On the pathogenesis of canine cranial cruciate ligament rupture: The role of stress induced ligamentocyte death.

Graduate School for Cellular and Biomedical Sciences
University of Bern
PhD Thesis

Submitted by
Simone Forterre
from Cologne, Germany

Thesis advisor
Prof. Dr. David Spreng
Department of clinical veterinary medicine
Division of small animal surgery
Vetsuisse Faculty of the University of Bern

Original document saved on the web server of the University Library of Bern

This work is licensed under a Creative Commons Attribution-Non-Commercial-No derivative works 2.5 Switzerland License. To see the license go to http://creativecommons.org/licenses/by-nc-nd/2.5/ch/ or write to Creative Commons, 171 Second Street, Suite 300, San Francisco, California 94105, USA.
Copyright Notice
This document is licensed under the Creative Commons Attribution-Non-Commercial-No derivative works 2.5 Switzerland.
http://creativecommons.org/licenses/by-nc-nd/2.5/ch/

You are free:

- to copy, distribute, display, and perform the work

Under the following conditions:

- **Attribution.** You must give the original author credit.

- **Non-Commercial.** You may not use this work for commercial purposes.

- **No derivative works.** You may not alter, transform, or build upon this work. For any reuse or distribution, you must take clear to others the license terms of this work.

Any of these conditions can be waived if you get permission from the copyright holder.

Nothing in this license impairs or restricts the author's moral rights according to Swiss law.

The detailed license agreement can be found at:

http://creativecommons.org/licenses/by-nc-nd/2.5/ch/legalcode.de
Accepted by the Faculty of Medicine, the Faculty of Science and the Vetsuisse Faculty of the University of Bern at the request of the Graduate School for Cellular and Biomedical Sciences

Bern, Dean of the Faculty of Medicine

Bern, Dean of the Faculty of Science

Bern, Dean of the Vetsuisse Faculty Bern
Content
Acknowledgment 5
Summary 6
Zusammenfassung 7
Abbreviations 8
Introduction 9
  CCL rupture in dogs 9
  Partial and complete rupture of the CCL 10
  Caudal cruciate ligament rupture 10
  Problem of CCL disease 10
  Morphology of the stifle joint 10
  Function 12
  Pathology 12
  Histopathology of the CCL 13
  Remodeling and Repair 13
  Cruciate ligament matrix metabolism and degeneration 14
  Role of NO production in cruciate ligament degeneration 14
  Role of cell death in CCL rupture 15
  Role of cytokines and immune responses 15
Objectives of this thesis 16
Results 17
  Study I- In vitro effect of different mediators of apoptosis on canine cranial and caudal
  cruciate ligament fibroblasts and its reversibility by pancaspase inhibitor zVAD.fmkt 17
  Study II- Nitric oxide induces cell death in canine cruciate ligament cells by activation of
  tyrosine kinase and reactive oxygen species 24
  Study III- NSAIDs protect canine cruciate ligament cells against nitric oxide induced
  programmed cell death 35
Final discussion 43
  Apoptosis-induction in the CCL and CaCL 43
  Caspase inhibition in the CCL 44
  NO-signaling in the CCL 44
  COX and prostaglandin in the CCL 45
Outlook and future directions 46
References 47
List of Publications 53
Declaration of Originality 54
Acknowledgment

First of all, I would like to express my gratitude to Prof. Dr. David Spreng, whose encouragement, guidance and support from the initial to the final stages enabled me to develop an understanding of the subject.

I would also like to express my appreciation to my second supervisor, Prof. Andreas Zurbriggen. Without his valuable assistance and support this thesis would not have been possible. I owe him my deepest gratitude for giving me the opportunity to work in his lab.

It is a pleasure to also pay tribute to PD Dr. Giuseppe Bertoni for his good advice, support, and his willingness to share his bright thoughts with me, which were very fruitful for shaping up my ideas and research.

I would like to express my thanks to the members of the thesis committee, Prof. Dr. Andrew Hemphill, as my mentor, PD Dr. Giuseppe Bertoni, as my co-referee, Prof. Dr. Andreas Zurbriggen and Prof. Dr. David Spreng as my supervisors.

I have also benefited from advice and guidance from Prof. Dr. Thomas Brunner using his precious time to give his critical comments about my research. I am sincerely and wholly grateful to him for his unsurpassed knowledge about apoptosis.

I am much indebted to Philippe Plattet for his valuable advice during our discussions.

I would also like to thank Ljerka Zipperle for being the first person who taught me how to work with cell culture systems. I am sure it would have not been possible without her help.

I gratefully thank Diane Steber, Karla Vigiez, and Adam Michel for their constructive English comments on this thesis. I am thankful that in the midst of all their activities, they accepted to be a member of the reading committee.

Above all, I would like to thank my family for their personal support and great patience at all times. They boosted me morally and provided me with great information resources. My parents have given me their unequivocal support throughout, as always, for which my mere expression of thanks likewise does not suffice.

Lastly, I offer my regards and blessings to all of those who supported me in any respect during the completion of the project.
Cranial cruciate ligament (CCL) rupture is one of the most common orthopedic injuries in dogs, responsible for chronic osteoarthritis in the stifle joint. Previous studies mainly focused on biomechanical factors, which have been considered the main cause of CCL rupture. But evidence that an intrinsic mechanism in the ligament is responsible for the gradual degradation of the matrix in CCL disease is increasing.

This thesis is focused on one of the intrinsic factors: increased apoptosis in CCLs of affected dogs. Several *in vitro* experiments were performed to elucidate the pathway of apoptosis induction as well as details of the signaling of nitric oxide (NO) induced cell death. Moreover, the possibility of apoptosis inhibition in CCL cells was investigated.

The first part of the thesis demonstrates that primary fibroblasts of canine cranial (CCL) and caudal (CaCL) cruciate ligaments undergo apoptosis either by stimulating cell surface receptors or by activation of the mitochondrial apoptotic pathway in a dose-dependent manner. In contrast to CaCL fibroblasts, fibroblasts from the CCL were significantly more susceptible to apoptosis inducers. Strong apoptosis inducers showed the same effect in both ligaments. By contrast, stimulation with dexamethasone or TNFα could not induce apoptosis in CCL and CaCL fibroblasts. However, TNF receptors were identified on the surface of CCL and CaCL cells. Summarizing, CCL and CaCL cells exhibit essential differences from one another despite the fact that they are stifle ligaments within the same joint.

A subsequent study of this thesis focused on elucidating the mechanism of NO-signaling responsible for CCL and CaCL cell death. Data showed that ligament cell death was clearly linked to the activation of tyrosine kinase, as the inhibitor of tyrosine kinase reduced the level of ligament apoptosis very effectively. Moreover, NO-induced cell death was not solely related to the production of NO but also to the generation of reactive oxygen species such as peroxynitrite, hydrogen peroxide and/or superoxide.

Because NO and prostaglandins are involved in the same inflammatory and immune processes, the relation between the NO and cyclooxygenase enzymes were the objective of the third study. The protective effect of the tested NSAIDs was dependent on the magnitude of the cytotoxic effect of NO. Although the tested NSAIDs did not significantly suppress the endogenous prostaglandin E₂ release of treated CCL and CaCL cells, carprofen, meloxicam, and robenacoxib had a protective effect in canine cranial cruciate ligament disease through the inhibition of apoptosis in ligamentocytes.

In conclusion, our studies demonstrate that a single inhibitor cannot completely protect canine CCL cells from apoptosis. Consequently, it seems to be a combination of inhibitors that are required to disrupt the classical apoptosis pathway as well as up- or downstream kinase pathways, thus providing effective regulation of degenerative ligament rupture in dogs.
Die Ruptur des vorderen Kreuzbandes ist eines der häufigsten orthopädischen Verletzungen beim Hund und führt zur Arthrosebildung im Kniegelenk. Anfänglich wurden dieser degenerative Erkrankung, die vor allem mittelgrosse bis grosse Hunde betrifft, rein biomechanische Faktoren als Ursache zugrunde gelegt. Mittlerweile gibt es zahlreiche Hinweise, dass gleichzeitig Faktoren innerhalb des Ligaments, sogenannte intrinsische Faktoren, eine bedeutende Rolle bei der Matrixdegeneration des vorderen Kreuzbandes spielen und in Folge zu einem Funktionsverlust des Bandes führen.


Bei der Untersuchung der beteiligten Signaltransduktionswege konnte die zweite Studie darlegen, dass NO den Zelluntergang der Ligamentzellen unter anderem durch die Aktivierung der Tyrosinkinase auslöst. Darüber hinaus liess sich erkennen, dass ein durch NO induzierter Zelltod nicht alleine auf die direkte Wirkung von NO zurückzuführen war, sondern vielmehr die Bildung von sogenannten reaktiven Sauerstoffspezies (ROS) entscheidend ist.


Zusammenfassend liess sich erkennen, dass ein einzelner Inhibitor nicht in der Lage war die induzierte Apoptose vollständig bzw. massgeblich zu verhindern. Aufgrund verschiedener involvierter Signalprozesse innerhalb der Kreuzbandzellen schien vielmehr eine Kombination unterschiedlicher Inhibitoren notwendig zu sein, um eine Regulation der Apoptose vornehmen zu können.
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARC</td>
<td>apoptosis repressor with a CARD (caspase recruitment domain)</td>
</tr>
<tr>
<td>BAR</td>
<td>bifunctional apoptosis inhibitor</td>
</tr>
<tr>
<td>CaCL</td>
<td>caudal cruciate ligament</td>
</tr>
<tr>
<td>CCL</td>
<td>cranial cruciate ligament</td>
</tr>
<tr>
<td>e-FLIP</td>
<td>cellular FLICE-like inhibitory protein</td>
</tr>
<tr>
<td>CICD</td>
<td>caspase independent cell death</td>
</tr>
<tr>
<td>COX</td>
<td>cyclooxygenase</td>
</tr>
<tr>
<td>DNA</td>
<td>desoxyribonucleoid acid</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>Fas</td>
<td>death receptor of TNF superfamily</td>
</tr>
<tr>
<td>FasL</td>
<td>Fas ligand CD95L, type-II transmembrane protein</td>
</tr>
<tr>
<td>HC-gp 39</td>
<td>human cartilage glycoprotein 39</td>
</tr>
<tr>
<td>IgG</td>
<td>immunoglobulin G</td>
</tr>
<tr>
<td>IgM</td>
<td>immunoglobulin M</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>MAP</td>
<td>mitogen activated protein kinase</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>nitric oxide synthase</td>
</tr>
<tr>
<td>cNOS</td>
<td>constitutive nitric oxide synthase</td>
</tr>
<tr>
<td>iNOS</td>
<td>inducible nitric oxide synthase</td>
</tr>
<tr>
<td>NSAID</td>
<td>non-steroidal antiinflammatory drug</td>
</tr>
<tr>
<td>OA</td>
<td>osteoarthritis</td>
</tr>
<tr>
<td>PG</td>
<td>prostaglandin</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>TNFα</td>
<td>tumor necrosis factor alpha</td>
</tr>
<tr>
<td>TNF RI /II</td>
<td>tumor necrosis factor receptor I /II</td>
</tr>
<tr>
<td>zVAD.fmk</td>
<td>benzylxycarbonyl-Val-Ala-Asp (OMe)fluormethylketone</td>
</tr>
</tbody>
</table>
Introduction

CCL rupture in dogs

Cranial cruciate ligament (CCL) rupture, one of the most common injuries in dogs, is a major cause of osteoarthritis (OA) of the stifle joint [42]. First reports of dogs with CCL rupture focused mainly on surgical treatment of the unstable stifle more than 50 years ago [82]. As it has been a long-held clinical belief that the mechanism for CCL rupture in dogs is similar to anterior cruciate ligament rupture in humans [7]. The dog has served as a common animal model for human research. In the last 15 years, a great deal of attention has been concentrated on trying to identify the underlying cause that might lead to the structural failure of the ligament. The majority of dogs rupture their CCL during normal daily activities due to secondary progressive and irreversible degenerative changes within the ligament itself. This form of non-traumatic rupture is clinically termed spontaneous CCL rupture. Depending on the degree of affected fibers the rupture can be partial or complete (Figure 1).

Both partial and complete rupture result in stifle joint instability and contribute significantly to the development of OA subsequently with damage of the meniscus. In addition, 30% of all dogs surgically treated for CCL rupture subsequently sustained the same injury in the contralateral leg [11,29]. There are to date no consistently effective methods to prevent the development of CCL degeneration or to slow its progression. Although any breed may be affected by CCL disease, there are some breeds at high risk such as Newfoundland, Rottweiler, Labrador Retriever, Bulldog, and Boxer [100]. In addition, large breed dogs older than 4 years are significantly more likely to develop CCL rupture, as well large breed dogs appear to develop a rupture at a younger age than smaller breeds [104]. Neutered female dogs have the highest occurrence of CCL disease according to recent studies, and they may experience increased weight gain and body fat [28,92].

![Figure 1](image.png)

**Figure 1** Drawing of the right canine stifle joint. A) Partial rupture of the cranial cruciate ligament; B) complete rupture of the cranial cruciate ligament.
**Partial and complete rupture of the CCL**

Cranial cruciate ligament ruptures in dogs have been classified as partial or complete based on an anatomical definition. The canine CCL consists of two bundles, the craniomedial and the caudolateral component. Sectioning one of these bundles ex vivo induces a mild joint instability. On physical examination, however, partial rupture of the CCL needs not necessarily be associated with a positive cranial drawer motion due to periarticular fibrosis [89]. Thus, dogs with partial cruciate rupture often have a stable joint clinically, but are presented with lameness, effusion of the stifle joint, and synovitis [21]. A clinically obvious joint instability provides clear evidence of substantial disruption of the CCL. In dogs with partial CCL rupture, the remaining intact part is often found to be stretched out and slack. During the course of the CCL disease, an initially partial rupture can emerge as a complete rupture due to a small traumatic event during normal daily activities. A complete CCL rupture produces obvious instability of the stifle joint, resulting in more severe joint pain, and progressive degenerative changes within the joint [65].

