Protective effects of the COX-2 inhibitor Celecoxib on articular cartilage explants

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Abstract

Osteoarthritis is a degenerative joint disease that affects many dogs. It is caused by a shift in the normal dynamic balance to a more catabolic state. The damaged cells in the joint cause an inflammatory reaction and thus influx of macrophages and other inflammatory cells. The current therapy for dogs with osteoarthritis is focused on inhibiting this inflammation. It consists of a symptomatic treatment with nonsteroidal anti-inflammatory drugs, most often COX-2 selective inhibitors. However, these drugs are given orally and therefore have systemic side effects. A possible solution for this problem lies in the intra-articular injection of these drugs in joints with osteoarthritis. This local application, preferably a slow release formulation, will minimize the systemic side effects, while the inflammation in the osteoarthritic joint will still be inhibited. The local effect of the selective COX-2 inhibitor Celecoxib on the presence of COX-2 in cartilage and synovium explants of non-chondrodystrophic and chondrodystrophic dogs was examined in this study through immunohistochemistry. For 21 days, cartilage and synovial tissues were cultured in a monoculture and in a co-culture model with a chondrogenic medium. Statistical analysis showed there were no significant differences in COX-2 staining between the conditions the tissues had been exposed to. Two other scoring systems, the Krenn- and OARSI scoring system, had the same results. However, there were significant differences between the donors, according to the COX-2 explant study and the OARSI scoring system. No differences were found between the two culture methods (monoculture and co-culture), except for the OARSI scoring system. The COX-2 explant study showed there were no significant differences between the NCD and CD dogs. This was also the case for the cartilage monocultures evaluated by the OARSI scoring system. However, the cartilage co-culture tissues from the OARSI scoring system and the synovial tissues evaluated by the Krenn scoring system did show significant differences between NCD and CD dogs. Furthermore, the presence of COX-2 in knee joints of rats after intra-articular treatment with Celecoxib was analyzed. However, these results were primarily negative, so no statistical analysis was performed on this data. Finally, the CD-68 presence was compared in the osteoarthritic knee joints of rats that had received different doses of Celecoxib intra-articular or that had received no treatment. It showed no significant differences between the treatment groups. In conclusion, this study has not been able to prove a positive effect of Celecoxib on joint tissues in different conditions. Also, results concerning the possible differences between NCD and CD dogs were inconsistent. Finally, donor variation was present in this study.

Introduction

Osteoarthritis (OA) is one of the most common chronic joint diseases in dogs(1). There are several risk factors for OA, such as genetic predisposition, obesity and trauma(2,3). At this moment, there is no curative treatment available. Current therapy is symptomatic and consists of the administration of analgesics, like nonsteroidal anti-inflammatory drugs (NSAIDs), which also counteracts the inflammatory reaction that is present in osteoarthritic joints. In some cases, surgery is also part of the therapy. In obese dogs, promoting weight loss and low-impact exercise is important. Moreover, physical therapy may be effective for dogs with OA and there are also several dietary supplementations that may help inhibit the inflammation.

Anatomy of the joint

Important structures in the joint are the articular cartilage, the subchondral bone and the joint capsule(4). Also, a synovial membrane is present within the joint(1). As a support, there a several ligaments and tendons(4). In healthy dogs, enzymes maintain a dynamic balance of degradation and synthesis in the articular cartilage. Chondrocytes and extracellular matrix (ECM) together form cartilage (figure 1). The ECM consists of collagen (primarily type II), proteoglycans and water. Glycosaminoglycan chains (GAG chains), together with a central core protein, form the proteoglycans. GAG chains have anionic and hydrophilic properties, allowing them to attract and hold water. Because of the presence of collagen and GAG chains, articular cartilage is able to endure compression and shearing forces.

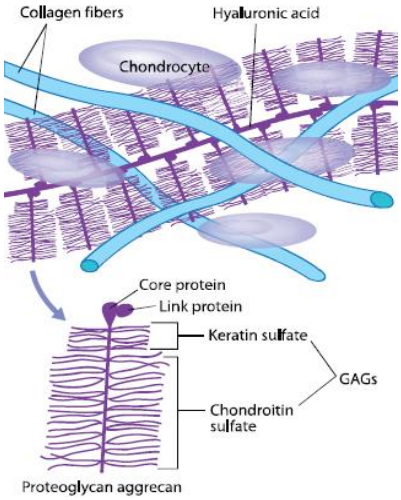

Figure 1 The structure of cartilage(4).

Pathogenesis of osteoarthritis

As previously mentioned, there is a continuous equilibrium of anabolism and catabolism in the joint(4,5). Due to abnormal use of these joints, this dynamic balance is disturbed, causing changes in the joint (figure 2)(5).

Due to IL-1β release, chondrocytes and synoviocytes produce inflammatory cytokines and increase the release of degradative enzymes, like matrix metalloproteinases (MMPs)(4-6). The increased production of MMPs by the chondrocytes causes degradation and thus loss of cartilage proteoglycans and type II collagen(1,2,7). This causes a shift of the equilibrium to a more catabolic state(4). The chondrocytes try to compensate for the high catabolism by producing new proteoglycans and collagen(4,8). However, this does not result in recovery of the joint tissue, because the proteoglycans and collagen are abnormal in quality(4). The ECM is abnormal and therefore more vulnerable to damage. Due to these processes, these structures are degraded and there is still no recovery of the joint tissue.

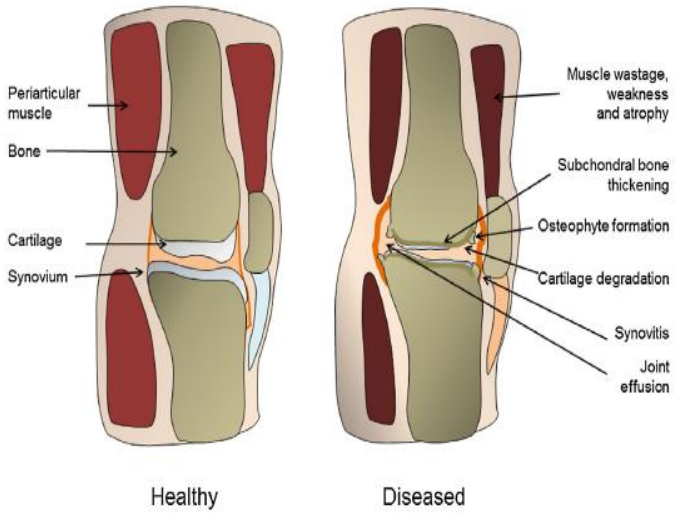

Figure 2 Comparison of a healthy and an osteoarthritic joint(6). Osteophyte formation and degradation of the cartilage are present in an osteoarthritic joint.

Synovitis probably plays a role in the onset of OA(7). Due to the inflammation of the synovium, there is an infiltration of synovial macrophages and T lymphocytes, indicated by the presence of CD-68+ and CD-3+, respectively(1,7). The inflammation causes hyperplasia of the synovium and will induce pain. Excessive production of cytokines due to the synovitis causes activation of chondrocytes(2). Like the synoviocytes, the chondrocytes are able to produce cytokines and chemokines. Cytokines have a number of functions in the process of OA(1). Pro-inflammatory cytokines induce enzymes that degrade the ECM, while synthesis of the ECM is suppressed(2,3). For example, IL-6 is able to inhibit the proteoglycan synthesis and increase the proteoglycan catabolism(1). It also reduces the chondrocyte proliferation and increases the activity of MMPs. It recruits osteoclasts and thus causes bone remodeling during OA(6).

