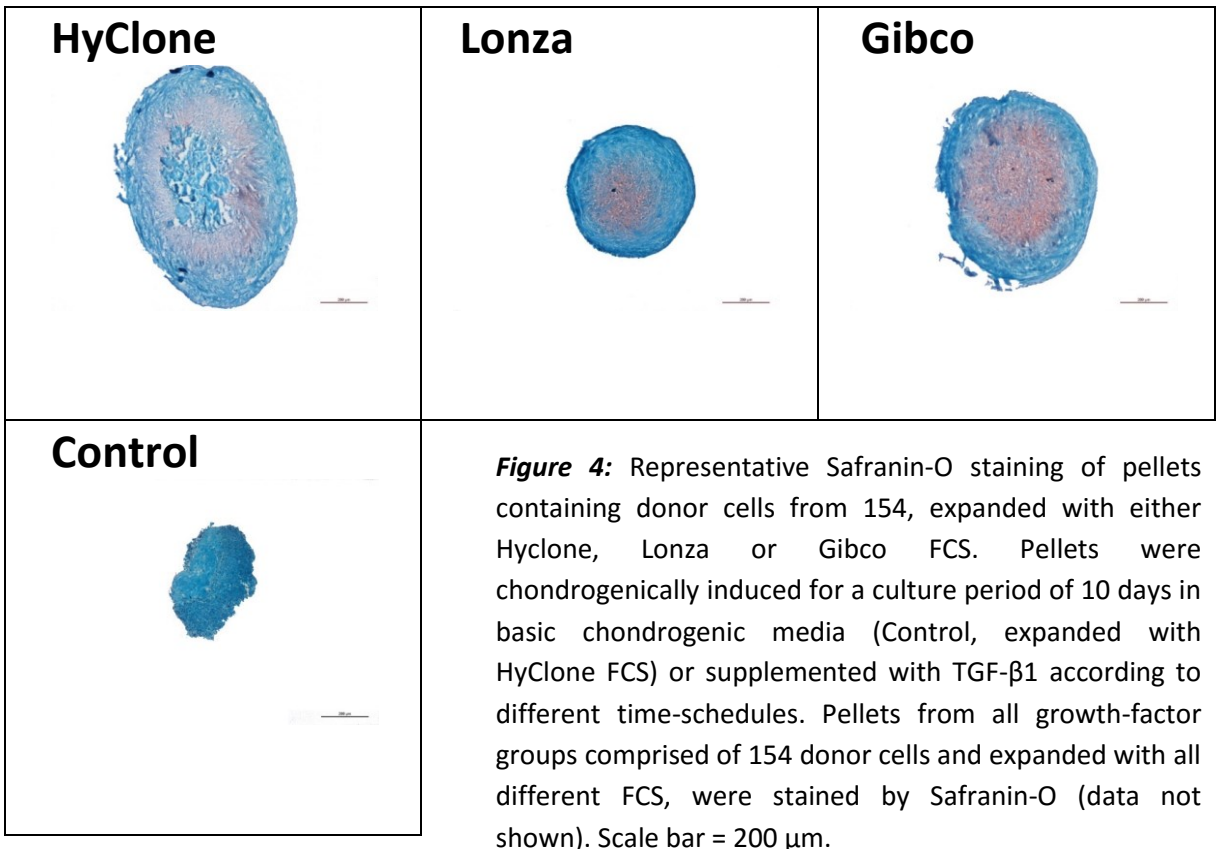

Figure 4: Representative Safranin-O staining of pellets containing donor cells from 154, expanded with either Hyclone, Lonza or Gibco FCS. Pellets were chondrogenically induced for a culture period of 10 days in basic chondrogenic media (Control, expanded with HyClone FCS) or supplemented with TGF-β1 according to different time-schedules. Pellets from all growth-factor groups comprised of 154 donor cells and expanded with all different FCS, were stained by Safranin-O (data not shown). Scale bar = 200 µm.

Interestingly, only one out of two donors stained positive for Safranin-O, indicating a GAG-rich matrix deposition, which was in line with GAG/DNA results. The other donor was not confirmed to be chondrogenically differentiated even though it showed CFUs during the expansion phase and did have increased GAG/DNA content compared to controls, which is indicative of GAG production and deposition in the pericellular matrix. Kuznetsov *et al*. 1997 showed that in contrary to what first was claimed, not all cells derived from CFUs have good differentiation potential. He demonstrated that only 58.8% of the cells derived from these colonies had the potential to form bone *in vitro* (11,28). These results were supported by Gronthos *et al*. 2003 (29) and these data suggested that BMSCs derived from CFUs are comprised of a heterogeneous cell population (11,28,29).

Another explanation could be that shortly after pellet formation, pellets were infected by a bacterial infection. The consequence could be that some pellets were more affected than others which could have influenced their response to TGF-β1 and also could have influenced GAG/DNA results. Zheng *et al*. showed that high DNA content resulted in false positive results in terms of GAG content when the DMMB-solution is used at a pH of 3.0 (30). Though, pellets were washed with HBSS before being digested and therefore it was less likely that bacterial DNA could have influenced these outcomes. Furthermore, the infection was introduced during the first and probably most crucial days for the cells in order to devote them towards the chondrogenic cell lineage.

Moreover, it could also be that some donors need a higher stimulus in order to get differentiated towards cartilage producing cells. Chondrogenic differentiation of BMSCs begins with condensation of stem cells, followed by differentiation (19). This denoted that there are two different phases in which one will succeed the other. However, there was still

a chance that the first phase will be successful and that it still will not result in chondrogenic differentiation (31). Therefore, it could be that in some cases an additional stimulus needs to be present to get more cells devoted.

Besides TGF-β1, there were also other potent factors that are able to induce chondrogenesis, among others Bone Morphogenetic Protein-2 (BMP-2) (4). BMP-2 was also able to induce transcription of transcription factor Sox9 (32-34). This factor was the master regulator of chondrogenic differentiation and is essential for ECM specific gene expressions, such as Collagen type 2α1 (COL2α1) and aggrecan (32,33). Therefore, it could be that BMP-2 has an additive effect on TGF-β1 creating a higher stimulus, which could result in more successful differentiated BMSC donors.

Future research recommendation

Following the obtained results from this experiment and the discussion of what may have influenced this outcome; it is recommended to investigate the (additive) effect of BMP-2 on TGF-β1 in terms of chondrogenic differentiation potential of BMSCs.

List of abbreviations

Not all abbreviations are mentioned below, due to that some are considered as common knowledge.

α-MEM	Minimum essential medium α
BMP-2	Bone Morphogenetic Protein-2
BMSCs	Bone Marrow derived Mesenchymal Stem Cells
CFU	Colony Forming Unit
COL2α1	Collagen type 2α1
CS	Chondroitin Sulphate
DMEM	Dulbecco's Modified Eagle's Medium
DMMB	Dimethylmethylene blue-assay
ECM	Extracellular matrix
FCS	Fetal Calf Serum
GAG	Glycosaminoglycan
HBSS	Hanks Balanced Salt Solution
OA	Osteoarthritis
PBS	Phosphate buffered saline
TGF-β1	Transforming Growth Factor bèta-1

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PROTOCOL OPTIMIZATION OF CHONDROGENIC DIFFERENTIATION OF CANINE BONE-MARROW DERIVED MESENCHYMAL STEM CELLS:

The (additive) chondrogenic effect of Bone Morphogenetic Protein-2 on Transforming Growth Factor-β1 on canine Bone Marrow derived Stem Cells

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Abstract

Introduction: Osteoarthritis (OA) is mostly diagnosed in the adult dog population in which it has a prevalence of 20%. OA often secondarily results of changed joint biomechanics. It is characterised by loss of extracellular matrix (ECM) components. Currently, many studies have been performed on regaining these components *in vitro* by using bone-marrow derived stem cells (BMSCs). These stem cells are multipotent and can be differentiated towards cartilage producing cells. In order to achieve differentiation, mostly the standard Transforming Growth Factor (TGF)-β1 based technique is used. However, this technique is not potent enough to induce chondrogenic differentiation in all canine BMSC-donors. Therefore, it could be that some donors need a higher stimulus in order to get devoted towards the chondrocytic lineage. This higher stimulus could be achieved by supplementing Bone Morphogenetic Protein (BMP)-2 in addition to TGF-β1 to the BMSC-pellet-culture. Consequently, the aim of this research was to investigate the (additive) effect of BMP-2 on TGF-β1 in terms of chondrogenic differentiation potential of canine BMSCs.

Methods: BMSC-pellets were cultured with either basic chondrogenic media or supplemented with either TGF- β1 (10 ng/mL) or TGF-β1 (10 ng/mL) combined with BMP-2 (100 or 250 ng/mL).

Samples were subjected to biochemistry assays for the quantification of glycosaminoglycan (GAG) and DNA content, histology and qPCR.

Results: Outcomes from all assays showed that pellets cultured with TGF-β1 and BMP-2 (250 ng/mL) were capable of successfully differentiating in at least two out of four donors in comparison to the other conditions. This concentration exerted the highest GAG production and also the highest gene expression regarding aggrecan and collagen type 2α1. In addition, this condition was also able to down-regulate gene expression of matrix remodelling enzymes, among others ADAMTS 5.

Conclusion: BMP-2 had an additive effect on TGF-β1 in terms of chondrogenic differentiation potential of canine BMSCs. This effect was most prominent when BMP-2 was added in the concentration of 250 ng/mL. Both growth factors were able to induce transcription of transcription factor Sox9, which is the master regulator of chondrogenic differentiation. Consequently, it was hypothesized that BMP-2 supplemented in the highest concentration had therefore the highest additive effect on TGF-β1.

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Introduction

Osteoarthritis (OA) is diagnosed in about 20% of the adult dog population and is an incurable disease. It is often a secondarily result of changed joint biomechanics and it is characterised by loss of extracellular matrix (ECM) components. Moreover, OA is the most common cause of pain and lameness in dogs (1-3). Currently, many studies have been performed on regaining these components *in vitro* by using bone-marrow derived stem cells (BMSCs). These stem cells have multipotent potential and can be differentiated towards cartilage producing cells. In order to achieve differentiation, researchers often used the standard Transforming Growth Factor (TGF)-β based technique (4). However, this technique was not potent enough to induce chondrogenic differentiation in all canine BMSC-donors during 21 days of culture (5). Therefore, it could be that some donors need an additional stimulus in order to get committed to the chondrocytic lineage. Such a stimulus could be achieved by supplementing Bone Morphogenetic Protein (BMP)-2 in addition to TGF-β1 to the BMSC-pellet-culture. Both growth factors are capable of inducing chondrogenic differentiation by, for instance activating the transcription of transcription factors Sox9 (6-10), which is the regulator of chondrogenic differentiation and is essential for matrix specific gene expressions, such as collagen type 2α1 (COL2α1) and aggrecan (6-8,10-12). Consequently, the aim of this research was to investigate the (additive) effect of BMP-2 on TGF-β1 in terms of chondrogenic differentiation potential of canine BMSCs. We focused on glycosaminoglycan (GAG) content which are part of proteoglycans; DNA content in order to determine proliferation rate and for normalizing GAG content for the amount of present cells; histology and finally gene expression profiles.

Materials and methods

Bio-Bank and expansion of BMSCs

Canine BMSCs were obtained from 4 donors from the Bio-Bank of the Faculty of Veterinary Medicine of Utrecht. Donor breeds were Beagle (2 donors) and Labrador (2 donors), aged from 4 months to 40 months (table 1). BMSCs were thawed in passage 1 and expanded in expansion media, containing minimum essential medium α (α-MEM; Invitrogen, 22561-021), 10% fetal calf serum (FCS; Gibco High performance, 16000-044), 1% penicillin-streptomycin (PPA cell culture company, P11-010), 1 ng/mL fibroblast growth factor (AbD Serotec, PHP105), 0.1 mM ascorbic acid 2-phosphate (Sigma Aldrich, A-8960) and 0.2% fungizone (Invitrogen, 15290). Cells were seeded with a density of 1.0 million cells per 175 cm^2 flask (Greiner Bio-One, 660175). During expansion, medium was replaced once. Cells were cultured under normoxic conditions, 5% CO_2 at 37 °C.

Passage 2 cells were prepared to form pellets consisting of 200,000 cells when subconfluency (60-100%) was reached. Cells were washed with HBSS and detached by trypsin. Hereafter, expansion medium was added to inactivate trypsin by the presence of FCS. Cells were centrifuged, followed by draining away the expansion media. Thereafter, chondrogenic media, containing Dulbecco's Modified Eagle's Medium, high glucose, GlutaMax™ pyruvate (DMEM; Invitrogen, 31966), 1% selenous acid (ITS+) premix (B&D, 354352), 1% penicillin-streptomycin (PPA cell culture company, P11-010), 0.04 mg/mL L-prolin (Sigma Aldrich, P-5607), 0.2% fungizone (Invitrogen, 15290), 0.0001 mM dexamethasone (Sigma Aldrich, D8893), 1.25 mg/mL Bovine Serum Albumin (Sigma Aldrich, A9418) and 0.1 mM ascorbic acid 2-phosphate (Sigma Aldrich, A-8960), was added. Cells were homogenized and counted by using a cell counting device. Subsequently, pellets were formed in which donor cells were unintentionally plated as pellets in a 96-wells-plate

(Costar, 7007). Pellets were divided into two groups: control group receiving basic chondrogenic media and three growth factor groups. Within 15 hours after pellet formation, pellets from the growth factor groups were transferred to 48-wells-plates (Greiner Bio-One, 677970) in which they received 500 µL of media. Media was changed on Monday, Wednesday and Friday. Pellets were cultured under normoxic conditions, 5% CO_2 at 37 °C.

Donor number	Dog breed	Age
154	Beagle	40 months
4225	Beagle	21.5 months
445	Labrador	4.3 months
492	Labrador	4.3 months

Table 1: Information about included BMSC donors.