**Caudal cruciate ligament rupture**

Rupture of the caudal cruciate ligament occurs less often. An isolated disruption of the caudal cruciate ligament (CaCL) is found in less than 2% of dogs with hindlimb lameness [41]. Dogs with a CaCL rupture usually have a history of trauma with avulsion fracture of an attachment site or other complicating injuries. The pathology of the CaCL is not of main interest in current research evident through the lack of studies on this topic.

**Problem of CCL disease**

The biomechanical properties of the cruciate ligaments are essential for stifle joint stabilization. It is the degradation of these properties that ultimately leads to cruciate rupture and the loss of joint stability. Consequently increased laxity contributes to abnormal knee biomechanics and abnormally distributed joint loads that likely lead to or accelerate arthritis.

There exist a large number of different surgical procedures mainly aimed at treatment of stifle instability and not at reconstruction or repair. Stabilization procedures are accomplished either by osteotomy of the tibia (tibial plateau leveling osteotomy, tibial tuberosity advancement) or by extracapsular stabilization. There is no consensus regarding which technique should be used for a patient, and depends on the surgeon’s experience and ability. Comparing these techniques, most studies to date have shown no differences in outcome [18,19]. To date, no surgical technique or medical treatment has been demonstrated to halt or slow the continued progression of osteoarthritis.

Until materials that display the key properties of ligaments are developed, or a means to reconstruct or replace the CCL is found, stabilization of stifles in dogs with CCL rupture will continue to be a challenge. The lack of understanding the mechanisms involved in the development of CCL rupture in affected dogs remains a barrier to the development of better treatments.

**Morphology of the stifle joint**

The stifle is a complex, synovial bicondylar hinge joint (Figure 2). It actually comprises three functional compartments: the femoropatellar articulation consists of the patella and the patellar groove on the front of the femur through which it slides; and the medial and lateral femorotibial articulations linking the femur with the tibia. The joint is bathed in synovial fluid which is contained inside the synovial membrane called the joint capsule.

There are two articular disks, the medial and the lateral meniscus which balance the incongruity of the joint. They consist of connective tissue with extensive collagen fibers containing cartilage-like cells. The menisci are flattened at the center of the knee joint, fused with the synovial membrane laterally, and can move over the tibial surface [90]. The two menisci are attached to each other cranially by the transverse ligament and caudally
by the meniscofemoral ligaments. The menisci serve to protect the ends of the bones from rubbing on each other and to effectively deepen the tibial sockets into which the femur attaches. They also play a role in shock absorption, and may be cracked, or torn, when the knee is forcefully rotated and/or bent.

Cartilage is a thin, elastic tissue that protects the bone and ensures that the joint surfaces can slide easily over each other. Cartilage ensures supple knee movement [90]. There are two types of joint cartilage in the knees: fibrous cartilage (meniscus) and hyaline cartilage. Fibrous cartilage has tensile strength and can resist pressure. Hyaline cartilage covers the surface along which the joints move. Cartilage has a very limited capacity for self-restoration and in such, will wear over years [55]. The newly formed tissue will generally consist of a large part of fibrous cartilage of lesser quality than the original hyaline cartilage. As a result, new cracks and tears will form in the cartilage over time.

The extracapsular collateral ligaments surrounding the knee joint offer stability by limiting movements and, together with several menisci and bursae, protect the articular capsule. In addition, the stifle joint is stabilized by a pair of cruciate ligaments. They are located intracapsularly and enveloped by a synovial membrane and thus they are extrasynovial. The **cranial cruciate ligament** stretches from a distal cranial medial orientation on the lateral condyle of the femur to the cranial interspinous area of the tibial plateau [4]. Therefore, its course extends diagonally across the stifle joint space in an outward spiral. The CCL is critically important because it prevents the tibia from being pushed too far cranial relative to the femur. It is often torn during twisting or bending of the knee. The **caudal cruciate ligament** stretches from the medial condyle of the femur to the caudal intercondylar area [4]. Injury to this ligament is uncommon but can occur as a direct result of forced trauma to the ligament [41]. This ligament prevents caudal displacement of the tibia relative to the femur.

The internal organization of cruciate ligaments is made up of a collection of longitudinal fiber bundles. Each fiber bundle has a hierarchical architecture composed of fascicular subunits, fibrils up to subfibrils and microfibrils [4]. The fascicles are enveloped in a thin connective tissue sheath, the endoligament. The endoligament itself is connected to the epiligament, a more vascular connective tissue that surrounds the entire ligament. Each fascicle appears hypocellular and the cells are aligned in rows interspersed between bundles of collagen. These fibroblasts, often referred to as ligamentocytes [98], are arranged to create the fibrils consisting mainly of collagen I (70-80%) and to a lower extent of collagen III (3-10%) [1]. Collagen fibers show varying amounts of crimp waves which serve the purpose of compensating load from joint motion. The cranial and caudal cruciate ligament consist each of two different bundles of fibers which are recruited differentially throughout stifle flexion: the cranial medial bundle and the caudal lateral bundle [23]. The cruciate ligaments are covered by a synovial membrane. In contrast to cruciate ligaments, the synovial membrane consists of numerous cells, primarily small fibroblast, so called synoviocytes, and a dense connective tissue.
The blood supply to both cruciate ligaments is predominantly of soft tissue origin [57]. Vessels coming from the osseous attachment are negligible. The infrapatellar fat pad and the synovial membrane are the most important sources of vessels. A network of epiligamentous vessels ensheathes the cruciate ligaments throughout their entire length forming an extra- and intraligamentous blood network. Interestingly, the CCL is less vascularized at the core of the mid-portion, the area where rupture mainly occurs. Various mechano-receptors and proprioceptive receptors have been identified within the substance of the cruciate ligaments.

Function

The knee permits flexion and extension about a virtual transverse axis, as well as a slight medial and lateral rotation about the axis of the tibia in the flexed position. The knee joint is called "mobile" because the femur and lateral meniscus move over the tibia during rotation, while the femur rolls and glides over both menisci during extension-flexion [90].

The center of the transverse axis of the extension/flexion movements is located where both collateral ligaments and both cruciate ligaments intersect. This center moves upward and backward during flexion, while the distance between the center and the articular surfaces of the femur changes dynamically with the decreasing curvature of the femoral condyles. The total range of motion is dependent on several parameters such as soft-tissue restraints, active insufficiency, and hamstring tightness [85].

With the stifle extended, both components of the CCL are taut and limit cranial translation of the tibia relative to the femur [4]. During extension, the femoral condyles glide into a position which causes the complete unfolding of the medial and lateral collateral ligaments which become the primary restraints of rotation. However, the cruciate ligaments, especially the caudolateral component of the CCL, prevent hyperextension.

In the flexed position, the collateral ligaments are relaxed while the cruciate ligaments are taut [4]. Rotation is controlled by the twisted cruciate ligaments. During medial rotation of the tibia, the two ligaments get twisted around each other, effectively reducing the amount of possible rotation, and they become unwound during lateral rotation of the tibia [47]. Because of the oblique position of the cruciate ligaments at least a component of one of them is always tense and these ligaments control the joint as there is loss of collateral ligament support.

Pathology

The etiopathogenesis of CCL rupture is still poorly understood and controversial. A definitive cause for CCL disease in dogs remains unknown but the presumed causal factors result in a final common pathway. Biological and biomechanical components are inextricably linked in health and disease of the CCL and should be considered both for the multifactorial process. One of the major limitations in most investigations is that the majority of studies on CCL disease to date have been carried out on dogs that already sustained a CCL rupture, which is the end-stage of the disease. Several risk factors have been identified such as breed, gender, bodyweight, stifle joint conformation, ligament extracellular matrix metabolism, and joint inflammation but none of them alone can satisfactorily explain it. There have been numerous studies evaluating the association of conformational variations of canine hindlimbs and CCL rupture [3,38,53]. Mechanical factors like straight tibial plateau angle, distal femoral torsion, femoral angulation, tibial torsion, and intercondylar notch stenosis have been associated with CCL disease [17,26,87]. However, mechanical factors have been assumed to be the major cause of CCL rupture, the true effect on the etiopathogenesis of CCL disease is currently unknown. Studies have shown that the tibial plateau angle is not significantly different in Labrador Retrievers with and without CCL disease, or between Greyhounds – a low risk breed – and Labrador Retrievers [101]. Researchers have
hypothesized that repetitive episodes of
subluxation under weight-bearing conditions
result in articular cartilage damage, meniscal tears,
and stretching of secondary capsular and
ligamentous restraints [26,38,53]. Such episodes
may result in additional trauma and progressive
deterioration of the joint. Altered gait caused by a
steep tibial plateau slope, or excessive internal
rotation of tibia associated with genu varum
measured by stifle joint kinematics may also
contribute to abnormal loads on the CCL of dogs
with a high risk of CCL disease [15,86]. It is
possible that abnormal biomechanics from various
causes initiates and perpetuates abnormal ligament
biology sustaining a cycle of stifle joint organ
failure.

Beside biomechanical factors several studies
are focused on the intrinsic process of the CCL
itself. This direction of research has gotten more
emphasis in the last 15 years. Developmental [27]
or immune-mediated [5] disorders, genetic
components [102,103] as well as cell
[39,46,58,74] and matrix [16,71,72,79] disorders
have been implicated in CCL disease. These
studies suggest that intrinsic alterations may be an
etiopathogenic factor leading to CCL disease
rather than a consequence of rupture of the
ligament. Abnormal ligament biology as a primary
player does not exclude abnormal biomechanics as
a driving force behind this abnormal biology. It is
the conjunction of both factors which have to be
considered for a comprehensive understanding of
CCL disease.

Histopathology of the CCL
Development of progressive CCL rupture appears
to involve a gradual degeneration of the CCL
itself, inflammatory disease in the stifle joint,
partial rupture and eventually complete rupture of
the ligament [45].

As described in the morphology section, the
CCL is a complex structure comprised of a
population of cells and an extracellular matrix
(ECM). The histological features are comparable
to tendons and other ligaments. The predominant
cell type of the CCL is the fibroblast. These
ligamentocytes are arranged in rows between
collagen fiber bundles and follow the crimp
waveform of the fibers. Similar to the human
anterior cruciate ligament, three different
phenotypes can be found in the canine CCL: (1)
fusiform or spindle-shaped, (2) ovoid, and (3)
spheroid [77]. It is unclear whether these cells
form different metabolic states or whether they are
distinctly different fibroblasts. Several histological
studies suggest that fusiform fibroblast were
located in the proximal one quarter of the ligament
as it coursed from the femoral to the tibial
attachment [76]. This zone is characterized by a
high number density clearly attached to the
extracellular collagen. Ovoid fibroblasts are more
densely arranged in the loose connective tissue of
the proximal one quarter. The third type, the
spheroid fibroblast, is located in a poor density in
the distal three quarters of the ligament.

As a consequence of chronic and irreversible
degeneration of the CCL, cell phenotype
undergoes a partial fibrocartilagenous
transformation [79,98]. Such changes are more
common in the mid-portion and the core region of
the CCL, the common site of rupture. The
superficial epiligamentous region and the regions
close to bony attachments deteriorate later. In the
ruptured CCL, more severe phenotypic
transformation to spheroid cells is described with
a decreased cellularity exclusively in the core
region [45]. However, inflammatory or reparative
responses are rarely observed [45].

Remodeling and Repair
In the process of extracapsular ligament healing –
for instance that of collateral ligaments – the gap
is filled initially by a blood clot, which is invaded
immediately by proliferating fibroblast from the
surrounding connective tissue [35]. Four days
after injury, new collagen fibrils form and after 2
weeks these fibrils bridge the gap. Finally, the
ligament shows rows of fibroblasts within parallel bundles of extracellular matrix composed primarily of collagen type I fibers [61]. In contrast, CCL injuries are unlikely to heal either with closed treatment or primary repair due to weak intrinsic healing potential or due to inappropriate ligament protection against excessive external forces during convalescence [82]. Other factors which adversely affect the healing capacity are the complex anatomy of the ligaments, nutritional delivery and environment.

There are major differences in healing potential between completely and partially ruptured CCLs [82]. A complete rupture of the CCL comes along with disruption of the synovial membrane. Synovial fluid flows around the ligament and prevents clot formation which is necessary to start the healing process. The injured ends of the completely ruptured CCLs retract and as a result of inadequate blood supply the ends become devitalized and disintegrate.

In partially torn CCLs, the healing process consists of intrinsic and extrinsic healing. Intrinsic healing occurs within the ligament itself, as a result of the activity of intrinsic fibroblasts and the increase in ligamentous blood supply from the synovium [48]. Extrinsic healing is due to several factors, such as the extrinsic peripheral fibroblasts, plasma and inflammatory cells, and extraligamentous vascular invasion. However, the CCL does not have sufficient extrinsic healing effects due to the articular cavity [10,81]. The repair process often extends from months to years but the injured ligament never fully recovers its original mechanical properties [81]. The major deficiencies are increased laxity and diminished tissue strength. Consequently, conservative treatments bear clear disadvantages, including the long-term wearing of a brace and a risk of osteoarthritis due to a continued state of disability.

**Cruciate ligament matrix metabolism and degeneration**

The mechanical properties of cruciate ligaments are dependent on the composition and structure of the ECM, which after deduction of water is composed mainly of collagen. Ligamentocytes are responsible for the homeostasis of the ECM; in particular, collagen turnover is a balance between synthesis and degradation. To date, most of the interest in ECM metabolism of the CCL has focused on degradation [16,72]. ECM degradation has been further characterized biochemically, revealing that ruptured CCL have significantly higher amounts of immature cross-links, total and sulfated glycosaminoglycans, and water content [16,69]. Elevated concentrations of gelatinase (matrix metalloproteinase-2) and a greater number of cells with proteinase tartrate-resistant protein and cathepsin K provide indications for increased matrix turnover and collagen remodeling [70,107]. However, it is unclear whether these ECM changes represent the initial phase before or whether it is a part of a reparative process after rupture.