The produced pro- and anti-inflammatory cytokines play an important role in the progression of OA(1). There are several cytokines involved in this inflammatory process. The synovial tissue produces cytokines like IL-1Ra, IL-6 and IL-8(7). In the study of *Venn et al*, the articular knee cartilage of twelve dogs was examined after resection of the cranial cruciate ligament in the right stifle joint(9). The other knee functioned as a control joint. The activity of IL-6 was measured in the synovial fluid. Eleven of twelve dogs had a significantly higher IL-6 level in the operated joint in comparison with the healthy joint, thus the expression of IL-6 in the joint is elevated during the process of OA.

There are also cytokines that are being produced by the cartilage tissues, for instance IL-1β, IL-4, IL-7, IL-10 and IL-13(7). IL-1β causes expression of nitric oxide synthase and as a consequence, reactive oxygen species accumulate(6). This causes apoptosis to be induced. IL-1β may also induce bone resorption. In vivo studies with rats have shown that serum- and urinary calcium concentration and the number of osteoclasts increase after injection with this interleukin. During the inflammatory reaction, TNF-α is also present(6,8). IL-1β and TNF-α are often used for in vitro studies(6). They are able to stimulate catabolism by inducing destructive enzymes, like collagenases and aggrecanases(8).

The use of glucocorticosteroids in osteoarthritis

In the study of *Beekhuizen et al*, triamcinolone was added to human cartilage and synovial tissues(7). This glucocorticosteroid decreased the activity of the MMPs in the synovial tissue monocultures and in the co-cultures of synovium with cartilage. Also, the release of cytokines by cartilage and synovial tissues was decreased. This suggests that triamcinolone has an inhibitory effect on the catabolic activity during OA, as it decreases the degradative enzymes and the inflammatory cytokines. Glucocorticosteroids inhibit the inflammation by decreasing the mRNA synthesis of cytokines and the receptors of these cytokines(10). Also, the mRNA synthesis of the COX-enzyme is inhibited. In addition, glucocorticosteroids are able to inhibit nuclear factor kappa B (NF-κB), which plays a role during the inflammatory process of OA(8,11). The inhibitory effect on MMPs may be caused by reducing the urokinase plasminogen activator activity, produced by activated chondrocytes through IL-1 and TNF-α(11). As a consequence, the activation of MMPs is decreased.

Van Middelkoop et al provide a meta-analysis that studied the effect of intra-articular administration of glucocorticosteroids in osteoarthritic joints in humans(12). At short-term, patients that had received glucocorticosteroids showed less pain than patients that had received a placebo. Also, there was a significant reduction of pain during glucocorticosteroid therapy at mid-term. These differences were not seen at long-term. Thus, although glucocorticosteroids may have an anti-inflammatory effect on joint tissue, intra-articular administration is probably not a suitable long-term symptomatic therapy for OA.

The use of nonsteroidal anti-inflammatory drugs in osteoarthritis

NSAIDs are widely used as a symptomatic treatment for OA because of their anti-inflammatory properties(8,13). This is due to the inhibition of COX-enzymes, also known as prostaglandin synthases(14). During an inflammatory reaction, prostaglandin synthase converts arachidonic acid to prostaglandin H_2, after which it is metabolized into prostaglandin E2 (PGE2) by prostaglandin E synthase (PGES)(8,13,15).

However, prostaglandins also have a number of physiological functions(14). They have a protective effect on the gastrointestinal mucosa and inhibition of prostaglandins can therefore cause gastritis and ulceration. These patients may show signs of anorexia, vomiting and diarrhea. Prostaglandins also regulate the blood flow to the kidneys. Thus, inhibition of prostaglandin production by NSAIDs can cause a decrease of the blood flow to the kidneys.

Of the two isoenzymes, COX-1 is produced in several tissues and is important in a number of physiological processes in the body(14). In most cases, COX-2 is produced during an

inflammatory reaction. This isoenzyme is induced by pro-inflammatory mediators, like cytokines(8). Because COX-1 has mainly physiological functions and COX-2 is important during an inflammation, it is favorable to administer NSAIDs that primarily inhibit COX-2. Thus, selective COX-2 inhibitors, like Celecoxib, have been developed(8). However, COX-2 can also be synthetized in the absence of trauma or inflammation, so it may also have a physiological function(14). Nevertheless, selective COX-2 inhibitors have less systemic side effects than non-selective COX-inhibitors(16).

The effect of Celecoxib on joint tissues

Celecoxib is a selective inhibitor of the activity of the enzyme COX-2(13,14,16). Therefore, it has a lower incidence of adverse gastrointestinal reactions in comparison with other, less selective, COX-inhibitors. However, it has been suggested that administration of Celecoxib causes an increased risk of myocardial infarction(17). Another study did not find an increased cardiovascular risk when treatment with Celecoxib was compared to the administration of a placebo or nonselective NSAIDs(18). Whether or not Celecoxib increases the risk of cardiovascular events, because of the systemic use, adverse reactions may occur. Therefore, local application of NSAIDs is preferred, because of the lack of systemic side effects(19).

It has been suggested that Celecoxib might have other functions besides the anti-inflammatory abilities(8). Its effect on cartilage, synoviocytes and subchondral bone has been reviewed in the study of *Zweers et al*. These possible effects will be discussed in the sections below.

There is an elevated COX-2 expression in chondrocytes in joints with OA(8). As previously mentioned, the production of prostaglandin H_2 by the COX-enzyme is followed by PGES-mediated PGE2 production. Therefore, a higher COX-2 expression will also result in an increase of PGE2(15). PGE2 has primarily catabolic effects on chondrocytes in osteoarthritic joints(20). Celecoxib might have an effect on the chondrocytes through its indirect inhibition of PGE2 (figure 3)(8). Because PGE2 stimulates the MMPs, Celecoxib might have an indirect inhibitory effect on those enzymes. It is also possible that this COX-2 inhibitor is able to inhibit the production of nitric oxide, although other studies did not have the same conclusion. Nitric oxide is able to destruct the cartilage during the process of OA. In addition, Celecoxib stimulates the synthesis of proteoglycans in degenerated and late-stage osteoarthritic cartilage. During an in vitro study, the proteoglycan level normalized when Celecoxib was added. However, other studies have shown different results of the effect of Celecoxib on chondrocytes, possibly due to the differences between the setups of these vitro models.

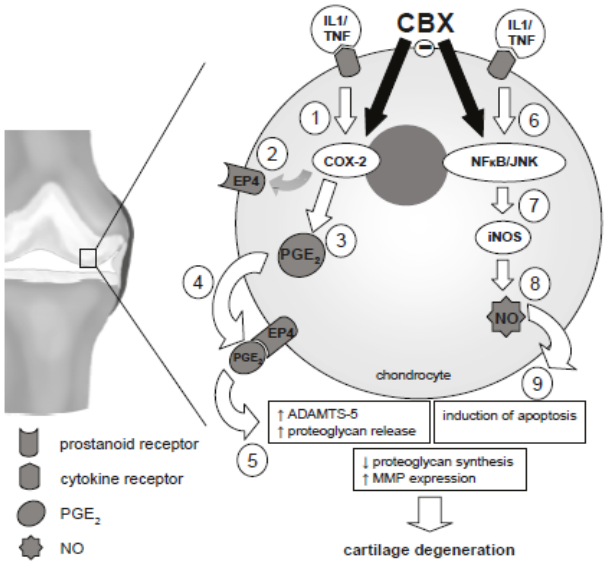

Figure 3 The in vitro effects of Celecoxib in the joint(8). This NSAID causes inhibition of COX-2 and thus a decrease of PGE2.

As previously mentioned, the synovium is an important structure during OA and synovitis with hyperplasia is present in osteoarthritic joints. Celecoxib induces apoptosis of synovial fibroblasts, which counteracts the hyperplasia(8). Also, Celecoxib is able to decrease the inflammatory mediators that are expressed during OA through inhibition of NF-κB. NF-κB is present in fibroblasts in the synovium. The MMPs that are released by the synovial cells are

inhibited by Celecoxib, although an increased expression of these enzymes due to this COX-2 inhibitor has also been seen.