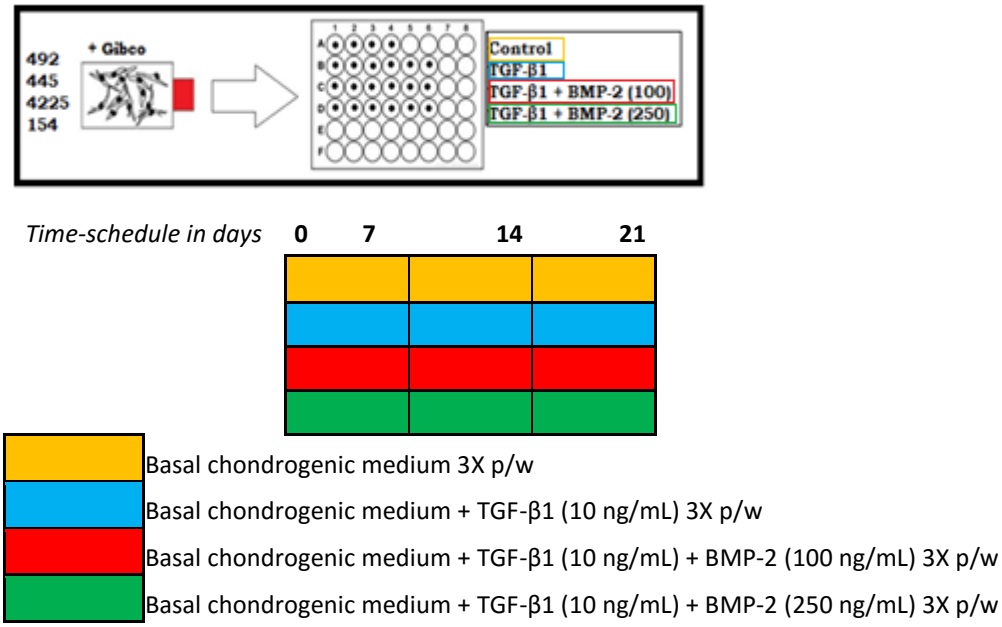


Figure 1: Experimental design. All donors were first expanded with FCS derived from the manufacturer Gibco. Thereafter, pellets were formed from all donors and were unintentionally plated in one of the four possible groups: control, TGF-β1, TGF-β1 + BMP-2 (100 ng/mL) or TGF-β1 + BMP-2 (250 ng/mL). All groups received chondrogenic media and the TGF-β1 and TGF-β1 + BMP-2 groups received either TGF-β1 or combined with BMP-2.

Microscopy

Pellets were observed under the microscope. Moreover, also the aspect of the culture media was studied.

***In-vitro* chondrogenic differentiation**

Three growth factor groups were used in this experiment for the induction of chondrogenesis of pellets comprised of canine BMSCs. These groups received chondrogenic media supplemented with TGF-β1 (10 ng/mL; R&D systems, 240-B-010) or TGF-β1 combined with BMP-2 (generous gift from TETEC AG) in the concentration of 100 ng/mL or 250 ng/mL (Fig. 1).

Biochemistry

GAG quantification

The dimethylmethylene blue (DMMB)-assay was used to quantify glycosaminoglycan (GAG) content within the pellet and in the media after the three weeks of culture. The dye binds to the repeating negative sulphates present on GAGs. Hereafter, a change in absorption spectrum can be measured (13) at wavelengths of 540 and 595 nm.

The DMMB-assay was performed as previously described (14). In brief, pellets (N = 3 per group) were digested overnight in a papain digestion solution at 60 °C. For the measurement, a standard curve was made using 50 times diluted chondroitin sulphate (CS, Sigma Aldrich, C4384; stock: 0.5 mg/mL) and phosphate buffered saline (PBS). The standard was made according to the manufacturer (Sigma Aldrich) and varied from 0 up to 10.0 µg/mL CS. Hereafter, pellet samples were measured in duplo in which 100 µL of 10 or 30 times of the diluted sample in PBS - ethylenediaminetetraacetic acid (EDTA) was pipetted in a 96-wells-plate with flat bottom (Greiner Bio-one 655191), followed by the supplementation of 200 µL of DMMB-solution pH 3.0 (Sigma Aldrich). For measuring GAG quantity in the culture media, media was collected (N = 2 per group) every time media was changed and it was collected per week. Media was measured undiluted in which 100 µL of media was pipetted in a 96-wells-plate with flat bottom (Greiner Bio-one 655191), followed by the supplementation of 200 µL of DMMB-solution pH 3.0 (Sigma Aldrich). Absorption levels were measured at 540 and 595 nm wavelengths by using a spectrophotometer. Results were extrapolated from the standard curve and both were corrected for the blank. The blank corresponds with the first result of the standard curve (0 µg/mL CS). Finally, absolute GAG (µg) content for each pellet and precipitated GAG content in the culture media were calculated as well as total GAG production which is the sum of the total GAG content precipitated into the medium and the GAG content calculated per pellet.

DNA quantification and GAG per DNA determination

Digested pellets were used for measuring DNA content. Samples were processed with Qubit™ dsDNA BR Assay Kit (Invitrogen, Q32853) and results were measured by the Qubit® 2.0 Fluorometer (Invitrogen, Q32866).

For this, samples were measured 20 times diluted and a calibration curve was obtained according to the manufacturer (Invitrogen). The obtained results were normalized for pellet content and absolute DNA (µg) per pellet was calculated, followed by determining absolute GAG per DNA (µg/µg) content.

Histochemical staining

Pellets (N = 2 per group) were stained histochemically with Mayer`s Hematoxylin, Fast Green and Safranin-O staining in order to confirm successful chondrogenic differentiation. Mayer`s Hematoxylin stain the nucleus of the cell blue. Fast Green is used as background staining and will stain the rest of the pellet blue-greenish. Safranin-O will stain GAGs in ECM orange-red (15). The preparation and staining of pellets was performed as previously described (14). Briefly, pellets were fixated overnight with buffered neutral formalin with 1% eosin and thereafter embedded in 2.4% alginate (Sigma Aldrich, A2033) and formalin containing 102 mM $CaCl_2$. Embedded pellets were stored in white embedding cassettes in 70% alcohol. Samples were dehydrated. Subsequently, pellets were embedded in paraffin and cut into 4µm sections. Sections were removed from paraffin and alginate and were hydrated to distilled water. Subsequently, sections were stained with Mayer`s Hematoxylin

for 10 seconds (J.T. Baker, 3870), 0.4% Fast Green FCF (Sigma Aldrich, F7252) for 4 minutes and 0.125% Safranin-O (Sigma Aldrich, 58884) for 7 minutes. Sections were dehydrated and mounted. Images of stained sections were taken with a digital microscopic camera (Leica, DF425C).

RNA-isolation and real-time quantitative polymerase chain reaction (RT-qPCR)

Pellets were harvested on day 1 (N = 1 per condition) and day 7 (N = 2 per condition) for RNA. Pellets were crushed with nitrogen and special RNAse-free pestles (Argos technologies, 9551-901), followed by adding 346.5 µL of RLT and 3.5 µL of β-mercaptoethanol. Thereafter, total RNA extraction was performed using miRNeasy Micro Kit (Qiagen) according to manufacturer`s manual. Hereafter, a total RNA-input of 300 ng was reverse transcribed to obtain cDNA according to the manufacturer (Bio-Rad, iScript™cDNA Synthesis Kit, 170-8891). cDNA was used as input for RT-qPCR. RT-qPCR was performed using iQ™ SYBR Green Supermix Kit (Bio-Rad, 170-8887) on genes related to chondrogenic differentiation (*SOX9; Collagen 2a1; Aggrecan),* hypertrophy (*Collagen X a1*), fibrosis (*Collagen 1a1*) and matrix degrading enzymes and inhibitors (*MMP-13, Adamts-5; TIMP-1*), according to manufacturer`s guidelines (Bio-Rad). Primer information can be found in table 3. Averaged results of housekeeping genes *B2MG, GUSB, HNRPH, RPL8, SDHA, SRPR* and *YWHAZ* were used for normalization. N-fold change of measured genes was calculated compared to the control group of day 1. Standard deviation belonging to the corresponding N-fold change was calculated.

Statistical analysis

Statistical analysis was performed by using the program R-studios, version 3.1.1 (www.rstudio.com). All PCR data was analyzed with the cox proportional hazard model, because certain genes were normally distributed while others were not and for this program it does not matter if data were or were not normally distributed. Furthermore, in this program it was also possible to censor data for samples that did not come up. Donor and day were assigned to be random effects. Significances are shown when there is more than at least a 2 N-fold difference between the significantly different groups. The Mann-Whitney U test was used as non-parametrical test to obtain significances corresponding to the results of the GAG, DNA and GAG per DNA content regarding the pellets. Though, not many significances were found and this is due to that we had one donor which outlaid the rest of the data and that we had only 4 donors. The Benjamini-Hochberg correction was used to correct for multiple testing. A P-value of less than 0.05 was considered to be significant.

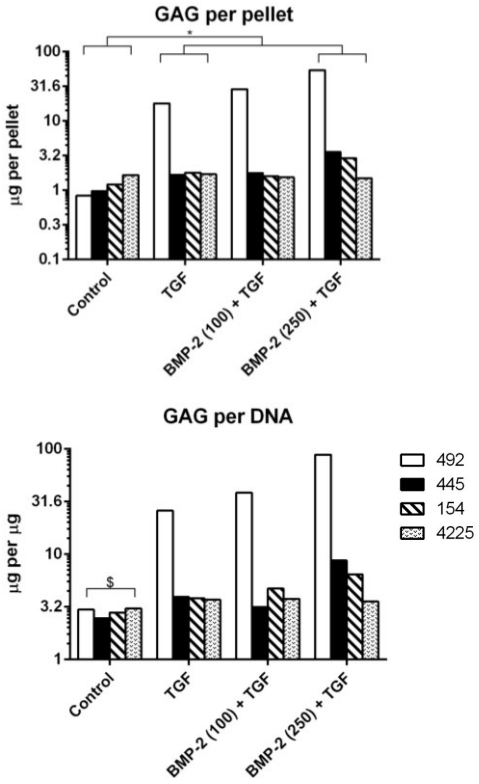
Results

Microscopy

During the chondrogenic differentiation phase, pellets from three out of four donors increased in pellet size when cultured with TGF-β1 + BMP-2 250 ng/mL. One donor also increased in pellet size when cultured with only TGF-β1 and also in the condition of TGF-β1 + BMP-2 100 ng/mL.

Genes	Extension	Sequence	Amplicon Size	EXON	Anealing temp (°C)	Accession no.
Reference genes						
SDHA	Forward	GCCTTGGATCTCTTGATGGA	92	6	61	DQ402985
	Reverse	TTCTTGGCTCTTATGCGATG		6		
HNRPH	Forward	CTCACTATGATCCACCACG	151	5	61,2	XM_538576
	Reverse	TAGCCTCCATAACCTCCAC		5-6		
RPL8	Forward	CCATGAATCCTGTGGAGC	64	4-5	55	XM_532360
	Reverse	GTAGAGGGTTTGCCGATG		5		
GUSB	Forward	AGACGCTTCCAAGTACCCC	103	4	62	NM_001003191
	Reverse	AGGTGTGGTGTAGAGGAGCAC		5		
SRPR	Forward	GCTTCAGGATCTGGACTGC	81	7	61,2	XM_546411
	Reverse	GTTCCCTTGGTAGCACTGG		7-8		
B2MG	Forward	TCCTCATCCTCCTCGCT	85	1	61,2-63	AB745507
	Reverse	TTCTCTGCTGGGTGTCG		2		
YWHAZ	Forward	CGAAGTTGCTGCTGGTGA	94	2	58	XM_843951
	Reverse	TTGCATTTCCTTTTTGCTGA		2-3		
Target genes						
SOX9	Forward	CGCTCGCAGTACGACTACAC	105	6	62-63	NM_001002978
	Reverse	GGGGTTCATGTAGGTGAAGG		6		
Collagen 2a1	Forward	GCAGCAAGAGCAAGGAC	150	52	60.5-65	NM_001006951
	Reverse	TTCTGAGAGCCCTCGGT		53		
Aggrecan	Forward	GGACACTCCTTGCAATTTGAG	110	13-14	61+62	NM_001113455
	Reverse	GTCATTCCACTCTCCCTTCTC		14		
Collagen X	Forward	CCAACACCAAGACACAG	80	1	61	XM_003639401
	Reverse	CAGGAATACCTTGCTCTC		2		
Collagen 1a1	Forward	GTGTGTACAGAACGGCCTCA	109	2	61	NM_001003090
	Reverse	TCGCAAATCACGTCATCG		2		
MMP-13	Forward	CTGAGGAAGACTTCCAGCTT	250	5	65	XM_536598
	Reverse	TTGGACCACTTGAGAGTTCG		5		
ADAMTS-5	Forward	CTACTGCACAGGGAAGAG	148	5	61	XM_846025
	Reverse	GAACCCATTCCACAAATGTC		6		
TIMP-1	Forward	GGCGTTATGAGATCAAGATGAC	120	2	66	NM_001003182
	Reverse	ACCTGTGCAAGTATCCGC		3		

Table 3: Information about the used canine qPCR primers.



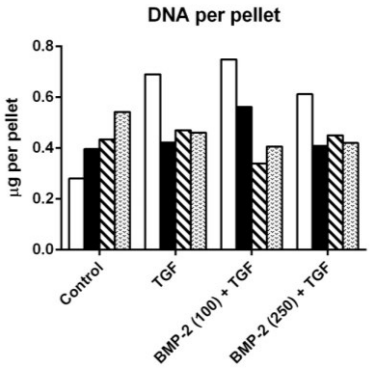


Figure 2: Results of GAG/DNA assay performed on pellets from different donors and conditions on day 21. Absolute values are shown. Note the logarithmic scale (Log 10) in figure "GAG per pellet" and "GAG per DNA". Significances are shown for the whole condition and not per donor; * = p <0.05 and $ indicates, that group is significantly different from all other groups.