**Role of NO production in cruciate ligament degeneration**

Nitric oxide (NO) is formed from the terminal guanidine nitrogen atom of L-arginine by nitric oxide synthase (NOS). Two major forms of NOS have been identified. Under normal physiological conditions, the constitutive, calcium-dependent NOS isoform (cNOS) is present in numerous cells including endothelium and neurons. In an inflammatory setting or in the presence of cytokines, the inducible, calcium-independent NOS isoform (iNOS) is expressed in numerous cell types [67]. Production of low levels of NO from cNOS functions to regulate a number of homeostatic processes, whereas generation of larger quantities of NO from iNOS accounts for its proinflammatory and cytotoxic effects by inhibiting mitochondrial respiration and inducing DNA alterations [6]. NO produced in macrophages or polymorphonuclear cells containing free oxygen radicals contributes to the generation of cytotoxic compounds such as peroxynitrite. NO is also involved in the pathogenesis of joint diseases such as osteoarthritis and rheumatoid arthritis in humans and dogs [2,83]. Although the exact signaling
cascade of NO in OA is unknown, it has been shown that NO mediates the expression of inflammatory cytokines and inhibits the synthesis of proteoglycans and collagen by up-regulation of matrix metalloproteinases [31,32,95]. In addition to ECM degrading effects, NO is known to mediate cell death in cartilage [56,59] and ligament tissue [74]. NO levels, usually measured as nitrite, in urine, blood, and joint fluid showed massive increases in rheumatoid arthritis patients and modest elevations in patients with OA. Nitrite levels were higher in joint fluid than in blood or urine of humans, indicating an elevated local synthesis in the diseased joints [51]. In dogs, NO production in OA cartilage was greater than in healthy cartilage [93]. Disease activity and NO levels were decreased in animal models by prophylactic administration of NO inhibitors (e.g. L-nitro-arginine methyl ester or aminoguanidine) [94]. However, these inhibitors had little or no effect when they were given after disease induction [52,94]. Within joints, NO can be produced by superficial cartilage chondrocytes, by cells contained in the inflammatory synovial membrane, including macrophages and synoviocytes as well as by ligamentocytes [30,93]. Measuring NO production in ligamentocytes, especially after partial or complete rupture, has a limited use. The reason is that the NO level could be influenced by the traumatic process of rupture or could be triggered by the original CCL pathology.

Role of cell death in CCL rupture

Ligamentocytes are the major cell type in cruciate ligaments and are responsible for the synthesis and the maintenance of the extracellular matrix. In normal mature ligaments, ligamentocytes synthesize sufficient amounts of macromolecules to maintain the integrity of the matrix, whereas in CCL disease an increase of proteolytic activity is present [12]. There is a decline in the number of ligamentocytes with age documented in intact CCLs [98] as well as in the core of ruptured CCLs [45]. Furthermore, there is increasing evidence suggesting that ligamentocyte cell death, whether through apoptosis or necrosis, may result in failure to maintain ECM integrity and contribute to the progression of ligament degeneration [46,58]. Using caspase-3 as a marker for apoptosis, significant more apoptotic cells were seen in the ruptured CCL compared to the intact CCL [39]. No differences could be shown when comparing the amount of apoptotic cells between the intact part of partially ruptured CCLs and completely ruptured CCLs [58]. These studies suggest that apoptosis may be an intrinsic initiating factor leading to CCL degeneration rather than a consequence of acute rupture of the ligament. However, the mechanisms regulating ligamentocyte death have not been characterized.

Role of cytokines and immune responses

Immune response and proinflammatory cytokines are additional biological factors that have received massive attention as possible candidates for induction and perpetuation of CCL disease [25]. Cruciate ligaments are enveloped by a synovial membrane and thus are extrasynovial. Upon damage of this structure, collagen antigens are released in the synovial fluid, capable of evoking an immune response. Antibodies against collagen I and II have been detected in dogs that sustained a CCL rupture [22]. Furthermore, significantly higher amounts of IgG and IgM have been detected in the synovial tissue of stifle joints of dogs with CCL rupture compared to normal ones. However, there was no evidence that anticollagen antibodies initiate CCL damage in dogs, since not all dogs with high antibody concentrations developed a contralateral CCL rupture [20].

There is increasing evidence that certain cytokines play an important role in joint inflammation and cartilage degradation in diseased and/or damaged joints. Studies examining various arthropathies in dogs and humans have found an imbalance between pro-inflammatory (TNFα, IL-1b, IL-6, IL-8) and anti-inflammatory cytokines (IL-1ra, IL-10) [12,33,63]. This imbalance is thought to have potential to induce cartilage loss and eventually lead to OA. IL-1 and TNFα initiate or suppress
gene expression of several proteins and promote cartilage loss [91]. TNFα has a function in the acute phase and in chronic joint inflammation. In dogs with rheumatoid arthritis, CCL rupture or with an experimentally transected CCL, the amount of TNFα varied from undetectable to elevated [13,37,49].

Objectives of this thesis

Many etiological factors have been proposed, including age-related deterioration, joint conformation, and synovial inflammation leading to degradation of the ligament. However, only a few studies focus on the processes taking place within the ligament such as a disregulation of cell death [39,58,75]. Our hypothesis is that beside biomechanical factors, an increase in different inflammatory cytokines and biological factors in the stifle joint leads to an imbalance between apoptosis-inducing and anti-apoptotic factors which finally favor the induction of cell death of ligamentocytes. Ligamentocytes are responsible for the homeostasis of the ligament matrix. Thus, an increased rate of ligamentocyte death may lead to decreased matrix production followed by an intrinsic weakness and finally the rupture of the CCL. Decreasing the rate of apoptosis could lead to less matrix degeneration and survival of the ligament.

The objective of this thesis was to get new insights on cell death in cruciate ligaments of dogs. To accomplish these goals we performed several in vitro experiments to elucidate the pathway of apoptosis induction and possibilities of apoptosis inhibition.

In the first study we tested different naturally and experimentally occurring substances on their potential to induce apoptosis in canine cruciate ligament cells. On the one hand those substances are molecules involved in joint inflammation and on the other hand they are chemical drugs known as potential apoptosis inducers in other cells. Furthermore, we compared the susceptibility of ligamentocytes from the CCL and the CaCL to apoptosis inducers. As a consequence of the first study we secondly focused on the characterization of the signaling cascade during NO-induced cell death in canine cruciate ligament cells.

Because NO and prostaglandins are involved in the same inflammatory and immune processes, the relations between the NOS and COX enzymes have attracted the attention of researchers. The goal of our third study was to evaluate the efficacy of anti-inflammatory drugs with regard to ligamentocytes apoptosis.

In contrast to human medicine we are interested in the healing capacities of still intact but microscopically altered ligaments and not in the healing of already macroscopically ruptured ligaments. The question as to why the CCL heals much more poorly than for instance the medial collateral ligament has long been a conundrum for orthopedic surgeons. For that reason getting information on pathways, potential inhibitors, or on intrinsic cellular differences between fibroblasts of the CCL and CaCL may give new therapeutic options for CCL disease.
Short communication

In vitro effect of different mediators of apoptosis on canine cranial and caudal cruciate ligament fibroblasts and its reversibility by pancaspase inhibitor zVAD.fmk

Simone Forterre a,*, Andreas Zurbriggen b, David Spreng a

a Division of Small Animal Surgery and Orthopedics, Vetsuisse Faculty Bern, Department of Clinical Veterinary Medicine, University of Bern, Länggassstrasse 30, 3012 Bern, Switzerland
b Division of Clinical Research, Department of Clinical Research and Veterinary Public Health, University of Bern, Bern, Switzerland

A R T I C L E   I N F O

Article history:
Received 13 April 2010
Received in revised form 6 September 2010
Accepted 28 September 2010

Keywords:
CCL rupture
Apoptosis
Caspases
Dogs
Stifle joint

A B S T R A C T

Primary fibroblast cultures of canine cranial (CCL) and caudal (CaCL) cruciate ligaments were stimulated with different apoptosis inducers with or without preincubation of the pancaspase inhibitor zVAD.fmk. In contrast to CaCL fibroblasts, fibroblasts from CCL were significantly more susceptible to apoptosis inducers of the intrinsic pathway like doxorubicin, cisplatin and nitric oxide (NO)-donors and to Fas ligand (FasL), an apoptosis inducer of the death receptor pathway. Apoptotic response to staurosporine and the peroxynitrite donor GEA was similar in both ligament fibroblasts. Stimulation with dexamethasone or TNFα could not induce apoptosis in CCL and CaCL fibroblasts, in spite of present TNFR1 and TNFR2 receptors. zVAD.fmk was able to prevent apoptosis in up to 66% of CCL cells when treated with FasL, cisplatin or doxorubicin but it had no effect on NO or peroxynitrite induced apoptosis. In conclusion, differential susceptibility to apoptotic triggers like FasL or NO between cranial and caudal cruciate ligament fibroblasts in vitro may be a reflection of the different susceptibilities to degenerative rupture of the ligament. These findings indicate that a general caspase inhibition does not completely protect canine CCL cells from apoptosis.

© 2010 Elsevier B.V. All rights reserved.

1. Introduction

Rupture of the cranial cruciate ligament (CCL) is one of the most important causes of hind limb lameness in dogs. The majority of dogs rupture their CCL without an acute traumatic injury suggesting CCL rupture as a final stage of progressive and degenerative changes within the ligament itself. Rupture of the CCL has been associated with age-related ligament deterioration (Vasseur et al., 1985), conformation abnormalities (Slocum and Devine, 1983), microinjury due to mechanical overload (Hayashi et al., 2003a) and also with idiopathic, probably immune-mediated, inflammatory arthropathy (Lemburg et al., 2004; Muir et al., 2002). Many of these mechanisms are still believed to be relevant in spontaneous CCL rupture, but none of them alone satisfactorily explains the ethiopathogenesis of this disease. The phenotypic stability and survival of the ligamentocytes are essential for the maintenance of a proper extracellular matrix. Ligamentocytes are specialized, fibroblast-like cells of mesenchymal origin that constitute the cellular component of intraarticular ligaments. Alterations in ruptured CCLs include a decrease in cell density, chondroid metaplasia of surviving fibroblasts and extensive disruption to the organized architecture of the ECM collagen (Hayashi et al., 2003a). These changes are associated with loss of ligament fibroblasts from the core region of the CCL. Moreover, a disregulation of cell death has been considered to be a central feature in

0165-2427/$ – see front matter © 2010 Elsevier B.V. All rights reserved. doi:10.1016/j.vetimm.2010.09.007
the canine cruciate ligament disease (Cyger et al., 2007). However, the mechanism by which the loss of cells occurs is presently unclear. The primary event might be a mechanical microinjury, excessive shear stress, or biochemical factors such as loss of growth factors, excessive levels of reactive oxygen species and cytokines. Information on the induction of cell death in articular tissues is limited. There are two possible mechanisms of cell death currently discussed in CCL disease. On the one hand, Hayashi et al. (2003b) found a high number of non-viable cells but not a great number of apoptotic cells suggesting necrosis as the main mechanism, but on the other hand, studies have shown the presence of a significant number of apoptotic cells in ruptured canine CCLs (Cyger et al., 2007; Krayer et al., 2008). Our hypothesis was that an increase in different inflammatory cytokines and biochemical factors, which may be associated with the microinjury during mechanical overload to the CCL, leads to an imbalance between apoptosis-inducing and anti-apoptotic factors and finally favors the induction of cell death. In the present study, we investigated the susceptibility of canine fibroblasts of the cranial (CCL) and caudal (CaCL) cruciate ligament to apoptosis and the influence of caspases in the presence of different apoptotic stimuli.

2. Materials and methods

2.1. CaCL and CCL fibroblasts isolation and culture

Specimens of the cranial and caudal crucial ligament were obtained from neonatal beagle dogs sacrificed by pentobarbiturate injection within 1 h of death. The ligaments were harvested under aseptic conditions and placed in sterile DMEM medium with 1% antibiotic solution. A portion of the ligament at both the tibial and femoral ends was trimmed and discarded. The outer synovial layer was removed via sharp dissection and the ligaments were cut into 0.1–0.2 mm pieces. The primary cells were harvested under aseptic conditions and placed in 25 cm² flasks for 3 days. The first passage was obtained after 3 days. The monolayer was washed twice with PBS, and the second passage was obtained within 1 h of sacrifice. A purity of the primary cell cultures was confirmed by >98%. Therefore, fibroblasts from the fourth passages were used in the following experiments.

2.3. Induction and inhibition of apoptosis

To induce apoptosis, cells were treated with serial dilutions of different apoptosis inducers for 24 h. For stimulation of apoptosis via the intrinsic pathway, ligamentocytes were treated with dexamethasone (10⁻⁷ to 10⁻³ M), cisplatin (3–200 μM) and doxorubicin (1–100 μM, Alexis Corporation, Switzerland). In order to investigate the effect of nitric oxide (NO) and peroxynitrite (ONOO⁻) on cell viability, cells were treated with the NO-donors DETA/NO (0.2–5 mM, Alexis) and SNAP (0.2–5 mM, S-nitroso-N-acetyl-d,l-penicillamine, Alexis) or the ONOO⁻ generator GEA 3162 (10–500 μM, Alexis). As apoptosis inducer of the extrinsic pathway rTNFα (1–200 ng/ml, recombinant canine TNFα, R&D systems, USA), rTNFβ (1–200 ng/ml, recombinant human TNFα, Alexis) and soluble human rFasL (0.1–200 ng/ml, SuperFasLigand, Alexis) were tested. In addition, some cells were treated by cycloheximide (CHX, 1 or 0.01 μg/ml) or actinomycin D (actD, 1 or 0.01 μg/ml) 2 h before and during TNF stimulation in order to investigate the mechanism of the apoptosis-modulating effect of TNFα. Staurosporine (STS, 0.03–10 μM) served as positive control. To assess the role of caspases on induced cell death via extrinsic and intrinsic apoptotic pathways, cells were preincubated with different concentrations of the pancaspase inhibitor, z-Val-Ala-Asp(OMe)-fluoromethylketone (zVAD.fmk, 10–100 μM, Alexis) for 2 h, followed by the coincubation with each apoptotic stimulus for 24 h. Concentration of the vehicle DMSO never exceeded 0.4%.