NF-κB is also involved in the synthesis and activation of osteoclasts(8). As a consequence, the destruction of subchondral bone is induced during OA. Celecoxib inhibits the osteoclast activity through this mechanism. This NSAID is also involved in other chondroprotective mechanisms. For instance, receptor activator of nuclear factor kappa-B ligand (RANKL) is produced by chondrocytes. It can stimulate the synthesis of osteoclasts and functions as an attractant for monocytes. Celecoxib causes inhibition of the RANKL expression and therefore inhibits the osteoclast activity.

Ou et al have studied the possible effect of Celecoxib on the collagen metabolism(21). As previously mentioned, OA is characterized by a loss of type II collagen. *Ou et al* observed whether long-term use of Celecoxib had an effect on collagen metabolism. This was tested in 130 Wistar rats, which were three to four months old. OA was induced in these rats by resection of the left Achilles tendon. The rats were divided in groups; each group received a NSAID, which was administered by an oral gavage once a day. There was also a control group that received saline. The expression of type II collagen did not change after nine months of treatment with Celecoxib. Also, the expression of type I collagen was not altered after nine months of treatment and there were no changes in the expression of type III collagen. The other NSAIDs that were used in the study, ibuprofen and indomethacin, did have effects on the expression of collagen. The observed effects were dependent on the duration of the treatment. After nine months of treatment, ibuprofen had a negative effect on the expression of type I collagen. For type II collagen, ibuprofen had a positive effect, while indomethacin had a negative effect on the expression. Ibuprofen also stimulated the expression of type III collagen after nine months of treatment. At six months of treatment, indomethacin had a positive effect on the expression of type III collagen. This study suggested that Celecoxib doesn't influence the collagen metabolism. However, *Zweers et al* suggested that Celecoxib inhibits PGE2, which inhibits collagen synthesis, and that Celecoxib thus may have an effect on collagen metabolism(8).

Thus, some studies may show other conclusions(8). This might be due to the differences in the experimental setup. Therefore, more studies are necessary to examine the effects of Celecoxib in healthy and osteoarthritic joints.

Intra-articular treatment

In order to develop an intra-articular treatment, it is necessary to create a formulation of Celecoxib that can be administered through that route. Intra-articular treatment with NSAIDs will not be successful if the drug leaves the joint soon after administration(22). The synoviocytes are positioned in such a way that gaps emerge between the cells. These intercellular gaps are in direct contact with the joint cavity, allowing drugs to exit the joint cavity quite easily. One solution for the intercellular gaps are drug carriers that have a higher radius than the size of the gaps (40 to 250 nm). Microspheres are drug carriers in which the drug can be incorporated. When the radius is bigger than 250 nm, the drugs will stay longer in the joint cavity. The macrophages in the synovial lining degrade the microspheres and the drug inside the microsphere is gradually released. Thus, the cell that plays a part in the pathophysiology, the macrophage, can also be used in the symptomatic treatment of osteoarthritis.

Sustained release formulation

In case of an intra-articular application, a sustained release formulation of Celecoxib is preferred, which causes the NSAID to be released over a longer period of time. As a consequence, less frequent intra-articular injections of Celecoxib are needed. This has already been studied in horses(19). Five horses were injected intra-articular with a 'low Celecoxib-loaded gel' (LCL gel) of 50 mg/g in the left talocrural joint and a 'high Celecoxib-loaded gel' (HCL gel) of 260 mg/g in the right talocrural joint. Also, a placebo was injected in the right

middle carpal joint and Hyonate (sodium hyaluronate) in the left middle carpal joint. The LCL gel contained a gel with fully dissolved Celecoxib, while the HCL gel was composed of a paste of undissolved Celecoxib. Before the intra-articular injection, the horses showed no signs of lameness.

In four out of five horses, the limb that had received the HCL gel showed signs of lameness for 72 hours after the intra-articular injection(19). Also, the white blood cell count and protein content in the joints were increased in some limbs. At 72 hours post injection, these values were all decreased to control levels. The glycosaminoglycan content was also increased, but like the white blood cell count and the protein content, it was back at baseline level 72 hours after the injection. The C2C epitope of type II collagen did not significantly changed, indicating that there was probably no damage to the cartilage after the injection. Histological staining of the cartilage confirmed this statement.

For the LCL gel, the maximum concentration in the synovial fluid was reached after 8 to 24 hours(19). For the HCL gel, this was 8 to 72 hours post injection. It was found that after 8 hours, the Celecoxib concentration in both Celecoxib-loaded gels was 20 to 25 ng/ml in the synovial fluid. This is approximately 50 times the dose after oral administration. At 24 hours post-injection, the Celecoxib concentration in serum was 75 to 145 ng/ml, which is 5 to 25 times lower than after oral administration. Celecoxib was not detected in the plasma at 7 days post-injection. This is beneficial, because a low Celecoxib concentration in the blood and a high concentration in the joint is preferred. The Celecoxib concentration in the joint was detectable until 28 days post-injection. Thus, there is a prolonged local exposure of Celecoxib to the joint, while systemic exposure is limited. However, four horses showed temporary signs of lameness after receiving the HCL gel. There was no lameness in the horses that had received the LCL gel. The authors suggest that the lameness of the horses is caused by an inflammatory reaction due to the crystalline Celecoxib in the HCL gel. The concentration of the C2C epitope of collagen and the histology did not indicate damage to the cartilage. Therefore, it is likely that the configuration of the HCL gel is the cause of the lameness and that it has no serious effects on the joint tissue. Because the LCL gel did not give signs of lameness, this gel is probably a suitable long term treatment for OA.

In vitro models

In order to evaluate the effect of a potential therapy for OA in dogs, a suitable in vitro model is essential(6). In OA, multiple tissues are involved. It is necessary that an in vitro model contains different joint tissues in order to most closely resemble the in vivo situation. Therefore, co-culture models have been developed with synovium and cartilage(6,7). Several in vitro models are useful to study the process of OA(6).

The review of *Johnson et al* discusses two in vitro models: the cytokine-based model and the load-based model(6). The explant-based model is an example of a cytokine-based model. It allows the response of cells to be studied. The other model is the in vitro load-based model. It is based on the principle that load is important to preserve the balance of anabolism and catabolism in the joint. Changes in load can disrupt this balance.

Beekhuizen et al provide a long-term co-culture model with human cartilage and synovial tissue explants(7). In this study, cartilage and synovium were cultured alone and in co-culture. Synovial tissues were cultured in three different culture media. However, there were no differences in viability between the culture media. In this study, an enzyme-linked immunosorbent assay was used to examine the release of pro- and anti-inflammatory cytokines by synovial tissue.

These in vitro models also have some disadvantages. The cultures of *Beekhuizen et al* showed that there was a high release of lactate dehydrogenase during the first days, indicating cell death(7). However, it lowered hereafter, thus the cell death was only limited. Also, *Johnson et*

al stated that cell death occurs at the edges of the explants and that is not possible to get a lot of samples from one source(6). These disadvantages have to be taken into account when these in vitro models are used.

Immunohistochemical analysis can be performed on the cultures to evaluate the presence of inflammatory cells(7). For instance, CD-68 staining may show the presence of synovial macrophages in osteoarthritic tissues. Synovial macrophages cause production of inflammatory mediators in the synovial fluid, thus playing an important role in the development of OA(2). In the study of *Blom et al*, the synovial macrophages were depleted from the joints of mice(23). As a result, there was a significant reduction in the production of MMPs in joints where the macrophages had been removed.