GAG and DNA quantification and GAG per DNA determination

Donor 492 responded very positive to all growth factor treatments in terms of GAG, DNA and GAG per DNA content (Fig. 2). GAG and GAG per DNA content increased when TGF-β1 was combined with BMP-2 compared to TGF-β1 alone. In addition, there was a dose-dependent effect: GAG and GAG per DNA increased more in the presence of TGF-β1 + BMP-2 250 ng/mL compared to TGF-β1 + BMP-2 100 ng/mL. Donors 445 and 154 only showed a difference in terms of GAG and GAG per DNA content in the condition TGF-β1 + BMP-2 250 ng/mL. Donor 4225 showed no evidence of GAG production regardless of the culture condition.

This pattern was also observed in the GAG content measured in the culture media (Fig. 3) in which donors 492, 445 and 154 produced most GAGs in the presence of TGF-β1 + BMP-2 250 ng/mL. Most GAGs were released in the media during the second week of culture.

Furthermore, donors 492, 445 and 154 showed increased total GAG production, composed of the GAG deposited in the matrix and the total GAG released in the media over the culture period (Fig. 3), in the condition TGF-β1 + BMP-2 250 ng/mL. Though, donor 492 responded positively in every growth factor condition.

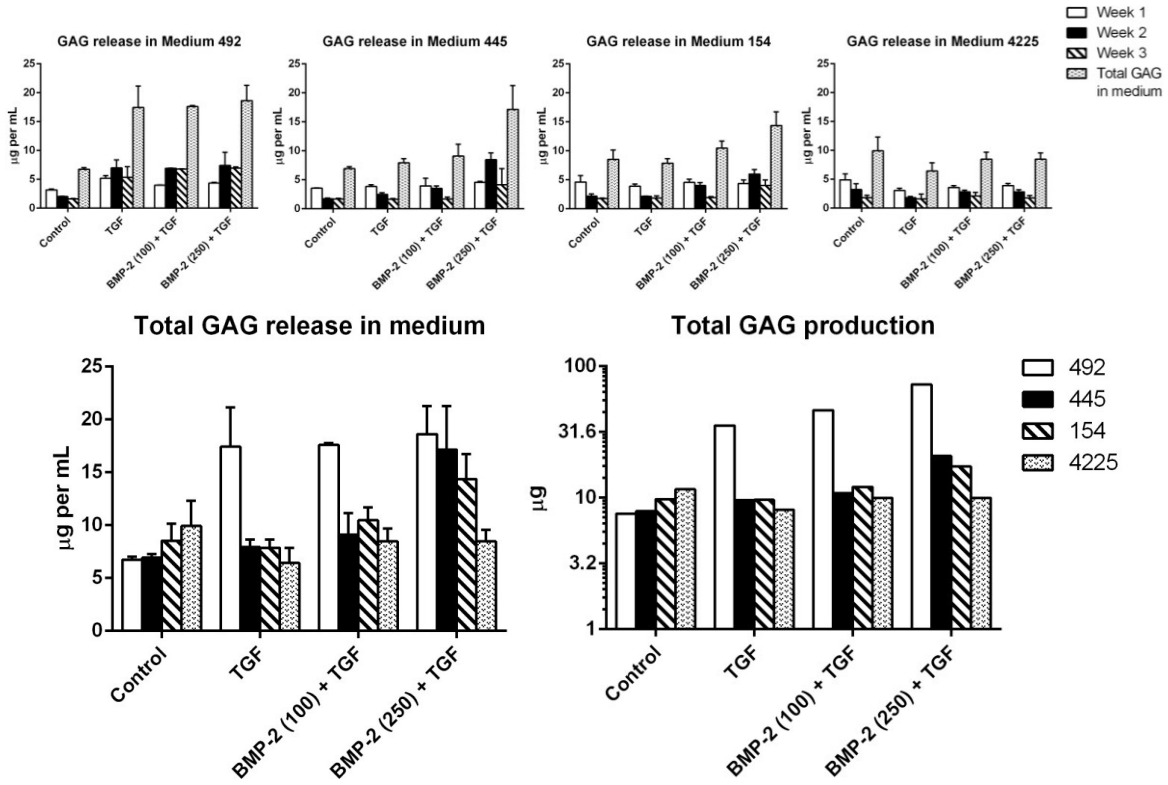


Figure 3: Precipitated GAG content in the medium and Total GAG production. µg precipitated GAGs was calculated per mL medium per culture week as well as total precipitated GAGs per condition. Graphics show GAG release in medium for each donor and total GAG release in medium concluding all donors after three weeks of culture. Total GAG production showed overall produced GAG content. Note the logarithmic scale (Log 10).

Histochemical staining (Fig. 4)

Donor 492 was positively stained by Safranin-O in all growth factor conditions, indicated that GAG-rich-matrix was deposited. The same accounts for donor 445 donor cultured with TGF-β1 + BMP-2 250 ng/mL, while the remaining of the donors did not stain positive for Safranin-O in the pericellular matrix.

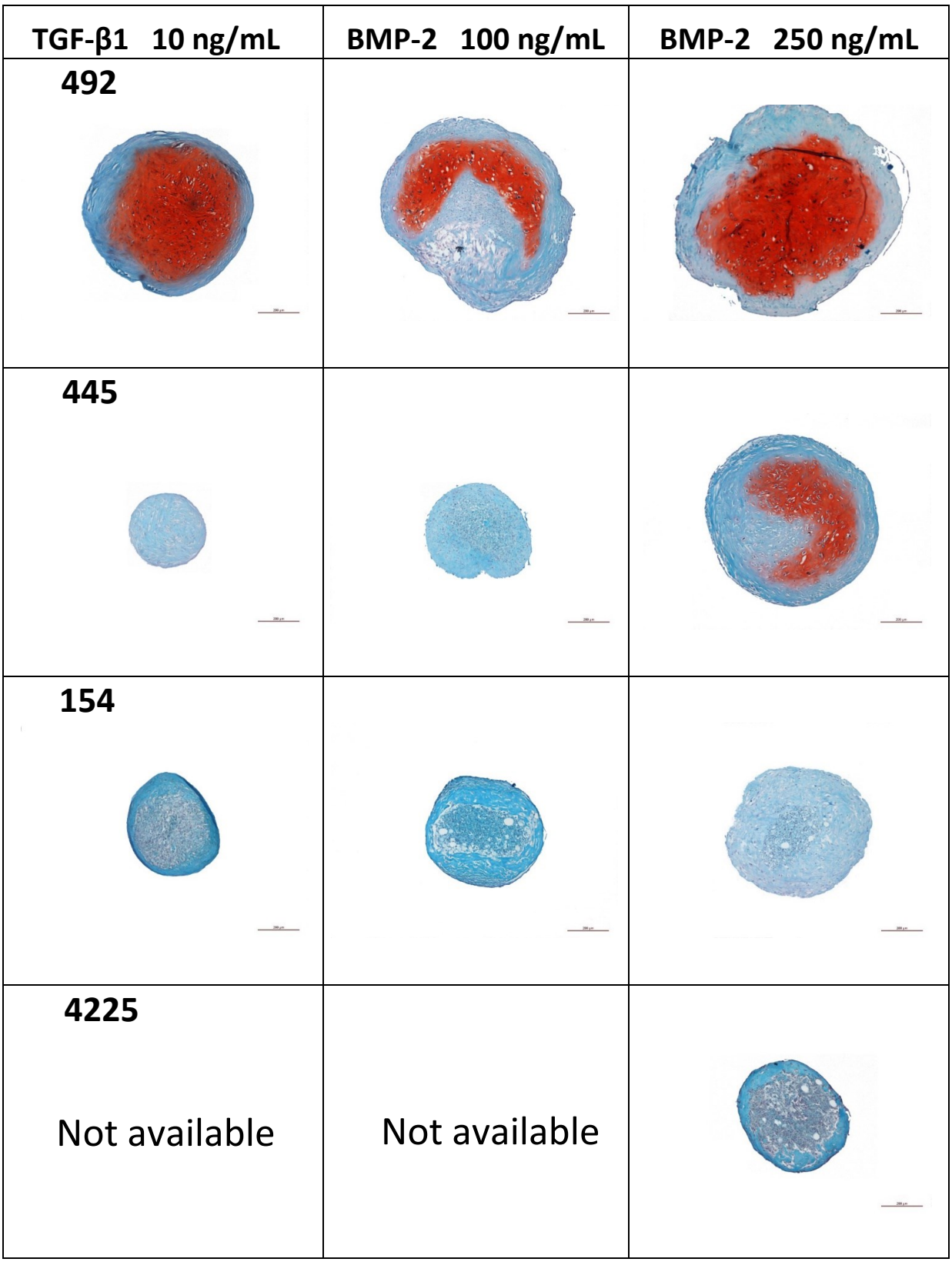


Figure 4: Stained pellets with Safranin-O for the detection of GAG-rich-matrix. Donor 492 was stained positive in all conditions for Safranin-O and the pellet containing 445 donor cells cultured with TGF-β1 combined with BMP-2 250 ng/mL, was also stained positive for Safranin-O. Scale bar = 200 µm.

qPCR

Regarding cartilage matrix production (Fig. 5), collagen type 2α1 (COL2α1) and aggrecan were significantly higher expressed for all growth factor groups on day 7 compared to day 1. Sox9 gene expression remained unchanged in the same time period. In addition aggrecan gene expression tended to be significantly increased in TGF-β1 + BMP-2 250 ng/mL on day 1 compared with TGF-β1 on day 1 ($p = 0.087$).

Furthermore, collagen type I gene expression in the TGF-β1 + BMP-2 groups on day 7 were significantly increased in comparison to the control group. In addition, there were no significant differences in expression between the growth factor groups.

With regard to genes implicated in matrix remodelling, ADAMTS 5 which degrades proteoglycans, was significantly lower expressed in TGF-β1 + BMP-2 250 ng/mL compared with the control. ADAMTS 5 tended to be significantly higher in the TGF-β1 group compared with the TGF-β1 + BMP-2 250 ng/mL ($p = 0.09288$) group on day 7. MMP-13 which degrades COL2α1, was also significantly lower expressed in TGF-β1 + BMP-2 250 ng/mL compared with TGF-β1 and TGF-β1 + BMP-2 100 ng/mL. Gene expression also tended to be higher expressed in the control compared with TGF-β1 + BMP-2 250 ng/mL ($p = 0.05813$). TIMP 1 was an inhibitor of MMP-13 and was not very high expressed (highest N-fold was 1). Though, the expression pattern was opposed to that of MMP-13: expression of TIMP 1 was higher on day 1 compared to day 7 and the expression of MMP-13 was higher on day 7 compared to day 1.

At last, collagen type X expression which was a hypertrophic marker was also measured on day 1 and day 7. No expression was detected by qPCR.

Discussion

GAG production, GAG per DNA content and gene expression profiles of aggrecan and COL2α1 showed that BMP-2 had an additive effect on the chondrogenic effects of TGF-β1 when added in the concentrations of 100 and 250 ng/mL. This observation was also confirmed by other studies. Mehlhorn *et al*. demonstrated that when human adipose derived stem cells were cultured with BMP-2 (50 ng/mL) and TGF-β1 (10 ng/mL), the combination of both growth factors had an additive effect on GAG production and on COL2α1 gene expression (16). Shen *et al*. showed that BMP-2 (100 ng/mL) increased TGF-β3 (10 ng/mL) stimulated chondrogenesis of human BMSCs in an alginate bead culture. They showed that the supplementation of both growth factors to the BMSC-culture provided a cell population with a better chondrocytic phenotype than the supplementation with TGF-β alone (4). Furthermore, Shintani *et al*. also confirmed the positive influence of TGF-β1 (10 ng/mL) combined with BMP-2 (200 ng/mL) on the chondrogenic potential of bovine synovial explants in which simultaneous administration of both growth factors resulted in improved hyaline-like characteristics of newly formed articular cartilage (17). Finally, Toh *et al*. reported that TGF-β1 (10 ng/mL) combined with BMP-2 (100 ng/mL) also had a positive influence on chondrogenesis of BMSCs derived from rabbits (18).