2.4. Cell proliferation assay

The inhibitory effects of the apoptosis inducers on proliferation and viability of ligamentocytes were measured by a modification of the MTT assay (Mosmann, 1983). Briefly, cells in 96-well plates were exposed to serial dilutions of the different apoptosis inducers as stated above. Controls without any inducer and controls with only diluents were also included in each experiment. For colorimetric MTT assay, 10 μl of MTT working solution (5 mg/ml in PBS) was added to each well with 100 μl medium, and incubated at 37 °C for 3 h. The supernatant was removed from wells and replaced with 100 μl of DMSO to solubilize the resulting crystals of MTT formazan. Quantitation was then conducted with a microplate reader (EL 800, BioTek, USA) to determine OD at 590 nm. The activity of the apoptosis inducers was expressed as a reduction of cell viability (% of control cell cultures) using the following formula:

\[
\text{Relative viability (\%)} = \frac{100 \times \text{Experimental absorbance} - \text{blank absorbance}}{\text{Absorbance of control} - \text{blank absorbance}}
\]

2.5. Detection of CCL fibroblasts apoptosis

Ligamentocytes were seeded at 4 × 10⁵ cells/well in a 24-well plate. After attaching, cells were treated for 24 h
with various apoptosis inducers as mentioned above. In case of inhibition studies, a treatment with the inhibitor zVAD.fmk with serial concentrations (10–100 μM) was carried out starting 2 h before apoptosis stimulation. Apoptosis was assessed by incubation with annexin V-FITC/propidium iodide followed by analysis in a flow cytometer (FACS LRII, BD Biosciences) \cite{vermes1995}. Apoptotic cell fraction was detected as cells stained FITC-annexin V positive and PI negative (early apoptosis) and cells FITC-annexin V and PI positive (end stage apoptosis and death). Data files were generated for 5 × 10^4 cells or more per sample using the FlowJo V7.1 Analysis Software (Treestar Inc., USA).

2.6. Detection of TNFα receptors

TNFα receptor expression was measured by flow cytometric analysis on the three trypsinized canine cells (a) CCL, (b) CaCL and (c) Mardin Darby canine kidney (MDCK) cells cultured with or without 100ng/ml rhTNFα for 24 h as described before \cite{aoki1998a}.

2.7. Statistical analysis

Each experiment was carried out using duplicate to triplicate samples as well as controls for a minimum of three replicates using primary cells derived from different donors, and the mean and standard deviation or standard error of the means of the combined data was calculated. Comparison between two groups was performed by a Student’s t-test. One-way ANOVA with Tukey’s HSD test was used to assess changes in drug effects on the proliferation and apoptosis of canine cruciate ligament fibroblasts. A P-value <0.05 was considered statistically significant. The concentrations of reagents that induced a 50% inhibition in cell viability (IC50) were determined from curves of reagent concentrations versus relative cell viability at 24 h of incubation for each primary cell culture.

3. Results and discussion

To our knowledge, studies dealing with apoptotic interventions on canine cruciate ligament fibroblasts in vitro have not been previously published. Apoptosis is a physiological process and is a highly selective way to eliminate aged and injured cells. In the present study, we used apoptosis inducers of the internal/mitochondrial and extrinsic/death receptor pathways to assess whether treatment with the different naturally and not naturally occurring substances can induce apoptosis in canine CCL and CaCL cells. The apoptotic cell rates of CCL fibroblasts measured by a flow cytometer with FITC conjugated-annexin V and PI were comparable with the rates obtained with the MTT assay, confirming that CCL fibroblasts undergo apoptosis with the different treatments mentioned above.

3.1. Nitric oxide and peroxynitrite

Several studies could demonstrate that excessive amounts of NO are overproduced in rheumatoid arthritis (RA) and osteoarthritis (OA) as well as in cranial cruciate ligaments of dogs with CCL disease \cite{murrell1996, pelletier1991, spreng2000}. A positive correlation between NO-production and the apoptotic cell rate in canine cruciate ligaments led to the assumption that apoptosis may be influenced by local NO-production \cite{gyger2007}. The present study showed for the first time that canine cruciate ligament fibroblast apoptosis could be mediated by NO-donors such as SNAP or DETA/NO. Interestingly, fibroblasts from the CCL have an intrinsically higher susceptibility to NO-induced apoptotic cell death than fibroblasts from the CaCL. NO-release from SNAP and DETA/NO treatment led to an IC50 at 3.7 ± 0.2 mM and 3.8 ± 0.3 mM in CCL fibroblasts. In contrast to CCL cells, SNAP and DETA/NO did not achieve an IC50 in CaCL cells; at 5 mM of SNAP and DETA only a 27 and 30% reduction of viability was observed. A higher susceptibility to NO-induced apoptosis was also documented for rabbit CCL cells compared to medial collateral ligament cells \cite{murakami2005}. Although the exact mechanism of NO-mediated cytotoxicity is still controversial, NO could induce cell death either directly or indirectly through reaction with superoxide to produce the powerful and toxic oxidant peroxynitrite (ONOO\(^-\)). The higher susceptibility of CCL cells to NO might result from a higher concentration of reactive oxygen species in the CCL compared to CaCL and/or a reduced activity of oxygen radical scavengers in the CCL leading to a difference in ONOO\(^-\) production. As a result of this study, a similar susceptibility of CCL and CaCL to apoptosis was found when ligament cells were directly induced with the ONOO\(^-\) generator GEA. Unfortunately, there are no studies available comparing cranial and caudal cruciate ligaments.

3.2. Dexamethasone

Due to its frequent application in humans as well as in dogs for treatment of a broad spectrum of musculoskeletal disorders and the possible side effects predisposing tendons to spontaneous rupture and cartilage to become thinner \cite{wong2003}, we tested dexamethasone for its potential to mediate apoptosis. Even addition of the highest dose of dexamethasone (10\(^-\) M) resulted in only a marginal, not significant reduction of viability in both cell types (Fig. 1A).

3.3. Cisplatin and doxorubicin

Cisplatin and doxorubicin were chosen due to their widely use for treatment of many malignancies inducing apoptosis in a variety of cell types. As a result after 24 h cisplatin exposure, the mean IC50 of CCL represented 95 ± 8 μM, whereas a concentration of 300 μM effected only a viability reduction of ~25% in CaCL cells. Similar significant differences in susceptibility were observed for doxorubicin-mediated apoptosis. Doxorubicin abated significantly viability of CCL cells resulting in an IC50 of 17 ± 3 μM compared to 61 ± 5 μM in CaCL fibroblasts.
3.4. Fas ligand

There are a lot of studies dealing with Fas/Fas ligand apoptosis in chondrocytes and OA, but investigations of Fas receptor/ligand related to canine cruciate disease are not available. Synovial cells are the most likely source of exogenous FasL, in form of soluble FasL as detected in synovial fluid of OA patients (Hashimoto et al., 1998). Alterations in the susceptibility of cells to Fas-induced cell death have been strongly implicated in the pathogenesis of inflammatory joint diseases such as RA. The present study demonstrates a significant higher magnitude of response to FasL in CCL than in CaCL fibroblasts (Fig. 1B). For the CCL fibroblasts, the IC50 value of FasL was obtained by 180 ± 8 ng/ml whereas the highest used concentration of 200 ng/ml FasL decreased the viability rate of CaCL cells only by 7%. An increased expression of Fas receptor by fibroblasts in CCLs compared to CaCLs would provide an explanation for the higher susceptibility. Our observation that nearly no cytotoxic effect was observed on CaCL fibroblasts implies that Fas-mediated apoptosis of CaCL cells – different to CCL cells – may be modified by functional changes in apoptosis-signaling molecules. A possible explanation might be that nuclear factor kappa B (NF-kB) mediated gene activation is involved in this anti-apoptotic mechanism, accompanied by upregulation of Bcl-2 expression or an interaction of caspase-8-inhibitory protein (FLIP) as reported for rheumatoid synoviocytes (Kobayashi et al., 2000).

3.5. Tumor necrosis factor

TNFα is thought to be one of the main cytokines responsible for cartilage loss as it stimulates chondrocytes and synoviocytes to produce various enzymes (Shinmei et al., 1990). TNFα mainly produced by macrophages was found in synovial fluids of dogs with OA resulting from a CCL rupture. Compared with dogs suffering from RA only a moderate TNFα expression of 1 ng/ml was measured in the synovial fluid (Hegemann et al., 2005). In our study, 20-fold higher concentrations of TNFα did not affect the apoptotic response of CCL and CaCL fibroblasts whether used alone or combined with actD or CHX (Fig. 1C). Biological activity of both recombinant TNFα was verified by the TNF bioassay with L929 and porcine kidney cells described by (Bertoni et al., 1993) (data not shown). TNFα has been shown to mediate two different responses by either eliciting apoptosis or facilitating cell survival and growth through the activation of the NF-κB and activator protein 1 transcription factors (Wajant et al., 2003). The dichotomy of cellular responses resides in the two different receptors TNFR1 and TNFR2 that are activated and in the downstream signal transduction molecules that interact with these receptors. Moreover, some human cell lines are sensitive to Fas-mediated cytotoxicity but not to that induced by TNFα, although they express both Fas and TNFR1 receptors (Wong and Goeddel, 1994). In this study, CCL and CaCL fibroblasts were not susceptible to TNFα mediated apoptosis although they both express TNFR1 and TNFR2 (Fig. 2). Compared to expression levels of MDCK cells, which are known to express TNFR1 and TNFR2 in moderate levels (Aoki et al., 1998b), CCL and CaCL cells showed significantly less of these surface receptors even after stimulation with TNF (Table 1). In the present study, exposure to 100 ng/ml TNFα for 24 h

<table>
<thead>
<tr>
<th>Cells</th>
<th>TNFR1 Untreated</th>
<th>TNFR1 +TNFα</th>
<th>TNFR2 Untreated</th>
<th>TNFR2 +TNFα</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCL</td>
<td>5.3 ± 1.9*</td>
<td>13.8 ± 3.5*</td>
<td>6.3 ± 0.7*</td>
<td>14.0 ± 1.2*</td>
</tr>
<tr>
<td>CaCL</td>
<td>13.5 ± 3.0*</td>
<td>21.3 ± 2.8</td>
<td>4.3 ± 1.6*</td>
<td>12.9 ± 1.8*</td>
</tr>
<tr>
<td>MDCK</td>
<td>41.4 ± 3.1*</td>
<td>51.3 ± 7.5</td>
<td>13.7 ± 1.2*</td>
<td>24.5 ± 1.1*</td>
</tr>
</tbody>
</table>

Expression of surface receptors: indicates the mean value of fluorescence intensity was 3- to 10-fold higher than the background staining observed in the absence of primary antibodies. Expression of TNFR1 receptors was determined by flow cytometry using anti-TNFR1 and R2 receptor polyclonal antibodies. Values were calculated as % of counted cells. Each value indicates mean ± SEM.

* P<0.05 R1 vs. R2 untreated.
# P<0.05 R untreated vs. R + TNF treatment.
3.6. Inhibitory effects of pancaspase inhibitor on apoptosis in CCL fibroblasts with different triggers

Preliminary animal model studies demonstrated fairly clearly that inhibition of caspases can reduce chondrocyte death and decreases OA severity (D’Lima et al., 2006; Dang et al., 2006). In the study presented, treatment with zVAD.fmk significantly inhibited FasL-mediated apoptosis in a dose-dependent manner (Fig. 3A). In particular, FasL-mediated apoptosis in CCL fibroblasts was partially blocked at 10 μM of zVAD.fmk and was more than halved by zVAD.fmk at a concentration of 100 μM (66% with 50 ng/ml FasL and 65% with 100 ng/ml FasL). These results clearly indicate that activation of caspases is involved in FasL-mediated apoptosis of cultured CCL fibroblasts, but additional signaling molecules might be responsible for the spread of FasL-mediated apoptosis. Compared to another study, where the caspase specific inhibitor completely abrogated apoptosis in cultured OA synoviocytes (Kobayashi et al., 1999), in the present study 35% of CCL cells could not be prevented from FasL-mediated apoptosis. Further, the apoptotic effect of 10 μM doxorubicin could be decreased from 30% to 17% with 10 μM zVAD.fmk. The inhibiting effect on doxorubicin mediated apoptosis could not be significantly increased with a higher zVAD.fmk concentration, leaving 55% apoptotic cells. Using 80 μM zVAD.fmk or more, finally reduced the apoptotic effect of 10 μM cisplatin (Fig. 3B). In contrast to rabbit CCL cells, where either caspase-3 and -9 inhibitors prevented NO-induced apoptosis (Murakami et al., 2005), in our study zVAD.fmk exhibited no significant protection against NO-induced or ONOO−-induced apoptosis in CCL cells (Fig. 3C and D). Taken together, these results indicate that one part of FasL, cisplatin and doxorubicin induced apoptosis was principally mediated by caspases, whereas CCL cells displayed less or hardly any contribution of caspases to NO or peroxynitrite toxicity supporting the fact that drug induced apoptosis varies with the cell type.

In conclusion, canine fibroblasts from the CCL and CaCL exhibit essential differences from one another despite the fact that they are ligaments within the same joint. In particular, CCL fibroblasts were more susceptible to apoptosis inducers, which might be relevant to the pathogenesis of a predominant rupture of the CCL. Although degenerative processes have been described in both, cranial and caudal cruciate ligaments of dogs (Vasseur et al., 1985), a degenerative rupture is almost exclusively seen in the CCL. Further investigations with ligament fibroblasts from adult dogs and especially from dogs, which are predisposed to CCL rupture would be helpful in finding out if these differences still exist. Employing the broad caspase inhibitor zVAD.fmk, we were able to distinguish between a caspase dependent and independent pathway, but it seems likely that other signaling mechanisms are concurrently involved because a general caspase inhibition alone was not sufficient to protect canine primary CCL cells totally from apoptosis. In this context, further research on the regulation of ligament apoptosis might give more details on the exact pathway and might subsequently provide new concepts for treatment of CCL disease in dogs.

resulted in a double to 3-fold increase of TNFR1 and TNFR2 receptor expression on CCL and CaCL cells. Similar effects of TNF were reported for synovial fibroblasts (Arend and Dayer, 1990). In the present study, there was no obvious correlation between sensitivity to TNF and expression of the corresponding receptors. There are different open questions to examine as following: it is still unclear, (1) if one or both of the signaling pathways were activated after ligand–receptor ligation, or (2) if the receptor expression was too low. An inappropriate concentration of downstream pro-apoptotic proteins as well as an upregulation of protective molecules, or an activation of protective pathways might be further possible reasons responsible for the unresponsiveness to TNF. CaCL and CCL fibroblasts were sensitive to killing by FasL, yet resistant to TNF treatment despite the presence of TNFR1. These results suggested that Fas and TNFR1 may couple to distinct signaling pathways and that these pathways are not necessarily functional in all cell types.