The study of *Beekhuizen et al* showed that the glycosaminoglycan production in osteoarthritic cartilage that was co-cultured with osteoarthritic synovium, was significantly reduced in comparison with cartilage that had been cultured alone(7). There was activity of MMPs present in the cartilage and synovium co-cultures and in the explants with synovium alone, but not in the cartilage explants that had been cultured alone. Thus, for an in vitro model that will resemble the in vivo situation most closely, it is important to involve both cartilage and synovium in the experiment.

Through the entire period of 21 days, the synovial tissue in the culture model of *Beekhuizen et al* was able to produce cytokines, which indicates the presence of viable cells(7). It makes this particular experimental setup useful to examine the production of inflammatory mediators in osteoarthritic joint tissue. *Beekhuizen et al* has thus provided a suitable in vitro model to evaluate the possible effects of Celecoxib in joint tissue with OA.

Evaluating the degree of synovitis and osteoarthritis
In order to semiquantify the severity of OA, there are scoring systems available. *Krenn et al* has provided a scoring system to assess the degree of synovitis in synovial tissues(24). Tissues are evaluated based on three characteristics: the lining cell layer, the synovial stroma and the degree of inflammatory infiltration. For each of these characteristics, a score of zero to three can be given to the tissue. The sum of these scores can give an indication of the presence and severity of synovitis.

Cook et al has created an OARSI scoring system for canine cartilage tissues(25). For this study, three characteristics were used to evaluate the cartilage. First of all, it was assessed whether the surface was smooth or showed fissures related to joint disease. Next, grading the chondrocyte pathology was based on whether small or large cell clusters were present. Using a safranin O fast green staining, the amount of loss in proteoglycan content was quantified. A score of zero to twelve can be assigned to each of these features. The sum of these scores can give an indication of the presence and severity of osteoarthritis in cartilage tissues.

In the present study, the effect of Celecoxib on cartilage and synovium explants was studied. Also, the influence of donor differences and different methods of culturing (cartilage and synovium monocultures and co-cultures) was examined. It is known that intervertebral disc degeneration (IVDD) has a different etiology in NCD and CD dogs(26). To study whether this might also be a factor in OA, these two groups of dogs were compared in this study. The same parameters were studied using the Krenn- and OARSI scoring system. In joint tissue of rats, the presence of COX-2 and CD-68 (indicating the presence of macrophages) was studied.

Materials and Methods
COX-2 explant study
The cartilage and synovium of six donor dogs of different age, breed and weight were used, of which three dogs were non-chondrodystrophic (NCD) and three chondrodystrophic (CD). The tibia plateau and the medial epicondyle of the femur were collected of five of these donors; of

one donor (donor 181), the joint tissue of the hip was used. The cartilage and synovial tissues of the joints were cultured individually and together for 21 days in a chondrogenic medium. TNF-α was added to a number of tissues to simulate an inflammatory environment. Other tissues did not received this cytokine and therefore served as control tissues. In some cultures with TNF-α, Celecoxib was also administered to study the effect of this NSAID in an inflammatory environment. Furthermore, Celecoxib was added to some tissues to determine the effect of Celecoxib in tissues without inflammation.

Through immunohistochemistry (IHC), a specific antigen (in this case COX-2) can be made visible using labelled antibodies. With the indirect method, a primary antibody will specifically bind to this antigen. After this, a secondary antibody is needed to make this binding visible as a brown-staining (figure 4). The secondary antibody binds to the primary antibody. After adding 3,3'-diaminobenzidine (DAB), Horseradish Peroxidase (HRP) catalyzes the reaction of DAB with hydrogen peroxide. This reaction will produce a brown, insoluble form of DAB, which is visible under a light microscope as a brown stained region. The staining indicates the location of the specific antigen.

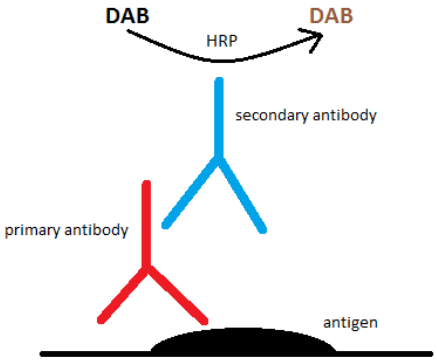

Figure 4 The indirect method of immunohistochemistry.

The following IHC protocol was used to study the presence of COX-2 in the cartilage and synovium explants of three NCD and three CD dogs.

For the cartilage, six tissues of the same donor and the same condition were placed on one slide. These tissues were divided in groups of two. Two groups received the COX-2 antibody. The third group received a mouse antibody and thus served as a negative control for the IHC. For the synovium, three tissues of the same donor and the same condition were placed on one slide. Two tissues received the COX-2 antibody and the third tissue received the mouse antibody.

The coupes stayed in a stove overnight. Next, they were placed on a SW 85 Eliwell IC 902 temperature controller for four to six hours. The paraffin on the coupes was resolved using Xylene I and II. The coupes were imbedded in these substances for five minutes each. After that, hydration was realized by using decreasing percentages of ethanol (96%, 80%, 70%, 60%, respectively), with also five minutes for each percentage of ethanol. Thereafter, Milli-Q water was used to rinse the coupes for five minutes. The water around the tissues was carefully removed. Circles were drawn around the tissues with a PAP pen to create a hydrophobic barrier.

One or two drops of an endogenous enzyme block (S2003 Dako Dual Endogenous Enzyme Block from Dako North America) at room temperature were placed on the tissues. Dako S2003 is a peroxidase and alkaline phosphatase blocking reagent. It suppresses endogenous alkaline phosphatase and peroxidase in the tissues. After ten minutes, the coupes were rinsed twice (for five minutes each) in Tris-Buffered Saline mixed with Tween (TBS-T), while being on a mini see-saw rocker (Stuart, SSM4) at about 30 rpm. Next, TBS-BSA 5% (TBS with 5% bovine serum albumin) was used for blockage. One or two drops were placed on the tissues within the circles that were made earlier with the PAP pen. After 60 minutes, the coupes were carefully dried.

A primary antibody was used for incubation. On every slide, there was one tissue (one pair of tissues for the cartilage slides) which received normal mouse IgG (SC-3877 from Santa Cruz Biotechnology) and thus served as a negative control. The other two tissues (two pair of tissues

for the cartilage slides) received COX-2 monoclonal antibody (Clone CX229 from Cayman Chemical Company) as the primary antibody. This primary antibody was diluted in TBS-BSA 5%, using a dilution of 1:25. Later in the study, a dilution of 1:50 was tested and this also gave positive results. After that, the 1:25 dilution was replaced by the 1:50 dilution. Next, the coupes stayed overnight at 4°C.

The following day, the slides were rinsed with TBS-T twice, for five minutes each. After that, the slides were incubated for 30 minutes with anti-mouse antibody (K4001 Dako EnVision + System + HRP Labelled Polymer Antimouse from Dako North America), which served as the secondary antibody (the antibody for the primary antibody, see figure 4).

Next, the coupes were rinsed twice with TBS, for five minutes each. After this, the tissues were incubated for five minutes with a solution that contained one drop of DAB diluted in 1 ml of substrate buffer (K3468 Dako Liquid DAB + Substrate Chromogen System from Dako North America). The slides were rinsed twice with Milli-Q water for five minutes each and then counterstained with hematoxylin QS H-3404 from Vector Laboratories for 10 to 15 seconds, causing staining of the cells. Hereafter, the coupes were rinsed with running tap-water for ten minutes. Dehydration was achieved by using ascending percentages of ethanol (70%, 80%, 96% twice and 100%, respectively). Next, the coupes were imbedded in Xylene twice. Each step with ethanol and Xylene lasted five minutes. Finally, the slides were covered with a coverslip for protection. Two or three drops of Vectamount Permanent Mounting Medium from Vector Laboratories were used to fix the coverslips on the slides.