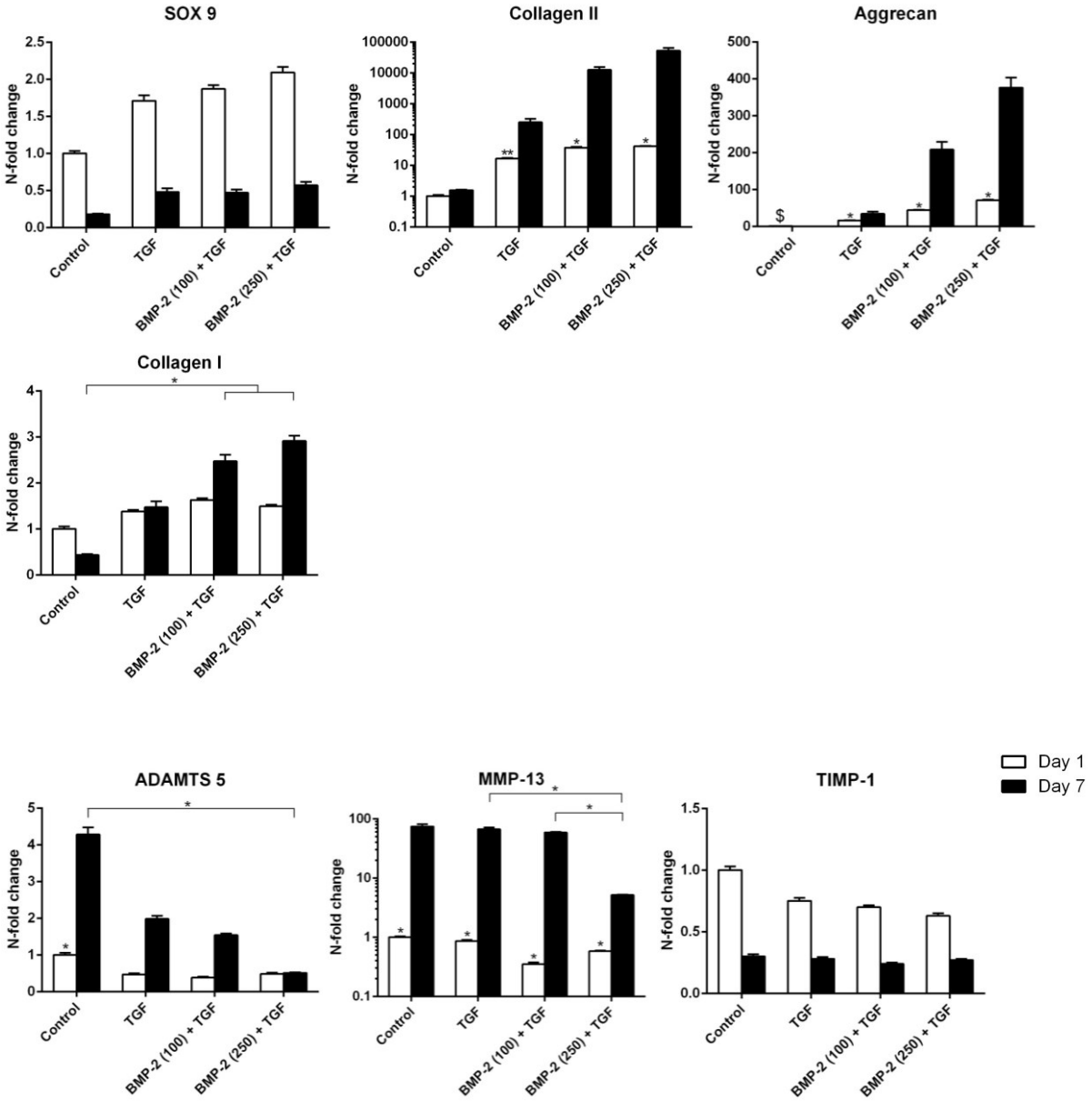


Figure 5: Relative gene expression profiles where the control of day 1 is set at 1. Teh treatment conditions are given in the X-axis: Control: control group; TGF: TGF-β1 (10 ng/mL); BMP-2 (100) + TGF: BMP-2 (100 ng/mL) + TGF-β1 (10 ng/mL) and BMP-2 (250) + TGF: BMP-2 (250 ng/mL) + β1 (10 ng/mL). N-fold changes are shown. Significances are shown when there was at least a two N-fold difference between groups; * = $p < 0.05$ and ** = $p < 0.01$, $ indicates, significantly different from all other groups for day 1 or day 7. Horizontal lines indicate differences between culture conditions. White bars represent gene expression measured on day 1 and black bars represent gene expression measured on day 7.

In this study BMP-2 was added in two different concentrations: 100 ng/mL and 250 ng/mL. Here we demonstrated that BMP-2 in the concentration of 250 ng/mL was more potent than in the concentration of 100 ng/mL, because it exerted a higher additive effect on TGF-β1 in terms of GAG production and GAG per DNA content (Fig. 2 and Fig. 3). Furthermore, histology (Fig. 4) and gene expression of aggrecan and COL2α1 supported these results and demonstrated that two donors were successfully differentiated when cultured with TGF-β1 + BMP-2 250 ng/mL. In addition, the supplementation of BMP-2 250 ng/mL to TGF-β1 was able to significantly down-regulate matrix remodelling enzymes

ADAMTS 5 and MMP-13 which means that there was less degradation of aggrecan and COL2α1 (19,20). Consequently, more aggrecan and COL2α1 could therefore be deposited in the matrix. Furthermore, no expression of collagen type X was detected by PCR on day 1 and day 7 which indicated that pellets were probably not in a hypertrophic state at those time-points. However, given that hypertrophic differentiation may still occur at a later time point, this should be further investigated with the aid of immunohistochemistry for collagen type X at the end of the culture period, i.e. 21 days.

Although, in the condition of TGF-β1 + BMP-2 250 ng/mL three out of four donors showed an increase in GAG content, this could not be confirmed by histology for donor 154. This suggests that donor 154 had not been successful in depositing GAGs in the matrix. However, some reports have been published about the sensitivity of the Safranin-O staining in which they showed that low amounts of GAG may not be detected by the staining (15,21-23), while it can be detected by using immunohistochemistry or biochemical analysis (21,23). On that account, it could be that donor 154 had as well been differentiated. To confirm this hypothesis, it is recommended to perform immunohistochemistry on Col2α1 and chondroitin and keratin sulphate (21,22,24), which are components of GAGs (25).

Moreover, donor 4225 showed no response which could be explained by the composition of the BMSC-culture and donor age. The most common way to select MSCs was by their ability to adhere to plastic and to form colonies (26). Consequently, the obtained population may be comprised of a heterogeneous cell population containing stem cells and other cell types (26-28). This could therefore have led to a decreased differentiation ability or to a restricted potential of BMSCs to chondrogenically differentiate (26,28). In addition, donor age could also have played a role. Increasing age had been reported to go hand in hand with a decrease in quantity of BMSCs that could be obtained from the tissue (29,30) and also in a reduced multipotent capacity (30-32). Increasing age had also a negative influence on the ability to form colonies (32).

In conclusion, BMP-2 had an additive effect on TGF-β1 in terms of chondrogenic differentiation potential of canine BMSCs. This effect was most optimal when BMP-2 is added in the concentration of 250 ng/mL. This concentration exerted the highest GAG production and also the highest gene expression regarding aggrecan and COL2α1. In addition, this condition was also able to down-regulate gene expression of matrix remodelling enzymes, among others ADAMTS 5. The latter, results into less matrix degradation and GAG release in the medium, and hence into an improved deposition of GAGs in the matrix.

Future research recommendation

Following the obtained results from this experiment, it is recommended to do first immunohistochemistry on COL2α1 regarding donor 154, in order to be able to draw a final conclusion whether this donor also had or had not been differentiated.

List of abbreviations

Not all abbreviations are mentioned below, due to that some are considered as common knowledge.

ADAMTS	A Disintegrin And Metalloproteinase with Thrombospondin Motifs
α-MEM	Minimum essential medium α
BMP-2	Bone-Morphogenetic Protein-2
BMSCs	Bone Marrow derived Mesenchymal Stem Cells
COL2α1	Collagen type 2α1
CS	Chondroitin Sulphate
DMEM	Dulbecco's Modified Eagle's Medium
DMMB	Dimethylmethylene blue-assay
ECM	Extracellular matrix
FCS	Fetal Calf Serum
GAG	Glycosaminoglycan
HBSS	Hanks Balanced Salt Solution
MMP	Matrix metalloproteinase
OA	Osteoarthritis
PBS	Phosphate buffered saline
TIMP	Tissue inhibitor Metalloproteinases
TGF-β1	Transforming Growth Factor bèta-1

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The differential chondrogenic effect of Transforming Growth-factor-bèta 1 and Bone Morphogenetic Protein-2 on Canine Nucleus Pulposus Cells derived from chondrodystrophic and non-chondrodystrophic dog breeds

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Abstract

Introduction: In intervertebral disc (IVD) degeneration the congenital cell population is replaced by chondrocyte-like-cells (CLCs). The replacement of the congenital cell population occurs differently in chondrodystrophic (CD-) and non-chondrodystrophic (NCD) dog breeds. In CD-dog breeds this replacement is completed after one year of age and in NCD -dog breeds after reaching middle to old age. Consequently, IVD degeneration can therefore be earlier clinical observed in CD- than in NCD-dog breeds in which also in CD-dog breeds the NP can be discovered to be calcified in contrary to NCD-dog breeds in which this phenomenon is rarely seen. Due to the differences in pathophysiology and clinical presentation, there could be a difference in cell response in growth factors employed for regenerative treatment strategies. Therefore the aim of this experiment was to investigate the influence of transforming growth factor Bèta 1 (TGF-β1) and bone-morphogenetic protein (BMP)-2 on CLCs derived from CD- and NCD-dog breeds in terms of matrix production.

Methods: CLCs derived from CD- and NCD-dog breeds were cultured in micro-aggregates, consisting of 35,000 cells. Aggregates received either basic chondrogenic media or supplemented with either TGF- β1 (10 ng/mL) or BMP-2 (100 or 250 ng/mL).
Samples were subjected to biochemistry assays for the quantification of glycosaminoglycan (GAG) and DNA content, (immuno)histochemistry and gene expression profiles.

Results: BMP-2 250 ng/mL was the most potent condition in terms of GAG-production and collagen type II and aggrecan gene expression, which was supported by immunohistochemistry. In addition, BMP-2 250 ng/mL induced a significantly lower collagen type I gene expression compared to TGF-β1. Immunohistochemistry also showed an overall decreased staining intensity for collagen I within the BMP-2 250 ng/mL groups compared to the other two growth factor conditions. Within the condition BMP-2 250 ng/mL the CD-group produced a significantly higher total GAG-content compared to the NCD-group.

Conclusion: BMP-2 250 ng/mL was the best condition for obtaining ECM production when focused on GAG-, collagen type II and collagen type I content combined with the results of the gene expression profiles obtained from day 7 and (immuno)histochemistry for both cell types. Micro-aggregates containing CLCs derived from CD-dog breeds were more productive and released more GAGs in the media when cultured with BMP-2 250 ng/mL compared to micro-aggregates containing CLCs derived from NCD-dog breeds. However, the NCD-group was more efficient in depositing GAGs in their matrix.

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Introduction

In intervertebral disc (IVD) degeneration the phenotype of the nucleus pulposus (NP) changes from a highly hydrated tissue containing the congenital notochordal cell (NC) population which produce high amounts of proteoglycans and low amounts of collagen type 2α1 (COL2α1), towards a more firmer structure containing a different cell population called chondrocyte-like-cells (CLCs) (1-3). These CLCs produce less amount of proteoglycans and a higher amount of disorganized collagen fibres which causes the change in phenotype of the NP. This process is called *chondrification* (4-8). As a consequence of the changed phenotype, the NP is impaired in its function as a load-bearing tissue, making it more prone to tissue damage (2,9).

The replacement of the congenital cell population by CLCs, is different in chondrodystrophic (CD-) dog breeds, such as the Beagle and non-chondrodystrophic (NCD-) dog breeds, such as the German Shepherd (1,10). Chondrodystrophic is a term which is used to refer to dog breeds in which an abnormal enchondral ossification occurs, leading to disproportionally short extremities (9,11). In CD-dog breeds the replacement of these cells is completed after one year of age, in contrast to NCD-dog breeds in which it is completed after reaching middle to old age (8,9,11). After the completion of this process, the NP can further calcificate in CD-dog breeds. This calcification is rarely observed in NCD-dog breeds (10,11).

Currently, there are only symptomatic treatments for canine IVD degeneration. In order to develop a curable treatment, researchers are focused on regenerative medicine, which has as main aim to biologically repair the degenerated tissue. This could be accomplished with help from regenerative cells, such as CLCs combined with the use of growth factors (9). However, IVD degeneration in canine is still for a greater part unexplored (2). For example, no research has been performed yet towards the response of CLCs derived from CD- and NCD-dog breeds in terms of *in vitro* matrix production with the employment of growth factors. CD- and NCD-dog breeds show a different pathophysiology in which the change in cell population occurs earlier in CD- than in NCD-dog breeds and the same accounts for the clinical presentation of the disease (9,11). In addition, there is also a difference between the clinical presentation in which the NP of CD-dogs breeds is often calcified in contrary to NCD-dog breeds in which this phenomenon is rarely observed (10,11). As a consequence, it could be that there is a difference in response in matrix production when these cells are cultured *in vitro* under the influence of specific growth-factors, such as transforming growth-factor Bèta 1 (TGF-β1) and bone-morphogenetic protein (BMP)-2. These growth factors are able to stimulate matrix production, such as COL2α1 and proteoglycans, namely aggrecan (12,13). Therefore, the aim of this experiment was to investigate the influence of TGF-β1 and BMP-2 on CLCs derived from CD- and NCD-dog breeds in terms of matrix production and the difference in cell response between CLCs derived from CD- and NCD-dog breeds. We focused on glycosaminoglycan (GAG) content which are part of proteoglycans; DNA content in order to determine proliferation rate and for normalizing GAG content for the amount of present cells; gene expression profiles and finally, (immune)histochemistry for visualizing GAG-, COL2α1- and collagen 1-rich matrix.

Materials and methods

Bio-Bank and expansion of BMSCs

NPCs derived from CD- and NCD-dogs were obtained from the Veterinary Bio-bank. Five CD- and five NCD-dog donors were employed (Table 1 and 2). Cells were thawed in P0 or P1 and

expanded per donor in expansion media, containing Dulbecco's Modified Eagle's Medium, high glucose, GlutaMax™ pyruvate (DMEM; Invitrogen, 31966), 10% fetal calf serum (FCS; high performance, Gibco, 16000-044), 1% penicillin-streptomycin (PPA cell culture company; P11-010), 1 ng/mL fibroblast growth factor (AbD Serotec; PHP105), 0.1 mM ascorbic acid 2-phosphate (Sigma Aldrich, A-8960), 0.2% Fungizone (Invitrogen, 15290) and 0.0001 µM dexamethasone (Sigma Aldrich, D8893) till reaching a confluency of 60-90% in P2. Hereafter, donor cells derived from CD- or NCD-dogs were pooled together in equal ratios and were cryopreserved in nitrogen.