Fig. 2. Flow cytometric analysis of expression of TNFα receptors (incubated with anti-human TNFRI or RII goat polyclonal antibodies; 50 μg/ml, R&D systems) on canine cells without TNFα incubation. CCL fibroblasts, CaCL fibroblasts and MDCK cells were stained with fluorescein isothiocyanate-conjugated (FITC) second antibody 1:200. MDCK cells served as positive control.
Fig. 3. Effects of caspase inhibitor zVAD.fmk on induced apoptosis in cultured canine CCL fibroblasts. An incubation with zVAD.fmk starting 2 h before apoptosis induction with (A) FasL 50 ng/ml, (B) cisplatin 10 μg/ml, (C) SNAP 2.5 mM and (D) GEA 250 μM as described in Section 2. Values are the mean and SD of three CCL cell samples obtained from three replicates. *P<0.05 apoptosis trigger vs. zVAD combined with trigger; **P<0.05 between zVAD treatments.

Acknowledgement

This work was supported by a competitive grant of the Department of Clinical Veterinary Medicine of the Vetsuisse Faculty of Berne.

References

Nitric oxide induces cell death in canine cruciate ligament cells by activation of tyrosine kinase and reactive oxygen species

Simone Forterre¹*, Andreas Zurbriggen², David Spreng¹

Abstract

Background: There is increasing evidence suggesting that development of progressive canine cranial cruciate ligament (CCL) rupture appears to involve a gradual degeneration of CCL itself, initiated by a combination of factors, ranging from mechanical to biochemical. To date, knowledge is lacking to what extent cruciate disease results from abnormal biomechanics on a normal ligament or contrary how far preliminary alterations of the ligament due to biochemical factors provoke abnormal biomechanics. This study is focused on nitric oxide (NO), one of the potential biochemical factors. The NO donor sodium nitroprusside (SNP) has been used to study NO-dependent cell death in canine cruciate ligament cells and to characterize signaling mechanisms during NO stimulation.

Results: SNP increased apoptotic cell death dose- and time-dependently as measured by MTT assay and FITC-conjugated annexin-V/propidium iodide flow cytometry. Caspase-3 processing in response to SNP was not detected. SNP-induced apoptosis in canine ligamentocytes was not blocked by the p38 inhibitor SP202190, the ERK1/2 inhibitor PD98059, or the protein kinase C inhibitor calphostin C. However, blocking the tyrosine kinase pathway completely abrogated the SNP-induced cell death. Moreover, data showed that NO-induced ligamentocyte death was not solely related to the production of NO, but also to the generation of reactive oxygen species such as peroxynitrite, hydrogen peroxide and/or superoxide indicated by the protective effects of ROS scavengers. Compared to other cells originating from joint tissues, ligamentocytes released only small amounts of PGE₂ in response to SNP and COX-2 expression was not detected by western immunoblotting.

Conclusions: These data support the hypothesis that canine ligamentocytes showed cell-type-specific responses which depend critically on the presence of individual components of the different signaling pathways and are distinct from other cells originating from joint tissue. The COX-2 system seemed to exhibit a less important regulator of NO-induced cell death in canine ligamentocytes than in human chondrocytes or synoviocytes whereas the activation of the tyrosine kinase and the generation of reactive oxygen species reveal as important signaling pathways. In perspective, new efforts to prevent the development and progression of CCL disease may include strategies aimed at reducing reactive oxygen species.

Background

Cranial cruciate ligament (CCL) rupture is one of the most common orthopaedic diseases among dogs and the subsequent cause of osteoarthritis. The majority of dogs rupture their CCL spontaneously due to secondary progressive degenerative changes within the ligament itself.

Mechanical factors such as straight tibial plateau angle, distal femoral torsion, tibial torsion, as well as intercondylar notch stenosis have been associated with CCL disease [1-3]. There is no doubt that biomechanical factors, among others, are likely to play an important role in the initiation of the disease process but the true effect on the ethiopathogenesis is currently unknown. There is strong evidence that the degenerative changes observed in CCL disease
are due to a combination of factors, ranging from developmental, immune-mediated disorders, genetic components, as well as impaired synthesis and turnover of cells and extracellular matrix have been implicated as biological factors. To date, knowledge is lacking to what extent cruciate disease result from abnormal biomechanics on a normal ligament or contrary how far preliminary alterations of the ligament due to biochemical factors provoke abnormal biomechanics. A lot of effort has focused on stifle joint stabilization as therapeutical interventions but markedly less studies investigated the role of biochemical factors.

Nitric oxide (NO) is one of these biochemical factors considered to be involved in the CCL disease [4, 5]. Under normal physiological conditions low levels of NO are produced by the constitutive nitric oxide synthase (cNOS) to regulate a number of homeostatic processes, whereas the generation of larger quantities of NO by the inducible NOS accounts for an inflammatory setting [6]. In osteoarthritis and in cruciate rupture arthropathy an excess production of NO has been documented [4, 7]. Excess production of NO in human osteoarthritis (OA) tissues has been linked to cartilage chondrocyte apoptosis both in vitro and in vivo [7-9]. NO-induced chondrocyte death has been studied by exposing the cells to high concentrations of exogenous NO donors such as sodium nitroprusside (SNP). NO has been shown to affect crucial intracellular signaling pathways in OA chondrocytes and synoviocytes [10, 11]. However, the intracellular signaling by which NO mediates apoptosis has not yet been completely elucidated in joint tissue. Interestingly, in the canine stifle normal CCL produces more NO compared to normal cartilage [4]. The objective of the presented study was to explore possible signaling pathways that contribute to apoptosis in canine cruciate ligament cells and to compare with known mechanisms in OA chondrocytes and synoviocytes of humans. For these reasons, we decided to stimulate canine cruciate ligament cells with the NO-donor SNP. The involvement of different cell signaling was tested by using specific inhibitors of the mitogen activated protein (MAP), tyrosine and protein C kinase, nuclear factor kappa B (NF-kB) and caspases. The production of additional reactive oxygen species (ROS) and their role in ligament cell death was evaluated by using specific reactive oxygen scavengers. Cell death in ligamentocytes isolated from canine ligaments was analysed using methods that measure various cell death parameters. We further examined if exogenous NO can activate the cyclooxygenase-2 (COX-2) system.

**Results and discussion**

Evidence documenting NO as a crucial mediator for canine CCL disease prompted our investigations to the underlying signaling of NO-induced cell death in ligamentocytes. Ligamentocytes are not only the sites of NO production but are also themselves targets for NO and undergo apoptosis upon exposure to high concentrations of NO [12, 13]. The rational for using cells from the cranial as well as from the caudal cruciate ligament (CaCL) was that we found in a previous study different susceptibilities to apoptosis [12]. Although CCL and CaCL have the same extrasynovial environment, apparently the same nutrition and blood supply, incidence of rupture differs between the ligaments.

**SNP causes caspase-independent apoptosis and bcl-2 down-regulation in CCL and CaCL cells**

Cultures of canine ligamentocytes were stimulated with increasing concentrations of SNP, and cell viability was assessed by MTT assay and flow cytometry. Using the double staining FITC-labelled Annexin V and propidium iodide flow cytometry, we could
Results – Study I

I corroborate that cells died by apoptosis (Fig. 1). A dose-dependent loss of cell viability was induced by SNP in CCL and CaCL cells (Fig. 2). Comparing CCL and CaCL cells, it became obvious that CaCL cells were less susceptible to NO-stimulated cell death. This effect was significant at 0.05 mM, 0.1 mM and 0.5 mM of SNP. In particular, at 0.5 mM of SNP, CaCL cells showed significantly less cell death compared to CCL cells (45% vs. 92%, P < 0.01). At concentration above 0.5 mM of SNP, the amount of dead cells was not different between CCL and CaCL. Measurement of the pro-survival bcl-2 protein showed that the level decreased in association with SNP treatment in a dose-dependent way (Table 1). Bcl-2 protein was measured by enzyme immunoassay. *, P < 0.05; **, P < 0.01 vs. control; †, P < 0.05; ††, P < 0.01 vs. SNP (0.5 mM); Dunnett’s multiple comparison test.

This dose- and time-dependent manner of apoptosis induction is closely in agreement with different studies using SNP-generated NO to stimulate various cell types originating from stifle tissue [10, 11, 15]. In these studies, cell death was clearly linked to the activation of the caspase cascade, as inhibitors of caspase-3 or caspase-9 prevented the cells from apoptosis. In our ligamentocytes, induction of cell loss by SNP was only marginally prevented when the cells were prestimulated for 2h with 100 µM of the pancaspase inhibitor zVAD.fmk (Table 2). Same results were achieved in a recent study with other NO-donors such as DETA or SNAP [12]. We then tested whether caspase-3 was activated by SNP. While caspase-3 processing was induced by staurosporine which served as positive control, no evidence for caspase-3 cleavage was found in SNP-treated ligamentocytes in the concentration range in which apoptosis was measured by flow cytometry (Fig 4). These observations suggest a participation of a caspase-independent cell death pathway (CICD), which has been noticed in the presence of the broad-spectrum caspase inhibitor [16]. Meanwhile, the absolute requirement for caspase activation in apoptosis is no longer considered dogma.

CICD occurs when a signal that normally

Table 1 - Dose-dependent effect of SNP with/without inhibitors on the Bcl-2 protein level in canine CCL and CaCL cells.

<table>
<thead>
<tr>
<th></th>
<th>Bcl-2 [ng/mg protein]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CCL cells (n=6)</td>
</tr>
<tr>
<td>Control</td>
<td>6.6 ± 0.3</td>
</tr>
<tr>
<td>SNP (0.3 mM)</td>
<td>6.5 ± 0.1</td>
</tr>
<tr>
<td>SNP (0.5 mM)</td>
<td>5.5 ± 0.3*</td>
</tr>
<tr>
<td>SNP (1 mM)</td>
<td>2.8 ± 0.2**</td>
</tr>
<tr>
<td>SNP (0.5 mM) + Genistein (50 µM)</td>
<td>8.8 ± 0.2‡‡</td>
</tr>
<tr>
<td>SNP (0.5 mM) + Uric acid (0.5 mM)</td>
<td>7.8 ± 0.1‡‡</td>
</tr>
<tr>
<td>SNP (0.5 mM) + Taxifolin (50 µM)</td>
<td>7.6 ± 0.3†</td>
</tr>
<tr>
<td>SNP (0.5 mM) + PTIO (5 µM)</td>
<td>5.8 ± 0.2</td>
</tr>
</tbody>
</table>

Data represent the means and standard deviations from two separate experiments, n refers to the number of donors, and each performed in triplicates. Bcl-2 protein was measured by enzyme immunoassay. *, P < 0.05; **, P < 0.01 vs. control; †, P < 0.05; ††, P < 0.01 vs. SNP (0.5 mM); Dunnett’s multiple comparison test.

Figure 2 Apoptotic fractions in canine CCL cells. CCL cells were stimulated with indicated concentrations of SNP for 18 hours. Apoptotic cells were measured by FITC-annexinV/propidium iodide double stained flow cytometry. The stacked bar graph is divided into two categories: ▲ indicates the early apoptotic fraction detected as cells stained annexin V positive and propidium iodide negative, ▲ indicates the end stage apoptosis and death detected as cells stained annexin V and propidium iodide positive. The graphs data represent the mean ± SD from at least three separate experiments of four different cell donors, each performed in triplicates. * P > 0.05, ** P < 0.01 CCL treated vs. control at each indicated concentration.
engages apoptosis fails to activate caspases, but the cell, nevertheless, dies. Typically, mitochondrial functions decline during CICD, although this is generally a slower process than is seen in apoptosis [17].

### Effects of mitogen-activated protein kinase, protein kinase C, tyrosine kinase, and NF-κB inhibitors on SNP-induced ligamentocyte death

MAP kinase is a family of enzymes that play an important role in converting extracellular signals to intracellular messengers that regulate several cellular phenomena, including apoptotic cell death [18]. Previous studies demonstrate that high concentrations of exogenously applied NO represent a severe stress factor for osteoarthritic chondrocytes and synoviocytes [10, 11]. They demonstrated that SNP can induce the activation of MAP kinase p38 and ERK1/2 (extracellular related kinase) because interruption of the kinase signaling by using ERK1/2 inhibitor (PD98059) and MAP kinase p38 inhibitor (SB202190) significantly reduced apoptosis. These cascades are thought to be required for the induction of apoptosis [19, 20]. To elucidate the role of potential upstream signaling molecules in canine ligamentocytes, we used the same cell-permeable inhibitors SB202190 and PD98059. In contrast to OA chondrocytes and synoviocytes, treatment with PD98059 (10 μM) and SB202190 (10 μM) caused death in our canine ligamentocytes.