Using a light microscope, it was visible whether a brown-staining was present. A brown-staining in the cytoplasm of the cells indicated the presence of COX-2.

A scale from 0 to 4 was used to evaluate the COX-2 staining in the cartilage and synovial tissues (table 1). Because there was a variation in the location of the staining in the synovial tissues, it was also noted where the staining was primarily present in these tissues (data not shown). If the tissue had a score of 0 or 1 for the COX-2 staining, it was not possible to mention were the staining was primarily located.

Table 1 Scoring system for the COX-2 staining of the cartilage and synovial tissues.

Score	
0	No cells are positive
1	< 5 cells are positive
2	5 cells -50% of the cells are positive
3	50-75% of the cells are positive
4	> 75% of the cells are positive

The Krenn- and OARSI scoring system

A modification of the synovitis score according to the study of *Krenn et al.* was used to study the degree of synovitis on Hematoxylin & eosin stained sections (table 2). A light microscope was used to evaluate the tissues. *Krenn et al* have described the evaluation of three characteristics in order to grade the severity of the synovitis. For these tissues, it was only possible to evaluate the synovial lining cell layer, so only this characteristic was used to grade the severity of the synovitis.

Table 2 Modified Krenn scoring system for the synovial tissues.

Score	
0	Lining cell layer consists of one layer
1	Lining cell layer consists of two to three layers
2	Lining cell layer consists of four to five layers
3	Lining cell layer consists of more than five layers

Cartilage tissues were used that had been previously stained with a Safranin O fast green staining. The presence and the severity of OA was evaluated according to the study of *Cook et al*, using a light microscope. For the evaluation of these tissues, three tables of the article of *Cook et al* were used (table 3). The sum of the scores led to a total score for the cartilage tissues.

COX-2 study in knee joints of rats

Rats with osteoarthritis had been previously injected intra-articular with either triamcinolone or Celecoxib in three dosages. Another group of rats had received no treatment. After euthanasia of these rats, sections were cut of the knee joints. For this study, only the tissues of the rats that had received Celecoxib or that had received no treatment were used. After this, the same IHC protocol was performed as the COX-2 explant study described before. A light microscope was used to see whether brown-staining was present.

CD-68 study in knee joints of rats

The knee joints of fourteen rats that had received different doses of Celecoxib or that had received no treatment, were used to study the presence of CD-68 staining.

Through IHC, the knee joints of these rats had been previously stained. Similar to the COX-2 explant study, a light microscope was used to examine the amount of staining in the joint tissues. A brown-staining in the synovium, caused by the presence of CD-68, indicated the presence of macrophages. A different scoring system was used for this study, since most tissues showed less staining in comparison with the COX-2 explant study (table 4). Therefore, more categories were made for staining below 50% of the cells.

Table 3 Modified OARSI scoring system for the cartilage tissues.

	Area of section affected			
	None	1/3	2/3	> 2/3
The cartilage structure				
Smooth surface	0	0	0	0
Fissures present	0	1	2	3
The presence of clusters				
No clusters	0	0	0	0
Occasional superficial clusters	0	1	2	3
Small cell clusters (2-4 cells/cluster) predominate	0	2	4	6
Large cell clusters (≥ 5 cells/cluster) predominate	0	3	6	9
Cell loss (apoptosis/necrosis) predominates	0	4	8	12
Proteoglycan content				
Normal	0	0	0	0
Decreased proteoglycan content in surface/upper zone	0	1	2	3
Decreased proteoglycan content into the mid zone	0	2	4	6
Decreased proteoglycan content into the deep zone	0	3	6	9
Ful depth decreased proteoglycan content	0	4	8	12

Table 4 Scoring system for the CD-68 staining of the joint tissues.

Score	
0	No cells are positive
1	< 10% of the cells are positive
2	10 - 50% of the cells are positive
3	> 50% of the cells are positive

Statistical analysis

IBM SPSS 24 Statistics Data Editor was used for statistical analysis of the results. The data of the COX-2 explant study was not normally distributed and therefore the Kruskal Wallis test was used for statistics. The results of the Krenn- and OARSI scoring system were not normally distributed, thus the Kruskal Wallis test was also used for this data. The correlations between the COX-2 score and the Krenn score of the synovial tissues, and the COX-2 score and the OARSI score of the cartilage tissues were analyzed using the Spearman's rank correlation analysis. No statistical analysis was performed on the data from the COX-2 rat study, since the tissues were primarily negative after IHC. The Kruskal Wallis test was used for the CD-68 rat study to examine the differences between the treatment groups, since this data was also not normally distributed.

Results

COX-2 explant study

Examples of the cartilage tissues after immunohistochemistry are displayed in figure 5. Examples of the synovial tissues are shown in figure 6.

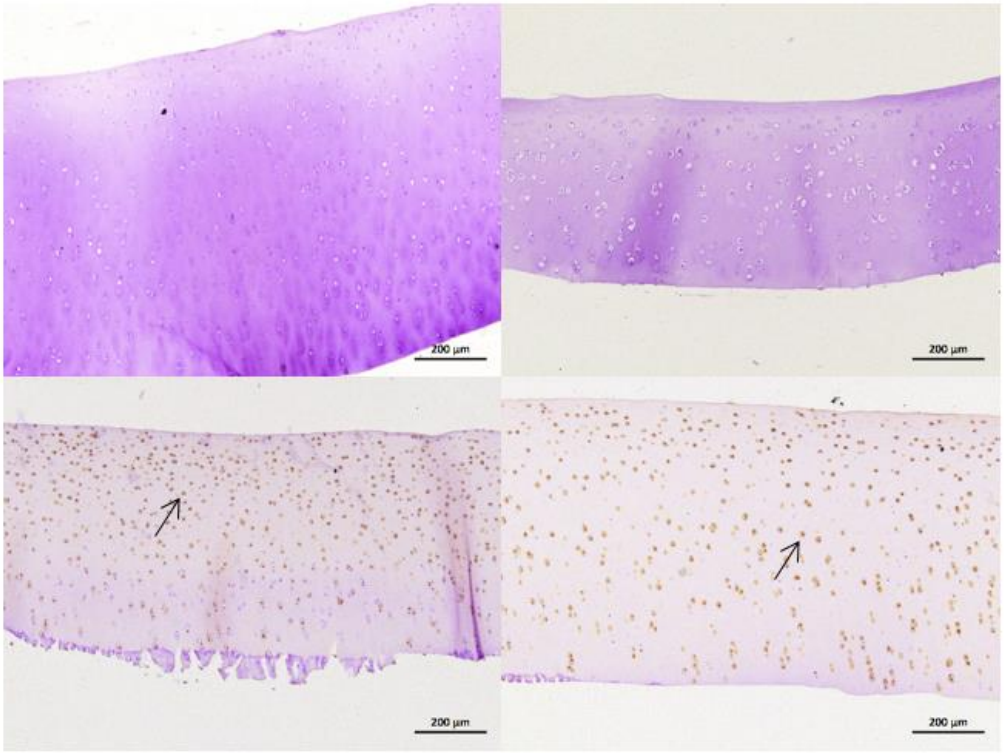

Figure 5 Cartilage tissues after immunohistochemistry. A and B show negative results, C and D show positive results. A: donor 181 at day 0, hip cartilage tissue. B: donor 181 at day 21, hip cartilage tissue (co-culture) with TNF-α, incubated with COX-2 antibody. C: donor 3516 at day 21, cartilage tissue (co-culture) with TNF-α and Celecoxib, incubated with COX-2 antibody. D: donor 3516 at day 21, cartilage tissue (co-culture), control tissue (nothing was added to the tissue), incubated with COX-2 antibody. Examples of positive cells are designated with an arrow.