For this experiment, the mixture of cells derived from CD- or NCD-dogs in P2 were thawed, expanded in expansion media and cultured under normoxic conditions, 5% CO_2 at 37 °C. During the expansion phase, medium was replaced twice per week. When cells had reached 70-80% confluence, they were prepared to form micro-aggregates consisting of 35,000 cells.

For the preparation of micro-aggregates, cells were washed with Hanks balanced salt solution (HBSS; Gibco by Life technologies, 14025-050) followed by tripsinization (Triple Ex; Gibco by Life technologies, A12177-01). Hereafter, expansion media containing FCS was added to inactivate trypsin. Cells were counted by using the Bio Rad TC 20 (Catalog #145-0101) cell counting device and diluted in basic chondrogenic media, containing Dulbecco's Modified Eagle's Medium, high glucose, GlutaMax™ pyruvate (DMEM; Invitrogen, 31966), 1% selenous acid (ITS+) premix (B&D, 354352), 1% penicillin-streptomycin (PPA cell culture company, P11-010), 0.04 mg/mL L-prolin (Sigma Aldrich, P-5607), 0.2% fungizone (Invitrogen, 15290), 1.25 mg/mL bovine serum albumin (Sigma Aldrich, A9418) and 0.1 mM ascorbic acid 2-phosphate (Sigma Aldrich, A-8960), was added. Subsequently, micro-aggregates were formed in which they were unintentionally plated as micro-aggregates in a 96-wells-plate (Costar, 7007). Micro-aggregates were divided into four groups: control group and three growth-factor groups and incubated under normoxic conditions, 5% CO_2 at 37 °C. Micro-aggregates were subjected to RT-qPCR, GAG- and DNA-assay, and (immune)histochemistry.

Donor number	CD-breed	Gender	Age
402932	Beagleton	Male	121 months
7200013	Beagle	Male	Unknown
7140314	Beagle	Male	40 months
7207859	Beagle	Male	58 months
7199261	Beagle	Male	62 months

Table 1: characteristics of CD-derived CLC-donors.

Donor number	NCD-breed	Gender	Age
879061	Mixed	Male	26 months
121114	German sheperd	Male	120 months
77662	Mixed	Male	14.3 months
881546	Mixed breed	Male	14 months
100913	Jack russel	Male	132 months

Table 2: Characteristics of NCD-derived CLC-donors.

Microscopy
During the entire chondrogenic differentiation period, micro-aggregates were microscopically observed in order to get a good overview of their response to the tested culture conditions.

In-vitro stimulation of matrix production
Four groups per cell type (CD- or NCD-derived CLCs) were used in this experiment which had a culture period of four weeks. The control group received 50 µL of basic chondrogenic media and the growth-factor groups received 50 µL of chondrogenic media supplemented with either TGF-β1 (10 ng/mL; R&D systems, 240-B-010) or BMP-2 (100 or 250 ng/mL; Generous gist by TETEC AG). Media changing was performed twice per week (Tuesday-Friday).

RNA-isolation and real-time quantitative polymerase chain reaction (RT-qPCR)
Micro-aggregates were harvested on day 7 (N=7 per group) and were crushed with nitrogen and special RNAse-free pestles (Argos technologies, 9551-901), followed by adding 346,5 µL of RLT and 3,5 µL of β-mercaptoethanol. Total RNA extraction was performed by using miRNeasy Micro Kit (Qiagen) according to the manufacturer`s manual. Hereafter, a standard RNA input of 10 µL was reverse transcribed to obtain cDNA according to the manufacturer (Bio-Rad, iScript™cDNA Synthesis Kit, 170-8891). cDNA was used as input for RT-qPCR.

RT-qPCR was performed using iQ™ SYBR Green Supermix Kit (Bio-Rad, 170-8887) on genes related to chondrogenic differentiation (*SOX9; Collagen 2a1; Aggrecan),* TGF-β-, BMP-2 and WNT-pathway *(ALK-5; ALK-1; Pai-1; ID-1; Axin-2*), proliferation and apoptosis (*Cyclin D1; Bcl-2; BAX; Casp-3*), hypertrophy (*Collagen X a1; PTH-R1; IHH*), fibrosis (*Collagen 1a1*) and matrix degrading enzymes and inhibitors (*MMP-13, Adamts-5; TIMP-1*), according to manufacturer`s guidelines (Bio-Rad). Primer information can be found in table 3.

Averaged results of housekeeping genes *GAPDH, HPRT, RPS19* and *SDHA* were used for normalization. N-fold change of measured genes was calculated compared to the CD control group. Standard error was calculated from the N-fold change.

Biochemistry
GAG quantification
The dimethylmethylene blue (DMMB)-assay was used to quantify glycosaminoglycan (GAG) content within the micro-aggregates and in the media after the four weeks of culture. The dye binds to the repeating negative charges, sulphates, on the GAGs. Hereafter, a shift in the absorption spectrum can be measured (14) at wavelengths of 540 and 595 nm.
The DMMB-assay was carried out as previously described (15). In brief, micro-aggregates (N = 7 per group) were digested overnight in a papain digestion solution at 60 °C. A standard curve was made using 50 times diluted chondroitin sulphate (CS, Sigma Aldrich, C4384; stock: 0.5 mg/mL) and phosphate buffered saline (PBS). The standard was made according to the manufacturer (Sigma Aldrich) and ranged from 0 to 10.0 µg/mL CS.

Genes	Extension	Sequence	AmpliconSize	EXON	Anealing temp (°C)	Accession no.
Reference genes						
GAPDH	Forward	TGTCCCCACCCCCAATGTATC	100	2	58	NM_001003142
	Reverse	CTCCGATGCCTGCTTCACTACCTT		2		
HPRT	Forward	AGCTTGCTGGTGAAAAGGAC	104	5-6	56-58	NM_001003357
	Reverse	TTATAGTCAAGGGCATATCC		7		
RPS19	Forward	CCTTCCTCAAAAAGTCTGGG	95	2-3	61-63	XM_005616513
	Reverse	GTTCTCATCGTAGGGAGCAAG		3		
SDHA	Forward	GCCTTGGATCTCTTGATGGA	92	6	61	DQ402985
	Reverse	TTCTTGGCTCTTATGCGATG		6		
Target genes						
SOX9	Forward	CGCTCGCAGTACGACTACAC	105	6	62-63	NM_001002978
	Reverse	GGGGTTCATGTAGGTGAAGG		6		
Collagen 2a1	Forward	GCAGCAAGAGCAAGGAC	150	52	60.5-65	NM_001006951
	Reverse	TTCTGAGAGCCCTCGGT		53		
Aggrecan	Forward	GGACACTCCTTGCAATTTGAG	110	13-14	61+62	NM_001113455
	Reverse	GTCATTCCACTCTCCCTTCTC		14		
ALK-5 (TGFβR1)	Forward	GAGGCAGAGATTTATCAGACC	116	4	59,5	XM_538750.4
	Reverse	ATGATAATCTGACACCAACCAG		5		
ALK-1 (ACVRL)	Forward	CCTTTGGTCTGGTGCTGTG	107	9	61	XM_005636802.1
	Reverse	CGAAGCTGGGATCATTGGG		10		
Pai-1	Forward	AAACCTGGCGGACTTCTC	98	5	61,5	NM_001197095
	Reverse	ACTGTGCCACTCTCATTCAC		6		
ID-1	Forward	CTCAACGGCGAGATCAG	135	2	59,5	XM_847117.3
	Reverse	GAGCACGGGTTCTTCTC		3		
Axin-2	Forward	GGACAAATGCGTGGATACCT	141	1	60	XM_548025
	Reverse	TGCTTGGAGACAATGCTGTT		1		
Cyclin D1	Forward	GCCTCGAAGATGAAGGAGAC	117	2	60	NM_001005757.1
	Reverse	CAGTTTGTTCACCAGGAGCA		3		
Bcl-2	Forward	GGATGACTGAGTACCTGAACC	80	2	61.5-63	NM_001002949
	Reverse	CGTACAGTTCCACAAAGGC		3		
BAX	Forward	CCTTTTGCTTCAGGGTTTCA	108	2-3	58-59	NM_001003011
	Reverse	CTCAGCTTCTTGGTGGATGC		3		

Casp-3	Forward	CGGACTTCTTGTATGCTTACTC	89	8	61	NM_001003042
	Reverse	CACAAAGTGACTGGATGAACC		9		
Collagen X	Forward	CCAACACCAAGACACAG	80	1	61	XM_003639401
	Reverse	CAGGAATACCTTGCTCTC		2		
PTH-R1	Forward	GACCACATCCTTTGCTGG	217	5	51	NM_001003155
	Reverse	CAAACACCTCCCGTTCAC		6/7		
IHH	Forward	TCACCACTCAGAGGAGTCG	172	2	60	XM_545653
	Reverse	GTGCTCAGACTTGACGGAG		2/3		
Collagen 1a1	Forward	GTGTGTACAGAACGGCCTCA	109	2	61	NM_001003090
	Reverse	TCGCAAATCACGTCATCG		2		
MMP-13	Forward	CTGAGGAAGACTTCCAGCTT	250	5	65	XM_536598
	Reverse	TTGGACCACTTGAGAGTTCG		5		
ADAMTS-5	Forward	CTACTGCACAGGGAAGAG	148	5	61	XM_846025
	Reverse	GAACCCATTCCACAAATGTC		6		
TIMP-1	Forward	GGCGTTATGAGATCAAGATGAC	120	2	66	NM_001003182
	Reverse	ACCTGTGCAAGTATCCGC		3		

Table 3: Canine primers used for RT-qPCR.

Hereafter, samples were measured singular in which 100 µL of the diluted samples in PBS - ethylenediaminetetraacetic acid (EDTA) was pipetted in a 96-wells-plate with flat bottom (Greiner Bio-one 655191), followed by adding 200 µL of DMMB-solution pH 3.0 (Sigma Aldrich) to the wells. For measuring GAG content in the culture media, media (N = 7 per group) was obtained every time media was changed and it was collected per week. Media was measured diluted with PBS-EDTA in which 100 µL of diluted media was pipetted in a 96-wells-plate with flat bottom (Greiner Bio-one 655191), followed by adding 200 µL of DMMB-solution pH 3.0 (Sigma Aldrich). Absorption degrees were measured at 540 and 595 nm wavelengths by using a spectrophotometer. Results were extrapolated from the standard curve and both were corrected for the blank, which corresponds with the first outcome of the standard curve (0 µg/mL CS). Finally, absolute GAG (µg) content per micro-aggregate and precipitated GAG content in the culture media were calculated as well as total GAG production which is the sum of the total GAG content precipitated into the medium and the GAG content of the micro-aggregate.

DNA quantification and GAG per DNA determination

For measuring DNA-content, digested micro-aggregates obtained on day 0 and day 28 were also used to measure DNA-content. Digested samples were processed with Qubit™ dsDNA BR Assay Kit (Invitrogen; Q32853) and results were measured by the Qubit® 2.0 Fluorometer (Invitrogen; Q32866). Samples were prepared to be measured 40 times diluted and a calibration curve was obtained according to the manufacturer`s manual. The obtained results were calculated per µg/mL and further processed to obtain absolute DNA (µg) per micro-aggregate and per condition. Then absolute amount of GAG per DNA (µg) per condition was determined.

(Immuno)Histochemical staining

After four weeks of culture, micro-aggregates (N = 3 per group) were fixated overnight with buffered neutral formalin with 10% eosin. Following day, they were embedded in 2.4% alginate (Sigma Aldrich, A2033) and 100 mM $CaCl_2$ with formalin and stored in white embedding cassettes in 70% alcohol. Samples were dehydrated through graded alcohol steps.

After dehydration, samples were embedded in paraffin and cut into 4 µm sections. Sections were stained with either Safranin-O/Fast Green staining, which is used to indicate the presence of GAG-rich matrix (16) or stained immunohistochemically for COL2α1 and collagen 1. Sections were first deparaffinised, removed from alginate and hydrated to distilled water. For the Safranin-O staining, sections were stained with Mayer`s Hematoxylin for 10 seconds (J.T.Baker, 3870), 0.4% Fast Green FCF (Sigma Aldrich, F7252) for 4 minutes and 0.125% Safranin-O (Sigma Aldrich, 58884) for 7 minutes. For immunohistochemistry, sections were incubated with 0.3% H_2O_2 (Boom) solved in Phosphate Buffered Saline (PBS) for 10 minutes on a rocker (38 oscillations/minute). Thereafter, sections were washed twice for 5 minutes with PBS containing 0.1% Tween (PBS-T 0.1%, Boom). Then antigen retrieval was performed with Pronase (1 mg Pronase (Roche, 11459643001) solved in 1 mL PBS) for 30 minutes at 37 °C, followed by washing twice with PBS-T 0.1% for 5 minutes. Subsequently, second antigen retrieval was performed with Hyaluronidase (10 mg Hyaluronidase (Sigma, H3506) solved in 1 mL PBS) for 30 minutes at 37 °C, followed by washing twice in PBS-T 0.1% for 5 minutes. Sections were blocked with PBS/Bovine Serum Albumin 5% (PBS/BSA 5%) for 30 minutes and then incubated overnight at 4 °C with the

primary antibody for COL2α1: Col2α1 mouse monoclonal antibody (DSHB, II-II6B3; 42 µg/mL) diluted 1/2000 in PBS/BSA 5% and for collagen I: collagen I mouse monoclonal antibody (ab6308, 100 µg/mL) diluted 1/1500 in PBS/BSA 5%. The negative controls were incubated with normal mouse IgG_1 (Santa Cruz Biotechnology, 3877) diluted 1/750 in PBS/BSA 5%. Following morning, sections were washed twice in PBS-T 0.1% for 5 minutes and then incubated with the secondary antibody which was conjugated with HRP (Envision, Dako, K4001), for 1 hour. Sections were washed twice with PBS for 5 minutes and incubated with DAB peroxidase substrate solution (DAKO, K3468) for 1 minute. Sections were cleaned in MiliQ, counterstained with Hematoxylin (Vector Laboratories, H3404) for 1 minute and cleaned in running tap water for 10 minutes. Sections were dehydrated through graded alcohol steps: 70% and 80% for 3 minutes, twice in 96% and 100 % for 5 minutes, followed by twice in xylene for 5 minutes and mounted (*Based on our institutional immunohistochemistry protocol for collagen type I and II*). Images from stained sections were made with a digital microscopic camera (Leica, DFC425C).