### Table 2 - The effect of inhibitors on cell viability in Non-SNP treated or SNP treated canine CCL and CaCL cells.

<table>
<thead>
<tr>
<th>Inhibitor pretreatment</th>
<th>CCL cell viability SNP treatment</th>
<th>CaCL cell viability SNP treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>zVAD.fmk</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>100±6.0</td>
<td>75.5±3.1</td>
</tr>
<tr>
<td>100µM</td>
<td>92.5±5.3</td>
<td>84.5±4.3</td>
</tr>
<tr>
<td>CaC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>100±11.3</td>
<td>79.9±5.3</td>
</tr>
<tr>
<td>25 nM</td>
<td>93.5±6.9</td>
<td>73.4±3.3</td>
</tr>
<tr>
<td>PD98059</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 µM</td>
<td>100±5.6</td>
<td>75.8±6.4</td>
</tr>
<tr>
<td>SB202190</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>100±11.3</td>
<td>73.4±5.4</td>
</tr>
<tr>
<td>10 µM</td>
<td>74.2±2.1*</td>
<td>65.9±4.5</td>
</tr>
<tr>
<td>Genistein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>100±6.0</td>
<td>69.6±6.1</td>
</tr>
<tr>
<td>50 µM</td>
<td>101±6.4</td>
<td>97.4±9.3**</td>
</tr>
<tr>
<td>NS-398</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>100±5.4</td>
<td>64.9±3.6</td>
</tr>
<tr>
<td>50 µM</td>
<td>89.5±17.7</td>
<td>78.1±3.9*</td>
</tr>
<tr>
<td>100 µM</td>
<td>74.7±14.4</td>
<td>75.3±4.2*</td>
</tr>
<tr>
<td>SN-50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>100±8.3</td>
<td>74.6±3.8</td>
</tr>
<tr>
<td>50 µM</td>
<td>97.3±8.6</td>
<td>64.4±12.6</td>
</tr>
<tr>
<td>PDTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>100±8.3</td>
<td>74.6±3.8</td>
</tr>
<tr>
<td>10 µM</td>
<td>42.7±7.7**</td>
<td>5.5±1.9**</td>
</tr>
<tr>
<td>Uric acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>100±9.2</td>
<td>73.5±5.4</td>
</tr>
<tr>
<td>0.5 mM</td>
<td>103.6±9.5</td>
<td>102.7±12.1*</td>
</tr>
<tr>
<td>PTIO</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>100±6.2</td>
<td>64.9±2.6</td>
</tr>
<tr>
<td>5 µM</td>
<td>87.9±7.3</td>
<td>80.9±1.5*</td>
</tr>
<tr>
<td>Taxifolin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>100±3.7</td>
<td>76.2±4.2</td>
</tr>
<tr>
<td>100 µM</td>
<td>91.6±11.5</td>
<td>84.8±10.6</td>
</tr>
</tbody>
</table>

Values correspond to the average ± SD, calculated by using the formula from M&M section of three separate experiments of n different cell donors, each performed in triplicates. Ligamentocytes were preincubated with the indicated concentrations of the inhibitors for 2 h. SNP or none SNP were then added to the cultures and allowed to incubate for an additional 18-hour period. SNP concentrations used were 0.2 to 0.25 mM. Cell viability was assayed by MTT assay. P values indicate difference within the same type of cell and SNP treatment versus absent inhibitor: *, P < 0.05; **, P < 0.01.
Results – Study II

A parallel activation of the protein kinase C upon stimulation with NO was reported in human OA chondrocytes and synoviocytes [10, 11]. Blocking the protein kinase C, calphostin C was not able to significantly reduce cell death induced by SNP in our ligamentocytes. We performed the same experiments on human OA chondrocytes which served as positive controls and could verify the protective effect of these inhibitors as reported [10]. These findings support the view that protein kinases regulate very specific biological responses that differ from cell type to cell type and do not act as mediators of the NO-mediated cell death in canine ligamentocytes.

The activation of NF-kB can have both preventative and causative roles in the induction of apoptosis [21]. We used two inhibitors to investigate the potential role in canine ligamentocytes. SN-50 inhibits nuclear translocation of the activated NF-kB complex, whereas PDTC prevents the activation of NF-kB. In our cells, PDTC (10 μM) alone showed a significant reduction in viability and reduced the number of viable cells below 5% after SNP coincubation in CCL and CaCL cells. The effect was enhanced by addition of SNP. An apoptosis-enhancing capability was also seen when cells were treated with SN-50 (50 μM) following coincubation with SNP (Table 2) but the effect was less pronounced. This apoptosis–enhancing capability of NF-kB inhibitors is consistent with studies on human chondrocytes [10].

NO donors have the capacity to cause phosphorylation of various targets, for example the tyrosin kinase (TK). The TK family plays a key role in regulation of cell proliferation, differentiation, metabolism, as well as survival [22]. They catalyze the transfer of γ-phoshoryl groups from ATP to tyrosine hydroxyls of proteins. The phosphorylation of tyrosine residues modulate enzymatic activity and create binding sites for recruitment of downstream signaling proteins. The TK inhibitor genistein is involved in different cell processes such as inhibition of tumor cell proliferation, activation of tumor cell differentiation as well blocking oxidative DNA damage in vitro [23, 24]. Previous studies showed that blocking TK with genistein was very effective in preventing apoptosis in synoviocytes and chondrocytes after SNP exposure [10, 11]. Likewise, in canine ligamentocytes, genisten markedly and dose-dependently preserved cell viability in the

Figure 3  Time-dependent effect of SNP on canine ligamentocytes. The apoptotic cell number in canine CCL and CaCL cells was measured by flow cytometry. Cells were incubated in medium with 0.5 mM SNP for 6, 12, 18 and 24 h. Values correspond to the mean ± SD from at least three separate experiments of four different cell donor, each performed in triplicates. *, P<0.05, **, P > 0.01 CCL vs. CaCL at each indicated time.
Results – Study II

The protective effect was the highest of all used specific pharmacological inhibitors in our study. Even strong cytotoxic effects of high SNP concentrations (0.5 mM) were effectively degraded (from about 80% apoptosis to less than 20% in CCL and 13% in CaCL cells). Furthermore, addition of genistein to CCL and CaCL cells exposed to SNP resulted in a 1.6-fold and 2.2-fold increase in the bcl-2 protein level, relative to ligamentocytes exposed to SNP in the absence of the inhibitor (Table 1). Furthermore, the protection was associated with a complete inhibition of PGE2 secretion. This implied that TK is an important transducing pathway in regulation cellular susceptibility to NO in ligamentocytes, a finding similar to that reported in synoviocytes [11].

SNP induced ROS formation

In a previous study application of iNOS inhibitors was not able to reduce apoptosis in CCLs in vivo [25] suggesting NO does not feature the sole responsibility for CCL cell apoptosis. Several possible systems have been considered to explain the exact mechanisms of NO-mediated cytotoxicity. Because of the potential of NO to react with free radicals, we investigated whether SNP caused toxicity directly, due to NO, or indirectly, due to ROS formation. In the present study, ROS were found to be a major activation pathway in SNP-induced ligamentocyte cytotoxicity. We demonstrated that blocking generation of ROS significantly attenuate apoptosis. Taxifolin, a scavenger of O2- and H2O2, and the NO-scavenger carboxy-PTIO significantly reduced death in cranial as well as in caudal ligamentocytes (Table 2). The most pronounced effect was reached by using the ONOO- scavenger uric acid. Uric acid protected ligamentocytes against the toxic effect of NO in a dose-dependent manner and significantly increased their viability. In human OA, the uncontrolled production of free radicals is considered an important factor in the pathogenesis of osteoarthritis [26, 27]. Del Carlo and Loeser demonstrated that chondrocyte cell death from NO occurred only under conditions where other ROS were concurrently generated [28]. In human synoviocytes, only the NO-scavenger PTIO showed a protective effect on SNP induced cell death [11]. On the level of bcl-2, ROS inhibitors like uric acid and taxifolin prevented a downregulation of bcl-2 protein in SNP treated ligamentocytes (Table 1).

Cox-2 and PGE2 in SNP-mediated cell death

Recent studies have shown that NO-mediated cell death in OA chondrocytes and synoviocytes is dependent on the induction of PG synthesis and COX-2 expression [10, 11]. Based on the potential crosstalk between the two systems and the clinical usage of COX inhibitors in orthopaedic diseases in dogs, we were interested whether such a regulation exists in ligamentocytes as well. The COX-2 inhibitor NS-398 (0-100 μM) dose-dependently and significantly attenuated the cytotoxic effect of NO in ligamentocytes (Table 2), but it was less clearly than reported in human chondrocytes or synoviocytes where NS-398 totally abolished the effect of NO [10, 11].
Measurements of PGE$_2$ concentrations showed that endogenous PGE$_2$ release was increased dose-dependently by SNP-treatment in both cell types (Table 3). However, the amount of PGE$_2$ in ligamentocytes was significantly lower compared to the amount in human OA chondrocytes measured in parallel (Table 3). This may explain the slight effect of NS-398 in our ligamentocytes compared to chondrocytes. The lower concentrations of PGE$_2$ can be an indication for the lower metabolic activity of ligamentocytes compared to highly active synoviocytes and chondrocytes. Regarding the individual results in terms of COX-inhibitor and PGE$_2$ release, we suspect a potential linkage of these systems, although we were not able to identify COX-2 expression in ligament cells by immunoblotting (Fig. 5). In contrast, human OA chondrocytes showed a distinct COX-2 expression and endogenous PGE2 production after SNP stimulation [10]. These data suggest that the COX-2 system exhibit a less important regulator of NO-induced cell death in CCL cells than in human chondrocytes or synoviocytes.

**Figure 5** Effect of SNP on COX-2 expression. Cells were cultured with or without indicated concentrations of SNP for 18h. The COX-2 expression was analysed by Western immunoblotting as described in the M&M section. COX-2 expression was not observed in CCL or CaCL cells at the different concentrations of SNP as shown in this representative immunoblot. hChondro indicates human chondrocytes as positive controls, cCCL, cCaCL indicate canine cranial, caudal cruciate ligament cells.
Conclusions
Comparison of canine cruciate ligament cells with other cells originating from joint tissue clearly showed cell-type-specific responses which depend critically on the presence of individual components of the different signaling pathways in a particular cell. NO-induced ligament cell death seemed to be mediated multi-plain, whereupon TK and ROS play a major role. New efforts to prevent the development and progression of OA may include strategies and interventions aimed at reducing oxidative damage. Further studies are necessary to clarify if the inhibition of apoptosis in ligamentocytes induced by NO or other stressors would be effective in preservation ligament homeostasis. Until now the standard of care for CCL disease is surgical. With this study in perspective, we open the discussion to the use of the novel concept of disease-modifying therapy as a less invasive procedure offering a potential solution in enhanced primary repair in cruciate treatment.

Materials and methods

Materials
All reagents for the tissue preparation and assays were purchased from Sigma-Aldrich (Buchs, Switzerland) unless otherwise stated. Sodium nitroprusside (SNP), SB-202190, SN-50, and NS-398 were purchased from Enzo Life Sciences (Lausen, Switzerland). Carboxy-PTIO, taxifolin, genistein (4,5,7-Trihydroxyiso-flavone), calphostin C, and uric acid were the products of Calbiochem (Merck, Switzerland). PD98059 was obtained from Cell Signaling (BioConcept, Allschwil, Switzerland).

Cell preparation
Canine cranial and caudal cruciate ligaments (CCL and CaCL) of 8 one day-old beagle dogs (5 male, 3 female) were obtained within 1 hr of death in accordance with review board approval by the Animal Care and Experimentation Committee of the Canton of Bern, Switzerland (No 56/06). Ligaments were harvested under aseptic conditions and placed in sterile DMEM medium. A portion of the ligament at both the tibial and femoral ends was trimmed and discarded. The outer synovial layer was removed via sharp dissection and the ligaments were cut into 0.1 – 0.2 mm pieces. For ligamentocyte isolation, canine CCL and CaCL pieces were digested with collagenase type IV and cultivated in DMEM supplemented with 15% fetal calf serum (FCS), 250μM ascorbic acid and antibiotics as previously reported.5 At 80% confluence, cells were harvested after trypsin/EDTA treatment and frozen in 20% FCS and 10% DMSO until use. After thawing and passaging again, cells from the fourth passage were synchronized by 10% FCS-DMEM for 1 day and then used in the following experiments. Cell cultures up to the fifth passage were investigated to confirm purity using specific fibroblast antibodies. Ligamentocytes were identified by immunohistochemistry with antibodies against fibronectin (F3648) and collagen I (6308, Abcam, USA) and compared to the staining in explants of cranial and caudal crucial ligaments of dogs as described before [12]. Human OA chondrocytes were a gift from S. Kohl and served as positive controls for PGE₂ measurements.

Experimental culture conditions
Because of its ability to induce apoptosis in ligamentocytes, SNP was chosen as a pro-apoptotic agent. For the experiments on the SNP dose response, ligamentocytes were treated with various concentrations of SNP in 10% FCS-DMEM for 18 h. A time course of response was also investigated by incubating cells with SNP for the indicated time period (6-24 h). To explore signaling cascades in SNP-induced cell death, inhibitors of different pathways were used as mentioned below. Therefore, ligamentocytes (40 x 10³ cells/96-well for MTT or 10⁶ cells/6-well for flow cytometry and immunoblot) were preincubated with each inhibitor for 2 h, and then SNP concentrations (0-0.5 mM; as indicated) was added directly to the cultures and allowed to incubate for an additional 18-hour period in 10% FCS-DMEM. Cytotoxicity and cell death were always determined 18 hours after SNP incubation (except for time course assay), using the assays described below.

Inhibitors
The role of caspases was investigated by using a pancaspase inhibitor zVAD.fmk (0-100 μM). To assess the involvement of protein kinases, we used calphostin C (25 nM), SB 202190 (10 μM), and PD98059 (10 μM). SN-50 (0-50 μM) and PDTC (0-10 μM) were applied to investigate the role of nuclear translocation of NF-kB. Genistein (50 μM) was used as a tyrosine kinase inhibitor. To explore the effect of oxidative stress/free radicals in NO-mediated ligamentocyte
apoptosis, scavengers for peroxynitrite (ONOO\(^-\)) (uric acid, 0-2 mM), NO (carboxy-PTIO, 0-
12.5 \(\mu\)M), and superoxide/ hydrogen peroxide
\((O_2^-/H_2O_2)\) (taxifolin, 0-100 \(\mu\)M) were used. The
COX-2 inhibitor NS-398 (0-100 \(\mu\)M) was used to
to examine the role of endogenously synthesized
PGE\(_2\). Preliminary experiments were used to find
inhibitor concentrations inducing maximal
response in our study (data not shown). These
different inhibitors were tested up to the maximal
concentrations that were practicable under the
actual experimental conditions.