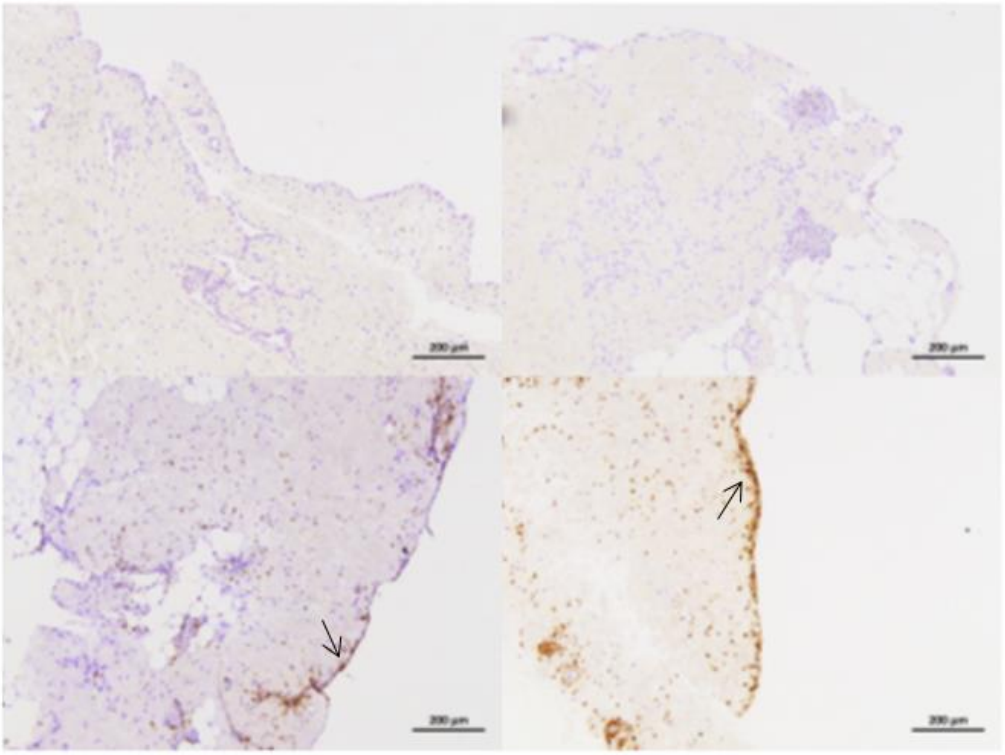

Figure 6 Synovial tissues after immunohistochemistry. A and B show negative results, C and D show positive results. A: donor 3516 at day 0, synovial tissue. B: donor 17316 at day 21, synovial tissue (monoculture), control tissue (nothing was added to the tissue), incubated with COX-2 antibody. C: donor 3516 at day 21, synovial tissue (co-culture) with TNF-α, incubated with COX-2 antibody. D: donor 3516 at day 21, synovial tissue (monoculture), control tissue (nothing was added to the tissue), incubated with COX-2 antibody. Examples of positive cells are designated with an arrow.

Differences between the conditions

Since the data was not normally distributed, the Kruskal Wallis test was used to study whether there were significant differences in COX-2 staining between the conditions to which the cartilage and synovial tissues had been exposed to. The coupes were divided in four groups: cartilage from monocultures, cartilage from co-cultures, synovium from monocultures and synovium from co-cultures. Statistics were performed on these four groups and resulted in P-values of 0.464 for the cartilage monocultures, 0.350 for the cartilage co-cultures, 0.753 for the synovium monocultures and 0.479 for the synovium co-cultures. All P-values were above 0.05, which means there were no significant differences between the conditions, whether the cartilage and synovial tissues had been cultured in a monoculture or in a co-culture (figure 7 and 8).

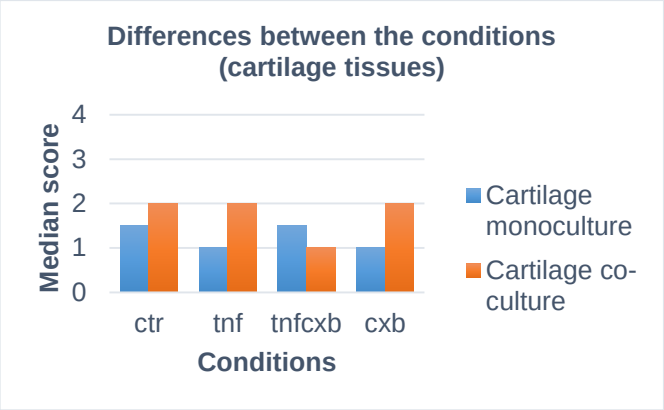

Figure 7 Differences between the conditions for the scores of both the cartilage monoculture and co-culture tissues. Ctr = control; tnf = TNF-α; tnfcxb = TNF-α with Celecoxib; cxb = Celecoxib.

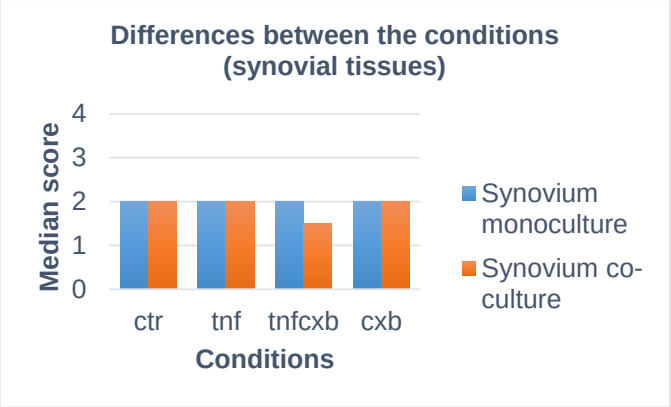

Figure 8 Differences between the conditions for the scores of both the synovium monoculture and co-culture tissues. Ctr = control; tnf = TNF-α; tnfcxb = TNF-α with Celecoxib; cxb = Celecoxib.

Differences between the culture methods

Statistical analysis was performed to examine whether there were differences between the culture methods. A Kruskal-Wallis test was performed and showed that the differences between the data of the cartilage monocultures and the data of the cartilage co-cultures were not significant (P-value of 0.115). The same conclusion could be made for the data of the synovium monocultures and co-cultures (P-value of 0.706). However, there was a significant difference between the scores of the cartilage and synovial tissues (P-value of 0.012). Thus, for further statistical analysis, it was not necessary to differentiate for the culture method.

Differences between NCD and CD donors

When the scores of the cartilage tissues of the NCD and CD dogs were compared using the Kruskal Wallis test, this difference was just above the significance level (P-value of 0.050). For the synovial tissues, the P-value was 0.790 and therefore also not significant. Therefore, no significant differences were found between the NCD and CD donor dogs for both tissues (figure 9).

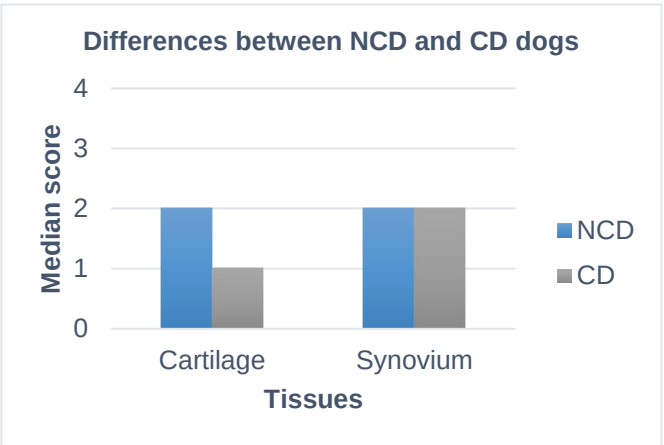

Figure 9 Differences between NCD and CD dogs, for the scores of both the cartilage and synovial tissues.