Statistical analysis

Statistical analysis was performed by using SPSS 19. Data were tested if they were normally distributed by performing *normality plots with tests* in SPSS 22. Normally distributed data were analysed with one-way ANOVA followed by Tukey`s post-hoc test to correct for multiple testing. Non-normally distributed data were analysed with Mann-Whitney test followed by the Benjamini-Hochberg correction to correct for multiple testing. All PCR data and the outcomes of the DMMB-assay are represented as mean plus standard error. A P-value of less than 0.05 was considered to be significant.

Results

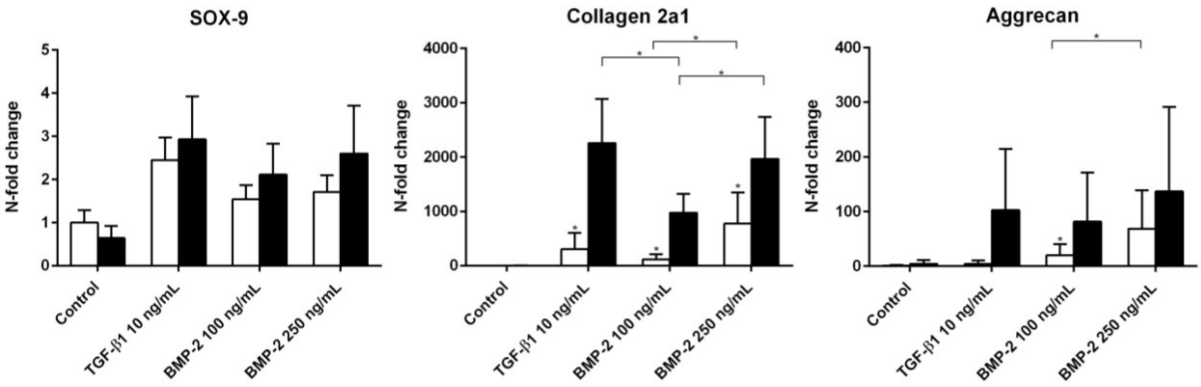
Microscopy

During the culture period, increase in size was observed in micro-aggregates from growth factor conditions and micro-aggregates from the growth factor groups from the CD-group were on the overall larger than the NCD-group.

RT-qPCR

Regarding gene expression patterns of matrix production (Fig. 1), it was seen from COL2α1 gene expression that there was a tendency towards higher expression in the CD-group when cultured with BMP-2 250 ng/mL in contrary to the NCD-group. To the contrary, the NCD-group had higher COL2α1 expression within the growth-factor groups compared with the CD-group when treated with the same growth-factors. Aggrecan expression tended to be higher expressed in CD-group as well as in NCD-group when cultured with BMP-2 250 ng/mL compared to the other growth-factor conditions.

Collagen type I gene expression, an indicator of de-differentiation and fibrosis, was significantly decreased in the condition of BMP-2 250 ng/mL compared with TGF-β1 within the NCD-group. Furthermore, within the growth factor conditions TGF-β1 and BMP-2 100 ng/mL on day 7, collagen type I was increased in the NCD-group compared with the CD-group, while this was not observed in the condition BMP-2 250 ng/mL.



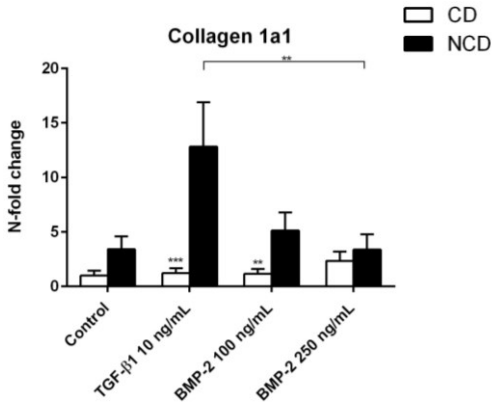


Figure 1: Gene expression profiles regarding ECM production by micro-aggregates comprised of CLCs derived from CD- or NCD-dog breeds on day 7. Aggrecan gene expression tended to be significantly higher in the BMP-2 250 ng/mL compared with TGF-β1 10 ng/mL within the CD-group ($p = 0.088$). Significances are shown when there was at least a two N-fold difference between groups: * = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$. Horizontal lines indicate differences between culture conditions. White bars represent the CD-group and black bars represent the NCD-group.

Gene expression profiles of matrix remodelling enzymes (Fig. 2) showed no differences in gene expression of ADAMTS 5 between the culture conditions and between CD-group and NCD-group. MMP-13 gene expression tended to be higher in the CD-group as well as in NCD-group in the condition BMP-2 100 ng/mL compared to the other conditions. This gene was significantly higher expressed in the CD-group cultured with BMP-2 250 ng/mL compared with the NCD-group. TIMP 1 tended to be higher expressed in the NCD-group compared with the CD-group within the first three conditions.

Furthermore, gene expression of hypertrophic markers: collagen type X, parathyroid hormone 1 receptor and Indian Hedgehog was undetectable.

In addition, gene expression of Pai-1, ALK-1 and ALK-5 was detected, which is indicative for TGF-β signalling. Gene expression of ALK-1 and ALK-5 were used to determine the ALK1-ALK5 ratio. Also expression of ID-1 was detected which is indicative for BMP-2 signalling. In addition, there was as well gene expression measured of Axin 2, which is indicative for WNT-signalling, though there was no significant difference regardless of the groups.

Markers regarding proliferation and apoptosis (Fig. 4): Cyclin D1, a proliferation marker, tended to be significantly higher in the condition TGF-β1 and tended also to be higher in the condition BMP-2 250 ng/mL, within the NCD-group compared with the CD-group. This was in line with the BAX-Bcl2 ratio which also showed that the ratio in the condition of TGF-β1 and BMP-2 250 ng/mL in the NCD-group compared with the CD-group was higher than 1.0 which indicated that there was more cell survival in the NCD-group compared with the CD-group. Casp-3 an apoptotic marker was not significantly different regardless of the conditions.

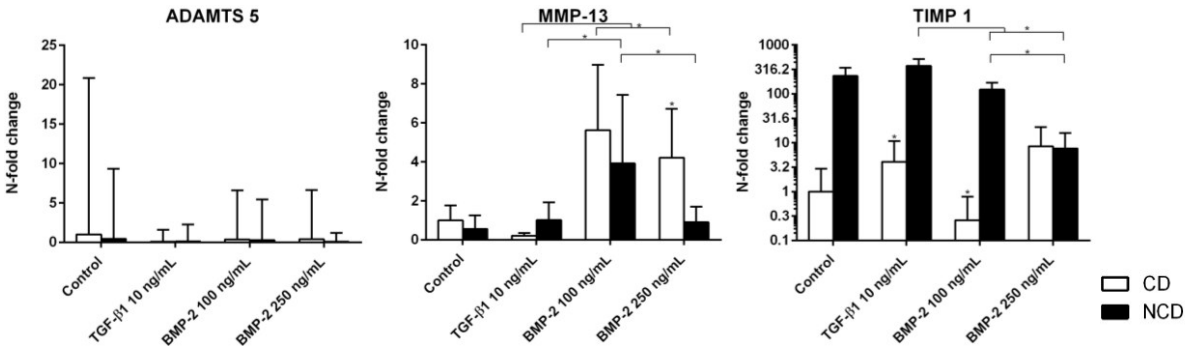


Figure 2: Gene expression profiles regarding matrix remodelling enzymes by micro-aggregates comprised of CLCs derived from CD- or NCD-dog breeds on day 7. Significances are shown when there was at least a two N-fold difference between groups: * = p <0.05. Horizontal lines, indicate differences between culture conditions. White bars represent the CD-group and black bars represent the NCD-group.

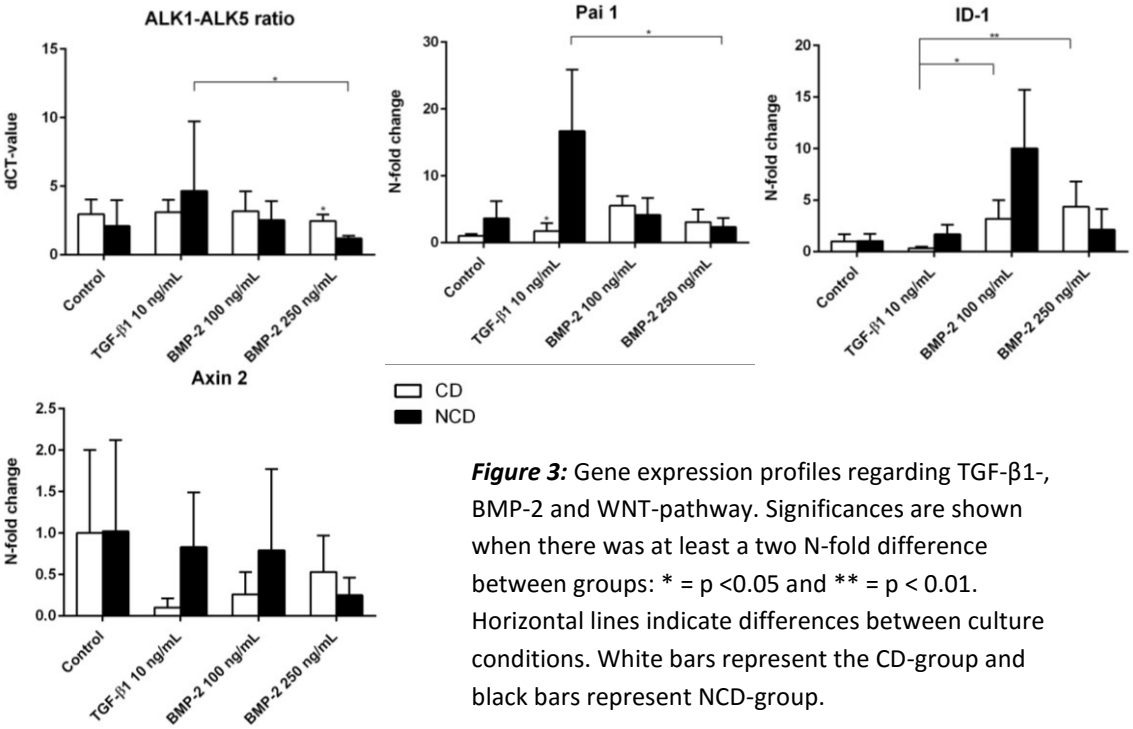


Figure 3: Gene expression profiles regarding TGF-β1-, BMP-2 and WNT-pathway. Significances are shown when there was at least a two N-fold difference between groups: * = p <0.05 and ** = p < 0.01. Horizontal lines indicate differences between culture conditions. White bars represent the CD-group and black bars represent NCD-group.

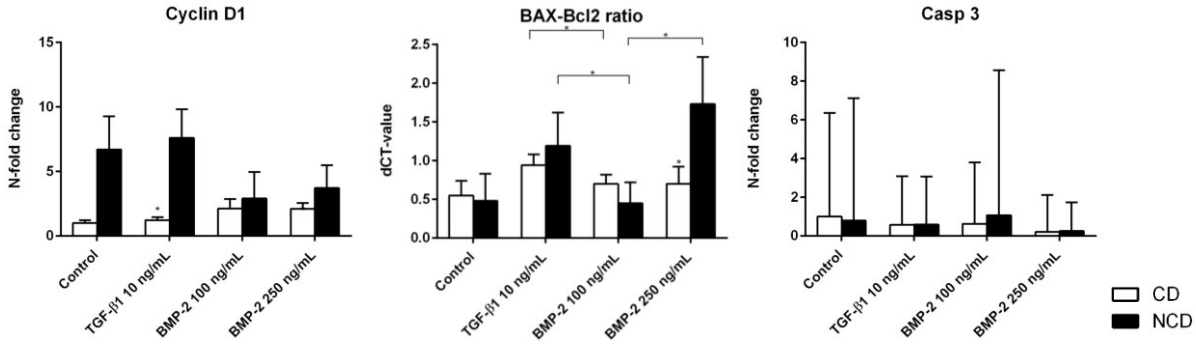


Figure 4: Gene expression regarding proliferation and apoptosis markers. Significances are shown when there was at least a two N-fold difference between groups: * = p <0.05. Horizontal lines indicate differences between culture conditions. White bars represent the CD-group and black bars represent the NCD-group.

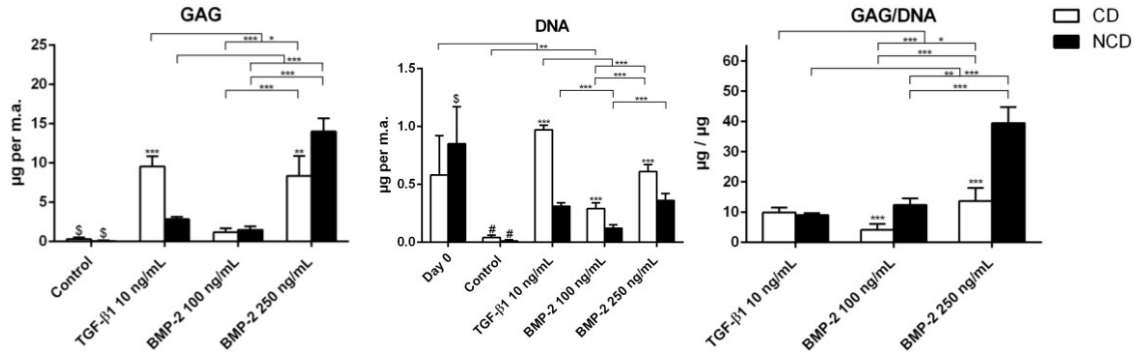


Figure 5: Results of the GAG, DNA and GAG per DNA content performed on micro-aggregates (m.a) containing CLCs derived from CD- or NCD-dog breeds cultured under different conditions for 28 days. Absolute values are shown. Significances are shown as: $ = significantly different from all other conditions within the CD or NCD group; # = significantly different from all other conditions within the CD or NCD group, except for day 0; * = p<0.05; ** = p<0.01 and *** = p<0.001. White bars represent CD-group and black bars represent NCD-group.