**Cytotoxicity assay**

Cytotoxicity was determined as a function of the
cellular conversion of the tetrazolium salt 3-(4,5-
Dimethylthiazol-2-yl)-2,5-diphenyltetra-
zoliumbromid (MTT) into a DMSO soluble
formazan product that was measured at 490 nm
in a microplate reader (EL 800, BioTek, USA) as
described before [12]. In all experiments using
this assay, results were expressed as a reduction of
cell viability (% of control cell cultures) using
the following formula with OD as optical
density: Cell viability (%) = 100 x (Sample OD –
Blank OD/ Control OD – Blank OD).

**Apoptosis assay**

Flow cytometry with FITC-conjugated annexin
V and propidium iodide double staining was used

used to identify apoptotic cell death (FACS LRII,
BD Biosciences). This assay based upon
Annexin V binding to the apoptotic cells,
executed simultaneously with propidium iodide
as a dye exclusion test [29]. Briefly, floating
cells were collected and adherent cells were
cautiously detached using trypsin/ PBS. The cells
were pooled, centrifuged at 1500 rpm and 4\(^\circ\)C
for 6 min. After decanting the supernatant, cells
were resuspended in 800 \(\mu\)L ice-cold annexin V
labeling buffer (10mM HEPES, 140 mM NaCl,
2.5 mM CaCl\(_2\)) for washing. After a second
centrifugation, the pellets were resuspended in
100 \(\mu\)L annexin V labeling buffer added with
FITC-conjugated annexin V to a final
concentration of 0.1\(\mu\)g/mL and incubated for 30
min at 4\(^\circ\)C. Counterstaining with 5 \(\mu\)g propidium
iodide/mL cell suspension was done 1 min
before analyzing. Cell stained FITC-Annexin V
positive and propidium iodide negative were
detected as early apoptotic cells, demonstrating
Annexin V binding and cytoplasmic membrane
integrity. Late apoptotic cells and necrotic cells
show Annexin V binding and propidium iodide
uptake due to loss of cell membrane integrity and
leakage of cellular constituents. Data files were
generated for 5x10\(^4\) cells or more per sample

using the FlowJo V7.1 Analysis Software
(Treestar Inc., Ashland, OR, USA).

**Western immunoblot of COX-2 and cleaved
Caspase-3**

For COX-2 and cleaved caspase-3 detection
ligamentocytes were lysed in RIPA buffer for
30 min. 20 \(\mu\)g supernatant proteins were
separated onto 12% Tris-HCl acrylamide gel and
transferred onto nitrocellulose membrane. The
membranes were blocked with 5% nonfat dry
milk in TBS with 0.1% Tween-20, for 1 h at RT.
The membranes were probed overnight at 4 \(\circ\)C
with a primary antibody (rabbit polyclonal anti
murine COX-2, Cayman, 1:200, or rabbit
monoclonal anti human cleaved caspase-3
(Asp175), Cell Signaling, 1:1000, both in 5%
nonfat dry milk, TBS, 0.1% Tween-20).
Theretofore blots were probed with the
corresponding secondary antibody, goat-anti-
rabbit Ig (HRP) (1:3000, DAKO, Switzerland).
Immunoreactive signals were visualised by the
ECL system (GE Healthcare, Switzerland)
according to the manufacturer’s protocol.

**PGE\(_2\) production**

PGE\(_2\) was determined in culture medium
supernatants by the PGE\(_2\) EIA Kit following the
company’s protocol (Cayman Chemical,
Michigan, USA).

**Quantification of Bcl-2 protein levels**

Ligamentocytes were seeded at 10\(^6\) cells per well
in a 6-well plate in 2mL 10% FCS-DMEM and
cultured until confluence. Cells were treated with
different inhibitors and various concentrations of
SNP. The Bcl-2 protein levels of adherent cells
were assayed using commercially available kits
(human Bcl-2 ELISA kit, Abnova, Germany)
according to the manufacturer’s instructions. The
level of Bcl-2 was expressed in ng per milligram
of total protein.

**Statistical analysis**

All statistical analyses were accomplished using
NCSS 2007 Statistical Software
(www.ncss.com). Each data point represented the
mean ± SD, of n different cell lines (i.e., donors)
each performed in triplicates. One-way analysis
of variance (ANOVA) with post hoc Dunnett’s
multiple comparisons was used for statistical
comparisons to the control treatment. \(P\)-values <
0.05 were considered as significant.
Acknowledgement
This work was supported by a competitive grant of the Department of Clinical Veterinary Medicine of the Vetsuisse Faculty of Berne.

Competing interests
The authors declare that they have no competing interests that could inappropriately influence or bias the content of the paper.

Author’s contribution
SF designed the study, carried out the experiments, interpreted the data, and drafted the manuscript. AZ participated in its design and helped in interpretation of data. DS conceived of the study and coordinated and helped to draft the manuscript. All authors read and approved the final manuscript.

References


23. Kobayashi S, Nishimura J, Kanaide H: Cytosolic Ca\textsuperscript{2+} transients are not required for platelet-derived growth factor to induce cell cycle progression of vascular smooth muscle cells in primary culture. 


**Results – Study III**

**NSAIDs protect canine cruciate ligament cells against nitric oxide induced programmed cell death**

Katrin Waldherr, MD, Andreas Zurbriggen, DVM, David Spreng, DVM, Simone Forterre, DVM

**Objectives** - To determine if ligamentocytes pretreated with non-steroidal anti-inflammatory drugs (NSAIDs) can be protected from apoptosis induction by nitric oxide (NO).

**Samples** - Primary cultures of canine ligamentocytes were established by the enzymatic dissociation of explants from the cranial (CCL) and caudal (CaCL) cruciate ligament of 8 one-day old beagle dogs.

**Procedures** - The protective effect of four NSAIDs were tested at three different cytotoxic degrees of NO-generator sodium nitroprusside (SNP): low, mean and high cytotoxic activity with >80%, 60-50% and <30% living cells, respectively. Cells were pretreated with NSAIDs for 2h following coincubation with SNP for 18h. Cell viability and apoptosis was analyzed by MTT assay and annexin V/propidium iodide flow cytometry. Prostaglandin E$_2$ concentrations were measured by ELISA.

**Results** - The protective effect of the tested NSAIDs was dependent on the cytotoxic degree of SNP. The strongest protection was present at a mean cytotoxicity of SNP. Acetylsalicylic acid, carprofen, meloxicam, and robenacoxib significantly prevented CCL and CaCL cells to undergo apoptosis when they were exposed to NO. Cell viability rose by 15 to 45% depending on the drug. Considering all cytotoxic degrees of SNP, carprofen (10µg/mL) showed the most effective protection against cell death in both cell types. NSAIDs slightly but not significantly suppressed the endogenous prostaglandin E$_2$ release of SNP treated ligamentocytes.

**Conclusions and Clinical Relevance** - The data suggest that carprofen, meloxicam, and robenacoxib, in addition to their anti-inflammatory and analgesic benefits, may also have a protective effect in canine CCL disease through the inhibition of apoptosis.

Non-steroidal anti-inflammatory drugs (NSAIDs) are widely used to alleviate the symptoms of orthopedic diseases. However, it remains controversial as to what effects these agents have on the progression of osteoarthritis (OA). More recent evidence indicates that some may favorably modify the metabolism of proteoglycans, collagen, and matrix and may decrease the release of proteases or toxic oxygen metabolites. Several NSAIDs have documented adverse effects on normal cartilage, ranging from decreased proteoglycan synthesis (e.g. aspirin) to chondrocyte death (e.g. phenylbutazone). Other drugs (more cyclooxygenase (COX)-2 selective NSAIDs) are recognized for their chondroprotective effects; they not only do not contribute to the degenerative process but also appear to protect the joint from some of the degenerative processes.

Generally, canine cranial cruciate ligament disease is considered to have an underlying degenerative process. Increased production of inflammatory mediators by the stifle synovial cells may be secondary to or may be an inciting cause of ligament degeneration. Nitric oxide (NO) has been identified as a major catabolic factor in joint diseases and is involved in the regulation of apoptosis.

Previous investigations have proposed a linkage between canine cranial cruciate ligament degeneration and apoptosis thereby altering the internal strength of the ligament. It is suggested that areas in ligaments with an increased number of cells undergoing apoptosis are subsequently the areas of mechanical injury leading to partial rupture and ultimately to the total rupture of the ligament. Manipulation of apoptotic mechanisms may thus represent a target for ligament protective therapy. The goal of the present study was to evaluate the efficacy of anti-inflammatory drugs as prophylactic treatment with regard to ligamentocytes apoptosis.

**Materials and methods**

**Cell preparation** - Primary cells were isolated from canine cranial and caudal cruciate ligaments (CCL and CaCL) of 8 one day-old beagle dogs (5 male, 3 female) sacrificed by pentobarbiturate injection within 1hr of death as previously reported.
Results – Study III

Briefly, ligaments were harvested under aseptic conditions and placed in sterile DMEM medium. A portion of the ligament at both the tibial and femoral ends was trimmed and discarded. The outer synovial layer was removed via sharp dissection and the ligaments were cut into 0.1 – 0.2 mm pieces. For ligamentocyte isolation, canine CCL and CaCL pieces were digested with collagenase type IVa and cultivated in DMEM supplemented with 15% fetal calf serum (FCS), 250µM ascorbic acid and antibiotics. At 80% confluence, cells were harvested after trypsin/EDTA treatment and frozen in 20% FCS and 10% DMSO until use. After thawing and passaging again, cells from the fourth passage were synchronized by 10% FCS -DMEM for 1 day and then used in the following experiments. All animal experiments were reviewed and approved by the Animal Care and Experimentation Committee of the Canton of Bern, Switzerland (No 56/06). Cell cultures up to the fifth passage were investigated to confirm purity using specific fibroblast antibodies. Ligamentocytes were identified by immunohistochemistry with antibodies against fibronectina (F3648) and collagen Ib (6308) and compared to the staining in explants of cranial and caudal crucial ligaments of dogs as described before.8

Experimental culture conditions - Because of its ability to induce apoptosis in ligamentocytes, sodium nitroprussidec (SNP) was chosen as a pro-apoptotic agent. Preliminary experiments on the dose response of SNP have shown that cell viability in CCL and CaCL cells decreased in a dose dependent manner (data not shown). Moreover, CCL cells were more susceptible to SNP induced cell death than CaCL cells, same as shown for NO-donors SNAP and DETA NONOate.8 Due to the different susceptibility between CCL and CaCL cells, we chose different SNP concentrations for the two cells types to induce a low, mean and high cytotoxicity with a maximal variation of 8% between CCL and CaCL cells. For low cytotoxicity (a) 0.1 mM and 0.15 mM SNP, for mean cytotoxicity (b) 0.2 mM SNP, and for high cytotoxicity (c) 0.35 mM and 0.5 mM SNP were applied for CCL and CaCL, respectively.

Table 1 – Relative cell viability (%) of CCL and CaCL cells. Cells were preincubated with NSAIDs for 2 h, followed by coincubation of SNP for 18 h. High cytotoxicity indicates a SNP treatment with 0.35 and 0.5 mM for CCL and CaCL; a mean cytotoxicity indicates a SNP treatment with 0.2 mM for CCL and CaCL; a low cytotoxicity indicates a SNP treatment with 0.1 and 0.15 mM for CCL and CaCL respectively.

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>µg/mL</th>
<th>High cytotoxicity (n=4) CCL</th>
<th>Mean cytotoxicity (n=6) CCL</th>
<th>Low cytotoxicity (n=3) CCL</th>
<th>Mean cytotoxicity (n=6) CaCL</th>
<th>Low cytotoxicity (n=3) CaCL</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
<td>25.9 ± 3.1</td>
<td>18.7 ± 2.2*</td>
<td>50.2 ± 2.6*</td>
<td>57.9 ± 1.1*</td>
<td>89.3 ± 3.2*</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>25.8 ± 1.9</td>
<td>15.7 ± 1.3</td>
<td>66.6 ± 3.9*</td>
<td>60.7 ± 1.5</td>
<td>88.6 ± 3.9*</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>23.0 ± 1.1</td>
<td>18.0 ± 1.2</td>
<td>59.0 ± 6.0</td>
<td>65.9 ± 3.9</td>
<td>82.9 ± 3.5</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>19.2 ± 1.4</td>
<td>16.2 ± 1.3</td>
<td>60.3 ± 4.9</td>
<td>72.2 ± 2.2*</td>
<td>80.7 ± 3.9</td>
</tr>
<tr>
<td>Acetyl-salicylic acid</td>
<td>0.1</td>
<td>22.7 ± 1.5</td>
<td>18.7 ± 1.4</td>
<td>64.6 ± 2.0*</td>
<td>58.8 ± 1.2</td>
<td>92.7 ± 4.4</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>24.6 ± 1.3</td>
<td>23.9 ± 0.8*</td>
<td>77.5 ± 2.1*</td>
<td>57.2 ± 1.1</td>
<td>102.7 ± 4.4*</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>31.0 ± 2.3</td>
<td>23.5 ± 0.7*</td>
<td>94.9 ± 2.3*</td>
<td>81.9 ± 1.1*</td>
<td>113.8 ± 4.7*</td>
</tr>
<tr>
<td>Carprofen</td>
<td>0.1</td>
<td>22.2 ± 1.5</td>
<td>19.2 ± 0.8</td>
<td>61.4 ± 5.4</td>
<td>59.6 ± 1.2</td>
<td>86.6 ± 3.8</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>25.0 ± 2.1</td>
<td>18.4 ± 1.4</td>
<td>68.1 ± 4.0*</td>
<td>52.0 ± 1.6</td>
<td>87.8 ± 2.8</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>24.0 ± 2.3</td>
<td>24.1 ± 0.9*</td>
<td>65.1 ± 2.3*</td>
<td>73.8 ± 0.5*</td>
<td>78.4 ± 3.3</td>
</tr>
<tr>
<td>Meloxicam</td>
<td>0.1</td>
<td>20.8 ± 1.7</td>
<td>17.9 ± 1.5</td>
<td>54.8 ± 2.1</td>
<td>66.9 ± 3.0</td>
<td>76.7 ± 1.8</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>27.7 ± 1.6</td>
<td>18.6 ± 1.2</td>
<td>56.1 ± 3.9</td>
<td>83.8 ± 10.1*</td>
<td>78.9 ± 2.4</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>33.0 ± 1.7</td>
<td>25.0 ± 0.5*</td>
<td>66.2 ± 3.4*</td>
<td>79.9 ± 6.2*</td>
<td>72.1 ± 2.4</td>
</tr>
</tbody>
</table>

Relative cell viability is calculated with the formula given in M&M section, viability of control cells cultured with neither pretreatment nor SNP equates 100 %. Values presented in the row NONE pretreatment correspond to viability of cells cultured only with SNP. Values are means ± SEM, n refers to number of different donors, each performed in triplicates. * denotes significant differences between the none group and the with pretreatment group within the different cytotoxic degrees and cell types, \( p \leq 0.05 \), Dunnett’s multiple comparison test.
To assess the protective role of NSAIDs on ligamentocytes against apoptosis induction by NO, cell cultures were preincubated for 2 h with either the non-selective COX inhibitor acetylsalicylic acid, or the preferential COX-2 inhibitors carprofen, meloxicam, or robenacoxib. The cultures were then challenged with SNP concentrations as aforementioned for 18 h in 10% FCS-DMEM. Preliminary experiments were used to monitor the cytotoxicity of the NSAIDs alone compared to control cultures (data not shown). In the following inhibition experiments NSAIDs were applied up to the maximal concentrations with regard to the therapeutic range that were possible under the actual experimental conditions without inducing cytotoxicity.