Differences between the donors

The median scores of the donors showed much variation (figure 10). Statistical analysis confirmed there were significant differences between the donors of this study. Using the Kruskal Wallis test for the cartilage and synovial tissues resulted in a P-value of 0.000 for both tissues. Using the Mann Whitney test, it was studied which donors had significant differences. Because there were many differences, it was not incorporated in figure 10.

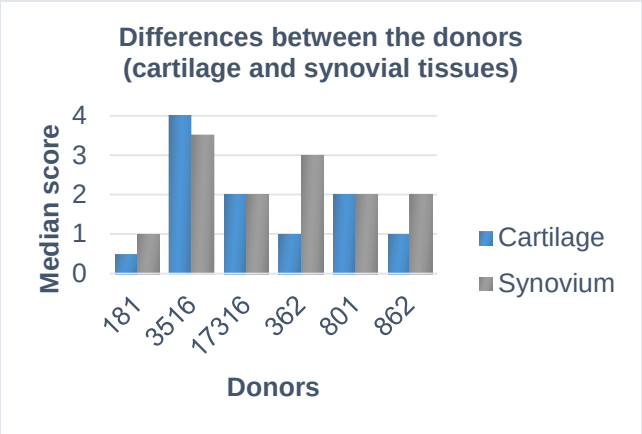

Figure 10 Differences between the donors for the scores of both the cartilage and synovial tissues.

The scores at day 0

The results of the Krenn- and OARSI scoring system and the IHC for COX-2 at day 0 are displayed in table 5. According to the IHC for COX-2, only donor 801 had healthy joint tissue, taking in consideration that the tissues of donors 362 and 862 were not available. The results of the Krenn- and OARSI scoring system showed that donors 3516 and 17316 had both healthy cartilage and synovial tissues at day 0.

Table 5 The results of the IHC and the Krenn- and OARSI scoring system for tissues at day 0. The numbers indicate the score. A question mark means there were no day 0 tissues available.

Donors	Results		
	IHC	Krenn score	OARSI score
181			
- Cartilage	0		0
- Synovium	1	2	
3516			
- Cartilage	2		0
- Synovium	1	0	
17316			
- Cartilage	2		0
- Synovium	0	0	
362			
- Cartilage	?		0
- Synovium	?	?	
801			
- Cartilage	0		?
- Synovium	0	0	
862			
- Cartilage	?		?
- Synovium	?	?	

The Krenn scoring system

The data of the Krenn scoring system was not normally distributed. Using the Kruskal Wallis test, there were no significant differences in Krenn score found between the conditions, whether the synovial tissues had been cultured alone (P-value of 0.225) or in a co-culture (P-value of 0.054). Furthermore, there were no significant differences in Krenn score between the tissues that had been cultured in a monoculture and in a co-culture (P-value of 0.967). When the scores of the synovial tissues of the NCD and the CD dogs were compared, there was a significant difference, indicated by a P-value of 0.029. In contrast to the COX-2 explant study, there were no significant differences in mean Krenn score between the donors (P-value of 0.254).

The OARSI scoring system

The data of the OARSI scoring system was also not normally distributed. Therefore, the Kruskal Wallis test was used to study the possible differences between the conditions. Whether the cartilage tissues had been cultured alone or in a co-culture, the differences between the conditions were not significant (P-value of 0.284 for the monocultures and 0.892 for the co-cultures). There were significant differences between the cartilage tissues that had been cultured in a monoculture and the tissues that had been cultured in a co-culture (P-value of 0.000). As in the COX-2 explant study, no differences were found between the scores of the NCD and CD dogs in the cartilage monoculture tissues (P-value of 0.096). However, the cartilage co-culture tissues did show a significant difference between the NCD and the CD

dogs (P-value of 0.009). Also, significant differences were found between the OARSI scores of the donors (P-value of 0.019 for the cartilage monocultures and P-value of 0.020 for the cartilage co-cultures).

Furthermore, the Spearman's rank correlation coefficient between the results of the Krenn scoring system and the synovial tissues of the COX-2 explant study was 0.223, which was not significant (P-value of 0.157). The correlation between the results of the OARSI scoring system and the cartilage tissues of the COX-2 explant study was also not significant, with a correlation coefficient of -0.005 and a P-value of 0.976.

COX-2 rat study
The tissues of the knee joints of nine rats that had received different doses of Celecoxib were stained for COX-2 using IHC. However, the tissues were primarily negative (data not shown). Therefore, no statistical analysis was performed on this data.

CD-68 rat study
Fourteen donor rats were divided in four groups: rats that had received no treatment (n=8), a low dose of Celecoxib (n=2), a middle dose of Celecoxib (n=2) and a high dose of Celecoxib (n=2). For the tissue of one rat that had received no treatment, it was not possible to evaluate the degree of staining. This donor rat was therefore not included in this study. The Kruskal Wallis test did not find significant differences between the treatment groups (P-value of 0.510) (figure 11).

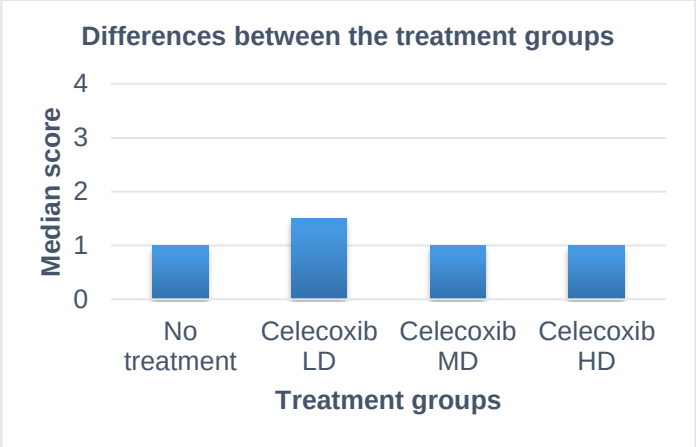

Figure 11 Differences in score between the treatment groups. LD = low dose; MD = middle dose; HD = high dose.

Conclusion
In the COX-2 explant study, there were no significant differences found between the conditions. The Krenn- and OARSI scoring system showed the same results. The COX-2 explant study and the Krenn scoring system also did not find significant differences between the culture methods. However, this was not the case for the OARSI scoring system. There were no differences in the COX-2 staining between NCD and CD donor dogs. The Krenn scoring system and the cartilage co-culture tissues of the OARSI scoring system showed that there were significant differences between the NCD and the CD dogs. The COX-2 explant study and the OARSI scoring system showed significant differences between the donors. No such differences were found when the Krenn scoring system was used. There was no correlation between the results of the COX-2 explant study and the Krenn- or OARSI scoring system. The data of the COX-2 rat study was not analyzed, since these results were primarily negative. There were no significant differences between the treatment groups in the CD-68 rat study.

Discussion
Differences between the conditions and the donors
Based on previous studies, it was expected that tissues with both TNF-α and Celecoxib would show less COX-2 staining than tissues with only TNF-α, since Celecoxib is a selective COX-2 inhibitor and TNF-α is an important inflammatory mediator in OA(6,14). However, statistical analysis of the results of the COX-2 staining in cartilage and synovial tissues showed no significant differences between the four conditions. Using the Krenn- and OARSI scoring system to evaluate the tissues showed the same results. A possible explanation is the limited number of donors used in this study. There were six donors (three NCD and three CD dogs) and it is possible that there were not enough donors in this study to obtain significant

differences between the conditions. Another possible explanation is the differences between the donors. *Beekhuizen et al* have previously described a donor variation in cytokine levels and chondrocyte metabolism in vitro(7). Moreover, *Cook et al* have advised to use donors with as little variation in gender, age and breed as possible, since these factors may influence the results(25). Also, OA is probably a polygenetic disease and using dogs that are closely related will minimize the variation(3,25). The donors used in this study differed in gender, age, breed and weight, so donor variation might have had an influence on the results of this study.