GAG and DNA quantification and GAG per DNA determination

BMP-2 250 ng/mL supplementation resulted in the highest GAG content deposited in the aggregates within the NCD-group (Fig. 5), while within the CD-group the higher GAG deposition was observed in the TGF-β1 aggregates. In addition, the CD-group deposited more GAGs in the aggregates cultured with TGF-β1 compared with the NCD-group. The opposite was true for the condition BMP-2 250 ng/mL. Furthermore, there was a significant increase in DNA content in the TGF-β1 group in the CD-group compared with the NCD-group. Correcting the GAG content of the aggregates for DNA content gave an indication of cell activity. GAG per DNA production was the highest in the condition BMP-2 250 ng/mL for CD-group as well as for NCD-group. This was also further supported for the CD-group by the GAGs released in the culture medium (Fig. 6). The NCD-group released more GAGs in the culture medium when cultured with TGF-β1 or BMP-2 250 ng/mL compared to the condition BMP-2 100 ng/mL. Total GAG production also showed that BMP-2 250 ng/mL produced overall most GAGs in the CD-group. TGF-β1 and BMP-2 250 ng/mL produced overall most GAGs in the NCD-group when compared to the condition BMP-2 100 ng/mL. The condition BMP-2 250 ng/mL produced overall more GAGs in the CD-group compared with the NCD-group.

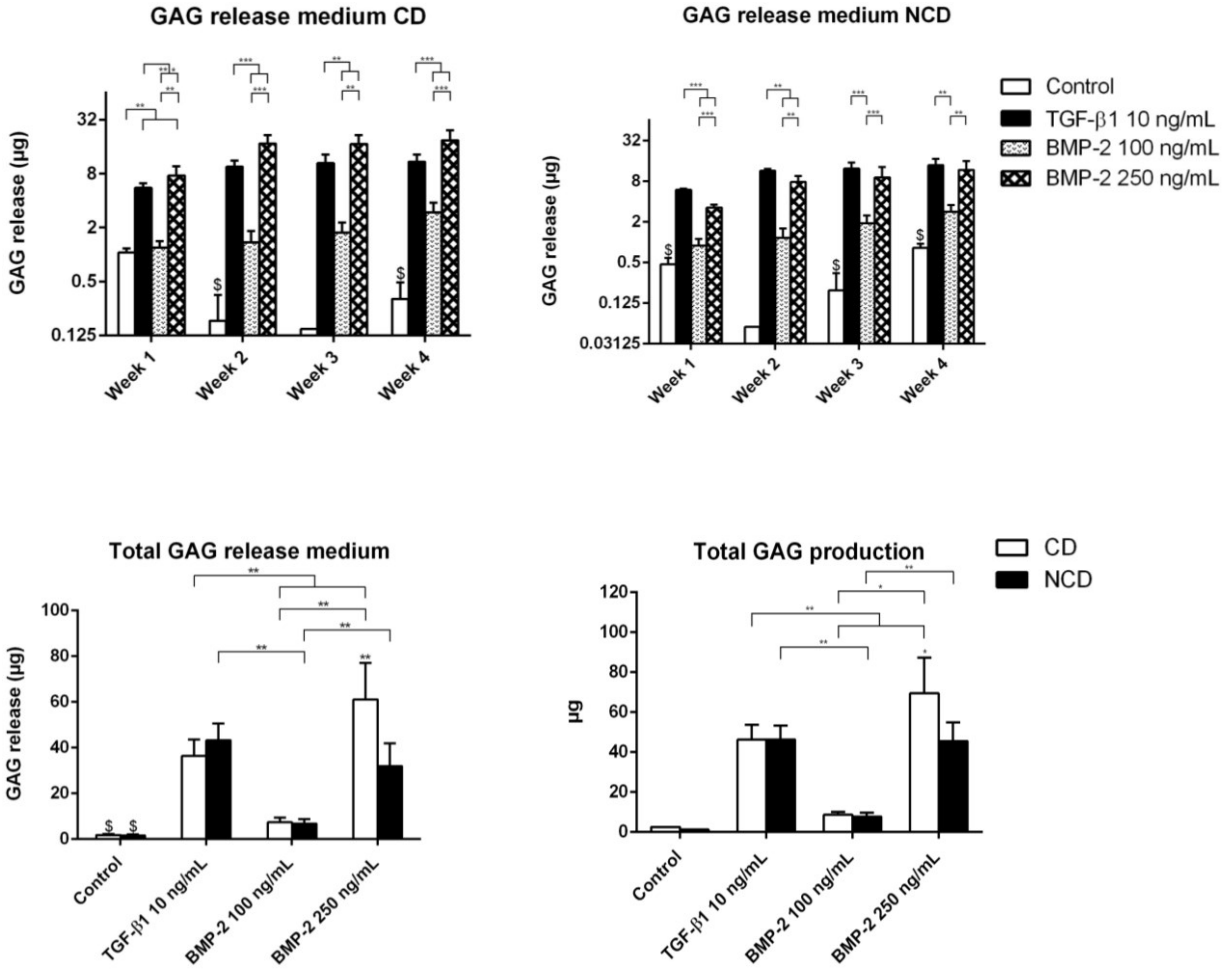


Figure 6: GAG-release in medium showed for the CD- and NCD-group comprised of micro-aggregates cultured under different conditions for 28 days. GAG-release in medium is first shown for all weeks and conditions for CD- and NCD-group alone. Total GAG release in medium over the total culture period is shown for CD- and NCD-group combined. Finally, Total GAG production (sum of total GAG release in medium + GAG content) is shown for CD and NCD combined. Significances are shown as: $ = significantly different with all other conditions within the group CD or NCD; * = $p<0.05$; ** = $p<0.01$ and *** = $p<0.001$. White bars indicate CD-group and black bars indicate NCD-group.

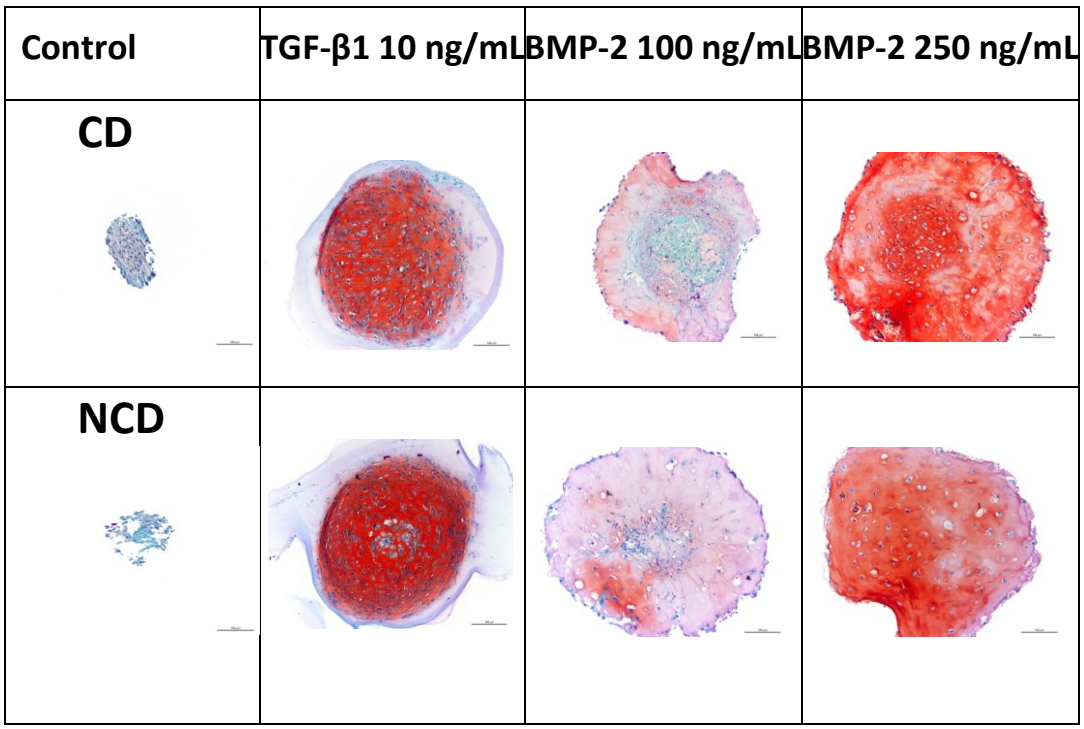


Figure 7: Representative Safranin-O/Fast Green staining of micro-aggregates containing CLCs derived from CD- and NCD-dog breeds after 28 days of culture in basic chondrogenic media or chondrogenic media supplemented with TGF-β1 10 ng/mL or BMP-2 100 ng/mL or BMP-2 250 ng/mL. Scale bar indicates 100 µm.

Immunohistochemistry

Safranin-O/Fast Green staining (Fig. 7) showed positive red-staining in the groups treated with growth factors, indicating that GAG-rich-matrix was produced and deposited (16). Furthermore, it was evident that staining intensity was increased in the pericellular environment.

Collagen type I immunohistochemistry (Fig. 8) confirmed the presence of collagen type 1 in all deposited matrix in all groups treated with growth factors, regardless of the type of growth factor. Less intense staining was present in the condition BMP-2 250 ng/mL compared to the other growth factor conditions for both CD- and NCD-groups. The controls did not stain positive for collagen type I.

COL2α1 immunohistochemistry (Fig. 9) confirmed the presence of COL2α1 in all groups treated with growth factors. The controls did not stain positive COL2α1.

Discussion

These results suggested that BMP-2 250 ng/mL was the best condition for obtaining ECM production based on GAG-, GAG per DNA, immunohistochemistry of collagen type I and COL2α1 on day 28 combined with the respective gene expression profiles obtained on day 7. Furthermore, it appeared that in the presence of BMP-2 250 ng/mL the CD-group was more active and produced overall higher amounts of GAG (total GAG content precipitated in the culture medium combined with GAG content of the aggregate) compared to the NCD-group. However, the NCD-group was more successful in depositing GAGs in their matrix in comparison to the CD-group. The proteoglycans are embedded between COL2α1 fibres which together formed a sheet-like structure (17). Hence, the higher release of GAGs in the medium seen in the CD-group could be explained by the fact that MMP-13 gene expression

was higher in the CD-group than in the NCD-group, resulted in respectively more COL2α1 degradation, followed by loss of proteoglycans into the media (Fig. 6).

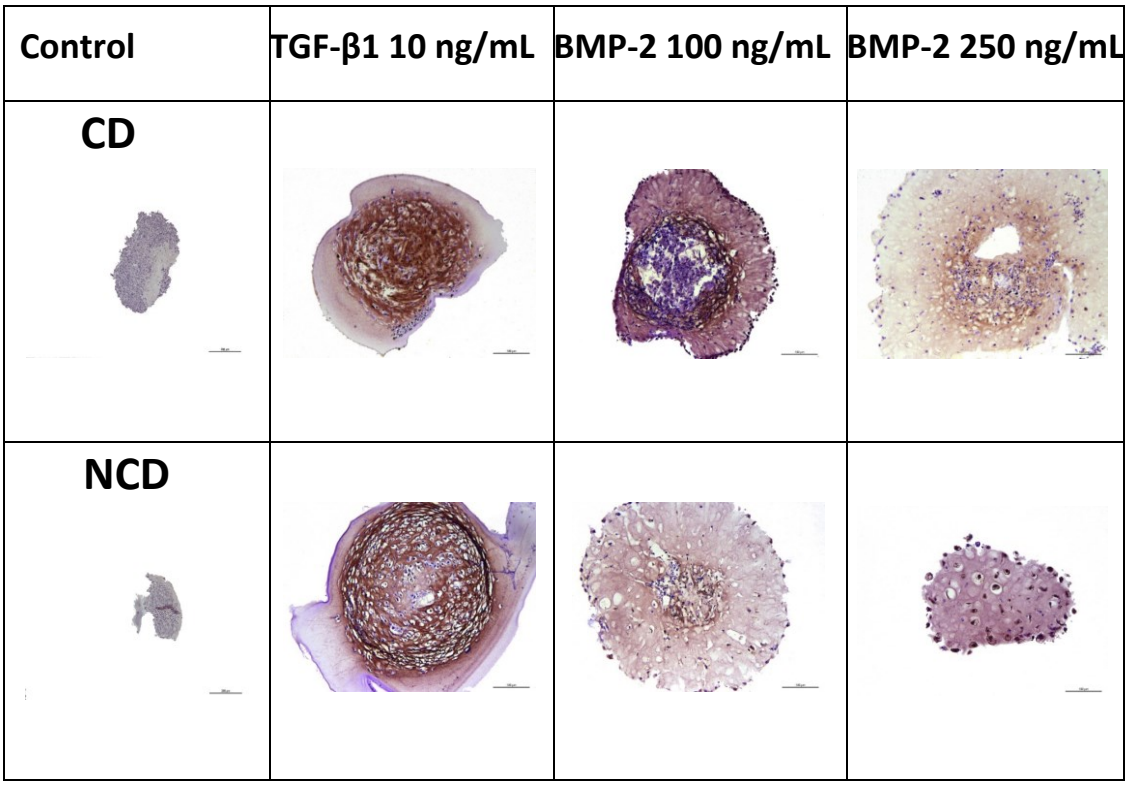


Figure 8: Representative collagen type I immunohistochemical staining of micro-aggregates containing CLCs derived from CD- and NCD-dog breeds after 28 days of culture in basic chondrogenic media or chondrogenic media supplemented with either TGF-β1 10 ng/mL, BMP-2 100 ng/mL or BMP-2 250 ng/mL. Scale bar indicates 100 µm.

The growth factors employed in this study did not only influence matrix production, they also had a differential effect in the composition of the deposited matrix. Collagen type I gene expression was significantly decreased in the BMP-2 250 ng/mL condition compared to the TGF-β1 condition within the NCD-group. Moreover, there was no significant difference in collagen type I expression between the CD- and NCD-group within the condition BMP-2 250 ng/mL. These findings were further confirmed on protein level. Immunohistochemistry of collagen type I showed different staining intensities in which the condition TGF-β1 was more intense stained than BMP-2 which could suggest that BMP-2 had a less fibrotic effect, while collagen type I is an indicator for fibrosis (18).

To our knowledge, this was the first study investigating the long term differential response of CLCs derived from CD- and NCD-dog breeds when cultured *in vitro* with growth-factors TGF-β1 and BMP-2. A previous study investigated the production of GAGs between CLCs and NCs derived both from CD-dog breeds and NCs derived from NCD-dog breeds when cultured for ten days *in vitro* in alginate beads <u>without</u> the presence of growth-factors. Cappello *et al*. 2006 showed that the NCs produced proteoglycans in the intercellular phase at a much faster rate than CLCs, which could explain the greater quantity of proteoglycans reserved by these cells in the pericellular compartment (7). We observed similar findings in our study (Fig. 6).

There are some studies investigating in either rat or human IVD cells the effect of TGF-β or BMP-2 (19-22). However, none of these studies compared the differential effects of these growth factors within the same experiment in terms of proteoglycan and collagen type II

production. Hiyama *et al*. had investigated the enzyme beta1,3-glucuronosyl transferase 1 which is an important regulator of chondroitin sulfate production in cells of the NP. They reported that BMP-2 was, if not more, at least as potent as TGF-β3 in controlling GAG and proteoglycan synthesis (22).

As indicated previously, there is a difference between CD- and NCD-dog breeds. This difference is based on the predisposition to chondrodystrophy which indicates an abnormal enchondral ossification (9,11). This can be explained by earlier performed experiments in which it was shown that chondrodystrophic dog breeds express a "Fibroblast Growth Factor (FGF) 4 retrogene" (23) which causes overexpression of the FGF-receptor-3. This receptor has a negative regulatory effect on enchondral ossification of long bones (24) causing the remarkable characteristics of CD-dogs. This difference between these dog breeds could therefore also play a role in the different responses obtained from the CLC-population derived from CD- and NCD-dogs breeds, used in this study. However, no studies have been performed yet, to identify if this mutation would indeed play a role.

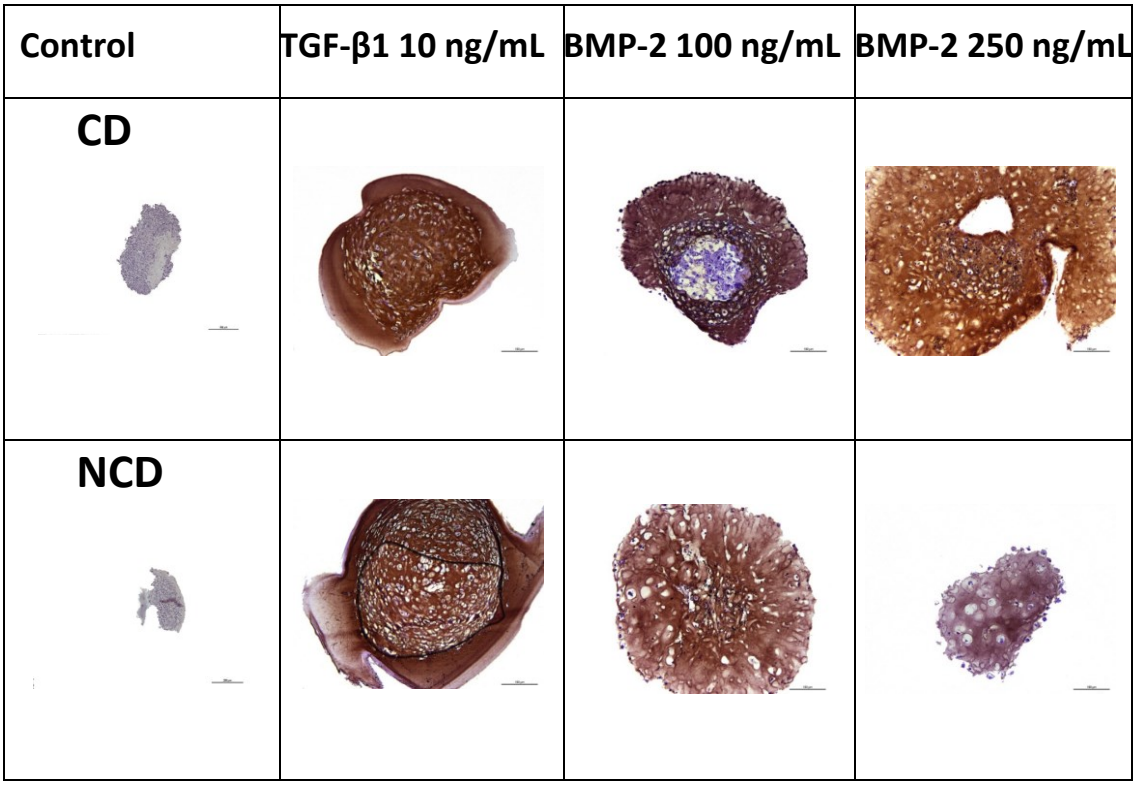


Figure 9: Representative collagen type II immunohistochemical staining of micro-aggregates containing CLCs derived from CD- and NCD- dog breeds after 28 days of culture in only chondrogenic media or supplemented with either TGF-β1 10 ng/mL or BMP-2 100 ng/mL or BMP-2 250 ng/mL. Scale bar indicates 100 µm.

Future recommendation

CLCs derived from CD and NCD-dog breeds showed that they have potential to be employed in regenerative treatment strategies for IVD degeneration. Currently, a hydrogel has been developed for IVD degeneration with positive results in a sheep model (25) and also recently in humans in clinical trials (26). A collaboration has started between the Department of Clinical Sciences of Companion Animals and TETEC in order to determine whether the same product would be feasible for veterinary medicine. Therefore, it is recommended to

investigate cell responses of CLCs derived from CD- and NCD-dog breeds when cultured in this hydrogel combined with BMP-2 250 ng/mL.

List of abbreviations

Not all abbreviations are mentioned below, due to that some are considered as common knowledge.

ADAMTS	A Disintegrin And Metalloproteinase with Thrombospondin Motifs
BMP-2	Bone-Morphogenetic Protein-2
CD	Chondrodystrophic
CLC	Chondrocyte-like-cell
COL2α1	Collagen type 2α1
CS	Chondroitin Sulphate
DMEM	Dulbecco's Modified Eagle's Medium
DMMB	Dimethylmethylene blue-assay
FCS	Fetal Calf Serum
GAG	Glycosaminoglycan
HBSS	Hanks Balanced Salt Solution
IVD degeneration	Intervertebral disc degeneration
M.a.	Micro-aggregate
MMP	Matrix metalloproteinase
NC	Notochordal cell
NCD	Non-chondrodystrophic
NP	Nucleus Pulposus
PBS	Phosphate buffered saline
TGF-β1	Transforming Growth Factor bèta-1
TIMP	Tissue inhibitor Metalloproteinases

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Chapter 3: General discussion

This general discussion focuses on which protocol has the best potential when used to achieve chondrogenesis of bone-marrow derived mesenchymal stem cells (BMSCs). As explained in chapter 1, cartilage has many significant tasks within the body and it is still very difficult to mimic. Osteoarthritis (OA) is a cartilage disease which is diagnosed in 20% of the adult canine population. It is often the result of altered joint biomechanics. As a consequence, the tissue is more prone to damage and when excessive damage occurs, the body is not capable of healing the damage by itself. Therefore, this disease is accompanied by a lot of pain and lameness in dogs (1-3). Currently, there are still only symptomatic treatments for this disease (4). As a consequence, researchers are focused on regenerative medicine which has as main goal to biologically repair the degenerated cartilaginous tissue (5) which might therefore lead to a curable treatment for OA. A lot of research has been performed by using BMSCs for this purpose (6). Though, it is still not known yet what protocol has the best potential to establish chondrogenesis of canine BMSCs. Therefore, my first aim was to optimize the protocol of chondrogenically differentiating canine BMSCs.

For this, three experiments were performed. In the first experiment, the influence of supplementing the growth factor transforming growth-factor-beta1 (TGF-β1) to the BMSC-culture according to different time-schedules was researched. Unfortunately, this led to negative results and no chondrogenic differentiation of canine BMSCs was achieved (7). As discussed previously (7), this could have been explained that during the expansion phase cells were very densely expanded combined with the supplementation of fetal calf serum (FCS). Due to the high expanding density, mesenchymal stem cells could be negatively influenced to form colony forming units (CFUs) (8-10). These colonies are a trait of stem cells and cells derived from theses CFUs appeared to have good differentiation potential (9,11). Furthermore, the use of FCS could also have been of influence, due to that this was an undefined medium and contained a lot of factors from which not all is known what their influence could be on the BMSC-culture (12,13). This led to the purpose of the second performed experiment in which the influence of FCS derived from three different manufacturers (HyClone, SV30160.03*;* Lonza, DE-14-801F*;* and Gibco 16000-044) and CFUs were investigated, followed by the influence of supplementing TGF-β1 to the BMSC-culture according to different time-schemes on the chondrogenic differentiation potential of canine BMSCs. This experiment was performed with two donors from which interestingly one out of two donors showed positive results in terms of GAG, GAG/DNA content and histology. The other donor showed increased GAG/DNA content in comparison to the control groups which is an indication of producing GAG-rich matrix, but this was not confirmed by histology. This experiment had a culture period of ten days due to the presence of an infection and this could be one of the explanations for the obtained outcomes. It could also be that some canine BMSC donors need an additional stimulus in order to get devoted towards the chondrocytic lineage (14). This could be achieved by supplementing TGF-β1 combined with other growth factors that also have potential to stimulate chondrogenesis, such as bone morphogenetic protein (BMP)-2 (15). The last performed experiment, regarding protocol optimization of chondrogenesis of canine BMSCs, investigated if BMP-2 had an (additive) effect on TGF-β1 (16). The results showed indeed that there was an additive effect of BMP-2 on TGF-β1 in terms of GAG, GAG/DNA content and gene expression profiles of aggrecan and collagen type 2α1 which was supported by other publications (17-

19). This experiment was performed with four donors from which two donors were confirmed to be chondrogenically differentiated by their GAG, GAG/DNA content and histology when cultured with TGF-β1 (10 ng/mL) combined with BMP-2 (250 ng/mL). Furthermore, another donor also showed evidence of being differentiated towards the chondrocytic lineage based on GAG and GAG/DNA content, but this was not confirmed by histology. This could be explained by that this donor produced low amounts of glycosaminoglycans which could not have been detected by the Safranin-O staining (20,21). In order to confirm this hypothesis, immunohistochemistry of collagen type II should be performed. Furthermore, one donor showed no response on the treatment when comparing the results from the growth factor conditions to the control group.

Based on the results obtained from all performed experiments, a conclusion can be made about what is the best way to obtain chondrogenically differentiated canine BMSCs. As mentioned before, culturing BMSCs begins by expanding them *in vitro*. My research showed that the cell density used for expanding BMSCs, may have an influence on the BMSCs prior to chondrogenesis. Expanding BMSCs with a clonal, optimal density could positively influenced the chondrogenic differentiation potential of canine BMSCs due to that the stem cells are triggered to form colonies. In addition, the supplementation of FCS could also positively influenced this potential and due to batch variations, it is necessary to perform an experiment in which the influence is investigated compared to other FCS in order to confirm that the FCS batch used, contains substances that do not have a negative influence on the chondrogenic potential of the BMSCs. After the expansion phase, BMSCs are subjected to chondrogenesis. In order to achieve successful chondrogenesis in most canine BMSC donors, TGF-β1 10 ng/mL + BMP-2 250 ng/mL appeared to be the most promising treatment.

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Appendix

Scientific activities

Throughout my minor Research I had the possibility to follow two courses and to attend several seminars/symposia:

Attended courses

- "Cartilage Mechanics" a two weeks during course
  Lectured by Dhr. C.C. van Donkelaar
  Followed from 6th of February till 7th of April 2015
  Grade: 7.

- "Academic writing" a one week during course
  Lectured by Ms. H.A. Buesseler
  Followed from 17th of February till 31st of March 2015
  Grade: Satisfactory

Attended seminars/symposia:

- Regenerative Medicine Lunch Seminar and Theme Day 13th of October 2014, Utrecht
- HP symposium 27th of October 2014, Utrecht
- Departure symposium for Dr. H. Hazewinkel 15th of November 2014, Utrecht
- Symposium International Stem Cell Forum: "Regenerative Medicine and Stem cells: Translation Challenges" 18th of November 2014, Amsterdam
- Veterinary Science Day 20th of November 2014, Driebergen
- Symposium of the Dutch Society of Stem Cell Research 24th of April 2015, Utrecht
- Annual meeting of the Dutch Society of Matrix Biology 28th and 29th of May 2015, Lunteren