The results of the NCD and CD dogs for COX-2 staining were compared with each other. There is a reason these two types of dogs were compared in this study. It is well known that intervertebral disc degeneration (IVDD) is more common in CD dogs than in NCD dogs(26). The etiology of IVDD is different between these types of dog breeds. NCD dogs show an age-related degeneration, called fibrous metaplasia(27). The degeneration in CD dogs is characterized by a chondroid metaplasia. In contrary to fibrous metaplasia, which often occurs in NCD dogs older than 7 years, chondroid metaplasia in CD dogs can start at a very young age. IVDD in CD dogs is rapid and likely to be genetic; probably multiple genes are involved(26). The same structures involved in IVDD also play a part in the process of OA in the joint. Some authors recommend not to use both NCD and CD dogs to avoid any possible differences in OA between these dogs(25). However, it is currently not known whether there is a difference in OA between NCD and CD dogs. To study whether there could be a difference, these two groups were compared. The COX-2 explant study and the cartilage monoculture tissues evaluated by the OARSI scoring system showed no significant differences between these two types of dog breeds. In contrary, the Krenn scoring system and the co-culture tissues evaluated by the OARSI scoring system did result in significant differences between NCD and CD dogs. Because of these contradictory results, it is not certain if there are differences in OA between NCD and CD dogs. However, the limited number of donors in this study might have been a reason why this difference was not found in every evaluation. Thus, it cannot be concluded with certainty that there are differences between OA in NCD dogs and OA in CD dogs.

Differences between the culture methods

In the joint, synovial fluid provides nutrients that reach the chondrocytes by diffusion(28,29). Synoviocytes are the source for the inflammatory mediators that are involved in OA(6). It has been shown that co-culturing chondrocytes with synoviocytes or synovial supernatant results in a reduction of collagen type II deposition, probably because of both suppression of the synthesis and stimulation of the degradation of the collagen by MMPs(28). The latter may be due to release of IL-1 and TNF-α by synovial fibroblasts. Also, proteoglycan metabolism is reduced when chondrocytes are co-cultured with synovium, caused by synovial fibroblast-mediated IL-1 release. Moreover, membrane damage is higher in chondrocytes that have been cultured alone. During the process of osteoarthritis, IL-1β causes reactive oxygen species to accumulate, which leads to cell death(6). Synoviocytes are able to protect chondrocytes against this damage with scavengers like glutathione peroxidase, since chondrocytes do not have much of these scavengers(29). Because there are several tissues involved in OA which interact with each other, using a co-culture provides an in vitro model that resembles the in vivo situation in the joint(6,7). However, in a co-culture model, it is necessary to compromise for the culture medium, because synoviocytes require other culture conditions than chondrocytes(6). In this study, all tissues had been cultured in a chondrogenic medium. It is possible that the culture medium was not optimal for all tissues, especially the synovial tissues, which may have influenced the results of the COX-2 staining.

Evaluation of the joint tissue at day 0

It is important that the joint tissues of the donor dogs were healthy at day 0. A joint that is osteoarthritic at the start of the experiment will already have high levels of COX-2, because there is an inflammatory environment(8,14). If the donors did not have healthy joints at day 0, this could explain the positive results in the control tissues. When the tissues were collected

from the donors, there was a macroscopic and microscopic evaluation to examine if the joints were healthy. In this study, Hematoxylin & eosin and Safranin O fast green staining was used to evaluate the day 0 synovial and cartilage tissues, respectively. Also, IHC was performed on the day 0 tissues of some donors. Of only three donors, the data of all three scoring systems were available. These three donors showed that when the IHC of cartilage tissue is positive, the OARSI score may still be 0. The same applied to the IHC of synovial tissues and the Krenn score. Therefore, it is not possible to conclude with certainty for every donor that the joint tissue was healthy at day 0 of the study.

Differences between the treatment groups in the CD-68 rat study
In the CD-68 rat study, significant differences between the treatments groups were not found. This is contradictory to a previous study in humans, where treatment with Celecoxib did cause less CD-68 staining in comparison with humans that had not received any treatment(30). As in the COX-2 explant study, the limited amount of donors (two rats per treatment and seven rats for the control group) and donor differences are possible explanations for this result.

Future studies
In this study, it was not possible to demonstrate the positive effect Celecoxib might have on cartilage and synovial tissues. It could not be concluded whether Celecoxib had an effect on the COX-2 and CD-68 staining in tissues that had been exposed to different conditions or treatments; donor differences and possibly other factors may have had an influence on the results. Therefore, there a several possibilities for future studies.

Donor choice

For further research on the presence of COX-2 in cartilage and synovial tissues in different conditions, it is necessary to use more donors and to have less donor variation(25). A possibility to reduce the donor variation in the present study is to use the results of the tissues at day 0 and compare these with the results after 21 days of culturing. In this way, a correction can be made for the differences between the donors. In future studies, donor dogs with the same gender, breed and the same weight class should be selected to limit donor variation(25). As previously mentioned, using donor dogs that are closely related will also minimize the variation.

Culturing tissues of OA patients

Instead of using the tissues of healthy donor dogs, tissues of OA patients can be used. However, this will likely increase the donor variation, since the severity of OA will be different in the patients. Different grades of OA will cause different tissue responses(7). The stage of OA is also an important factor, since certain mediators are active in specific stages. As previously mentioned, this variation can be reduced by scoring the tissues of all donors at day 0. In the present study, only TNF-α was used to induce inflammation in the tissues. TNF-α is often used to simulate OA in in vitro models(6,7). However, there are also other cytokines that are important in OA(2,6). For instance, besides TNF-α, IL-1β is often used in in vitro studies(6,7). It is possible that in this relatively new culture model, adding TNF-α to the tissues does not create an environment that is similar to OA in the in vivo situation.

Conditioned medium of synovium

In co-culture models, there is often a compromise concerning the culture medium(6). Therefore, the chondrogenic medium used in this study was probably not optimal for the synovial tissues. A possible solution for this problem is another culture method, called the conditioned medium of synovium. With this method, the cartilage and synovial tissues are cultured separately and the medium of the synovial tissues is added to the cartilage tissues(31). As previously mentioned, there is an interaction between synoviocytes and

chondrocytes(28,29). With this culture model, synoviocytes do not have to be cultured in an unsuitable culture medium, but are able to influence chondrocytes, as in the in vivo situation in the joint.

Quantitative measurement

In this study, the tissues were scored using a scale from 0 to 4. Each score indicated a range of percentages of positive cells. Another method of assessing the degree of COX-2 staining is to count the exact percentage of positive cells to obtain a quantitative measurement and a more accurate result for statistical analysis. This can be done manually, but also digital image analysis is now available(32).

The correlation of PGE2 and COX-2

Instead of measuring the presence of COX-2, it is also possible to study the presence of PGE2 in OA tissues, since this is the product of the activity of the COX-2 enzyme and PGES(33). Previous studies have found that the PGE2 level in OA tissues is increased when compared to healthy tissues(16,20,33). When Celecoxib is added to the tissues, it causes a decrease of the elevated PGE2 level(16,30,34). *Hardy et al* studied the combined activity of COX-2 and PGES by measuring the conversion of arachidonic acid to PGE2(33). The COX/PGES activity and PGE2 level were elevated in OA-induced joint tissue. The study of *Mastbergen et al* examined the effect of Celecoxib on the PGE2 production, which indirectly gave information about the effect of this NSAID on the COX-2 activity(16). In a future study, when joint tissues would be exposed to inflammatory conditions (for example by the addition of TNF-α and/or IL-1β) and would be stained for both PGE2 and COX-2, it could be possible to obtain a correlation for these two parameters.

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