**Assessment of cytotoxicity** - Ligamentocyte death was assessed using the colorimetric MTT assay as described before. Results were expressed as a reduction of cell viability (% of control cell cultures) using the following formula with OD as optical density:

Relative viability (%) = 100 x (Sample OD – Blank OD) / (Control OD – Blank OD).

In parallel, the apoptotic cell damage was confirmed by flow cytometry after double staining using on FITC-conjugated annexin V and propidium iodide followed by analysis in a flow cytometer, which allows discrimination between early apoptotic, late apoptotic and dead cells. Cultures were plated at 30,000 ligamentocytes per well in 96-well plates for MTT assay or at 10^6 cells in 6-well plates for flow cytometry.

**PGE2 production** - Prostaglandin (PG) E2 was determined in culture medium supernatants by the PGE2 EIA Kit following the protocol of the manufacturer.

**Statistical analysis** - All statistical analyses were accomplished using NCSS 2007 Statistical Software. Each data point represented the mean ± SEM, and n refers to the number of different cell lines (i.e., donors) each performed in triplicates or, respectively, the mean ± SD of at least two separate experiments each performed in triplicates. One-way analysis of variance (ANOVA) with post hoc Dunn’s multiple comparisons was used for statistical comparisons to the reference treatment. P-values < 0.05 were considered as significant.

**Results**

Protection of SNP-mediated cell death by NSAIDs - Beside the generation of NO, SNP releases cyanide ions. To exclude that cyanide release by SNP was attributable to death induction, thiosulfate (10:1) was added to the culture medium to detoxify cyanide. The level of ligamentocyte death was unchanged indicating that cyanide release played no role at SNP concentrations which were used (data not shown).

The protective effect of the tested NSAIDs was dependent on the cytotoxic degree of SNP. Corresponding data are presented in Table 1. At high cytotoxicity a pretreatment with the selective COX-2 inhibitors was able to protect CaCL cells from apoptosis but only by 5%. In CCL cells no significant protection was obtained. The strongest protective effect of NSAIDs on CCL and CaCL cells was obvious at mean cytotoxicity of SNP. In detail, a 50% SNP induced toxicity on CCL cells was significantly alleviated by 16 to 18% due to a pretreatment with acetylsalicylic acid (10 µg/mL) as well as with meloxicam (1 and 10 µg/mL) or with robenacoxib (10 µg/mL). Carprofen at a concentration of 10 µg/mL markedly decreased mean cytotoxicity of SNP from 50% to 5%. Protective effects of the NSAIDs were comparable in both, CCL and CaCL cells. In CaCLs, acetylsalicylic acid at a concentration of 200 µg/mL showed a slight but significant protection of 14%. A more considerable effect was detected by a pretreatment with carprofen (10 µg/mL) and robenacoxib (1 µg/mL) where cell viability rose by more than 20% to a viability of 82% for carprofen and 84% for robenacoxib. Pretreatment with meloxicam (10 µg/mL) led to an increase of 16% of cell viability in CaCL cells. In case of concentrations of SNP inducing low cytotoxicity only a pretreatment with carprofen was able to reduce toxicity in both cell types. Considering data from all cytotoxic degrees of SNP, carprofen (10 µg/mL) showed the most effective protection against apoptosis induction in both cell types whereas the non-selective COX inhibitor acetylsalicylic acid showed the weakest effect. The protective effects of the different NSAIDs were verified by flow cytometry (Figure 1). With this method it became obvious that in particular the amount of cells which have lost their cell integrity – late apoptotic fraction - was reduced by NSAID pretreatment.

PGE2 release influenced by NSAIDs - Since PGE2 has been demonstrated to induce apoptosis in articular chondrocytes through the stimulation of cAMP mediated pathways, the ability of the different NSAIDs to reduce PGE2 release after SNP stimulation was investigated. A stimulation of the ligamentocytes with SNP at a concentration of 0.2 mM was chosen due to the best inhibitory results of the different NSAIDs on apoptosis. SNP 0.2 mM was able to slightly increase the PGE2 concentrations in the CCL and CaCLs. NSAIDs slightly but not significantly suppressed the endogenous PGE2 release of SNP treated CCL and CaCL cells (Table 2). No significant differences were detected among the drugs with regard to the degree of change in PGE2 concentration.
Discussion

Several reports indicate a role of NO in the regulation of matrix synthesis, cytokine and MMP production leading to the hypothesis that NO has an important role in the degenerative process of canine cranial cruciate ligaments.\textsuperscript{11-14} In articular chondrocytes and cartilage, it was demonstrated that exogenous NO added in the form of SNAP induced metalloprotease activity in a dose-dependent fashion.\textsuperscript{15} Exposure to NO added as S-nitrosoglutathione inhibited attachment of chondrocytes to fibronectin and disrupted assembly of actin filaments.\textsuperscript{16} Further, endogenously produced NO from articular cartilage inhibited the synthesis of matrix proteoglycans such as aggrecans.\textsuperscript{17} There is high evidence that NO is produced within the inflamed joint in diseases such as rheumatoid arthritis or osteoarthritis.\textsuperscript{18-20} Different in vitro and ex vivo studies have shown that chondrocytes within the human\textsuperscript{20,21}, bovine\textsuperscript{22} as well as canine joint\textsuperscript{23,24} have the potential to generate NO in the presence of an appropriate pro-inflammatory cytokine stimulus. Investigations on explant cultures from ruptured ligaments of canine CCLs demonstrated that CCLs produce large amounts of NO originated from NO synthetase-induced NO-production supporting its role in the disease of canine CCL rupture.\textsuperscript{25} For this reason, a NO-donor was used in this study to induce apoptosis in canine cruciate ligament cells. It is generally accepted that susceptibility to drug-induced apoptosis varies with the cell type. Murakami and coworkers (2005) found an intrinsically different sensitivity between fibroblast from the cranial cruciate and the medial collateral ligament of rabbits.\textsuperscript{26} The rational for using CCL as well as CaCL cells was that we found a different susceptibility in the two groups of fibroblasts in a previous study\textsuperscript{8} as well as in a preliminary test of this study (data not shown). Although CCL and CaCL have the same extrasynovial environment, apparently the same nutrition and blood supply, incidence of rupture differs between the ligaments.

The standard therapy of canine cranial cruciate ligament disease includes analgesics/anti-inflammatory drugs and surgery. It has remained a matter of debate whether anti-inflammatory therapy, specifically NSAIDs, offers advantages or disadvantages over pure analgesics in OA management. Beside their analgesic and anti-inflammatory activities two conflicting effects are reported in the literature. On the one hand evidence suggests that NSAIDs exert apoptotic effects in a variety of cell lines, including colon cancer cells.\textsuperscript{27-29} On the other hand, a few papers have reported on the anti-apoptotic effects of NSAIDs.\textsuperscript{3,30-32} The involvement of NSAIDs on cell apoptosis is so complicated that the mechanism underlying NSAID apoptosis has not yet been well defined. In this study we found that the preferential COX-2 inhibitors carprofen, meloxicam, and robenacoxib as well as the general COX inhibitor acetylsalicylic acid prevented under certain conditions ligamentocytes from the cranial and the caudal cruciate ligament from SNP-mediated apoptotic cell death. Drug concentrations between 0.1 to 10 µg/ml for carprofen, meloxicam, and robenacoxib and

### Table 2 - Dose-dependent effect of SNP and effect of NSAIDs followed by SNP treatment on PGE\textsubscript{2} production in canine CCL and CaCL cells.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CCL cells</th>
<th>CaCL cells</th>
<th>human chondrocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>41.0 ± 8.4</td>
<td>32.0 ± 4.6</td>
<td>620 ± 10</td>
</tr>
<tr>
<td>SNP (0.2 mM)</td>
<td>95.7 ± 10.3*</td>
<td>87.3 ± 10.9*</td>
<td>995 ± 30</td>
</tr>
<tr>
<td>SNP (1 mM)</td>
<td>239.4 ± 12.6*</td>
<td>179.8 ± 8.2*</td>
<td>12790 ± 870</td>
</tr>
<tr>
<td>SNP (0.2 mM) + Acetylsalicylic acid (100 µg/mL)</td>
<td>72.3 ± 8.2</td>
<td>78.0 ± 7.3</td>
<td></td>
</tr>
<tr>
<td>SNP (0.2 mM) + Carprofen (10 µg/mL)</td>
<td>77.4 ± 6.9</td>
<td>67.2 ± 10.2</td>
<td></td>
</tr>
<tr>
<td>SNP (0.2 mM) + Meloxicam (10 µg/mL)</td>
<td>74.8 ± 6.2</td>
<td>77.7 ± 6.3</td>
<td></td>
</tr>
<tr>
<td>SNP (0.2 mM) + Robenacoxib (10 µg/mL)</td>
<td>78.6 ± 4.9</td>
<td>66.2 ± 1.6</td>
<td></td>
</tr>
</tbody>
</table>

Data are the mean ± SD, (n=2) from two separate experiments each performed in triplicates. PGE\textsubscript{2} release in pg of 2x10\textsuperscript{6} cells into 1mL medium was measured by enzyme immunoassay. \*, p < 0.01 vs. control; as determined by use of Dunnett’s multiple comparison test.
between 10 to 200 µg/ml for acetylsalicylic acid were chosen because they include the range of those concentrations that occur in synovial fluid of dogs after administration of a therapeutic dose of the different NSAIDs. In detail, a maximal concentrations of 0.5µg/mL for robenacoxib in synovial fluid of dogs following oral administration of 2.3 to 2.7 mg/kg were reported and a maximal concentration in synovial fluid of 0.6 µg/mL was obtained following an administration of 0.31 mg/kg meloxicam orally. The dosage necessary to maintain clinical control of various lameness in dogs in one study ranged from 23 to 86 mg/kg aspirin twice daily, resulting in plasma drug concentrations ranging from 71 to 281 µg/mL. Synovial concentrations have not been estimated. Toxicity occurs if serum salicylate concentration exceeds 300 µg/ml. Concentrations that occur in synovial fluid of dogs after administration of a therapeutic dose of carprofen have not been determined. However, 0.5 µg/mL carprofen was achieved in horses receiving 0.7mg/kg of racemic carprofen.

In this study, carprofen as well as meloxicam at pharmacological concentrations ranging from 0.1 to 1 µg/mL led to a significant reduction in the apoptotic fraction of CCL cells. Robenacoxib at a concentration of 1µg/mL significantly decreased apoptosis in CCL cells. However, stronger antiapoptotic effects were achieved at the higher concentrations of the NSAIDs. Using different SNP concentrations leading to a dose dependent amount of apoptosis, we observed that the antiapoptotic effect of the NSAIDs became significant only in case of a moderate fraction of apoptosis. Strong apoptotic effects of high SNP concentrations could not be influenced in CCL cells and only slightly by 5% in CaCL cells. Likewise, at a low reduction of viability on CCL and CaCL cells protective effects of NSAIDs became not obvious except in case of carprofen. Carprofen in a concentration of 10µg/mL, lying at the upper range of therapeutic attainable synovial fluid levels, increased cell viability over 100% compared to the control. Although these results suggest additional antiapoptotic potencies of these NSAIDs via inhibition of NO induced cell death, extrapolation from in vitro to in vivo must be carried out with caution. Further investigations have to clarify if under therapeutic concentrations of NSAIDs inhibition of apoptosis might occur in vivo due to the fact that the more effective concentrations of NSAIDs in our study lie and respectively go over

**Figure 1** - Effect of NSAIDs on the apoptotic fraction in SNP treated canine CCL cells. ☐ indicates the early apoptotic fraction before loss of cell membrane integrity assessed by flow cytometry detected as annexin V positive and propidium iodide negative. ☐ indicates the end stage apoptosis and death after loss of cell membrane integrity detected as cells stained annexin V and propidium iodide positive. Cells were cultured with or without NSAID pretreatment following a coincubation of 0.2 mM SNP for 18 h. Cell viability and the apoptotic fraction were measured by FACS. The graphs data represent the mean and SEM from at least three separate donors, each performed in triplicates. *, p < 0.05, **, p< 0.01 NSAID pretreatment vs. non pretreatment. ASC indicates acetylsalicylic acid.
the limit of therapeutic attainable synovia fluid levels.

Additionally, we measured the PGE₂ level in the SNP-treated cells in the presence or absence of NSAIDs due to a potential crosstalk between NO-mediated cell death and the induction of PG synthesis. The results of this study showed that stimulation with the NO-donor SNP was significantly capable to stimulate PGE₂ release in CCL and CaCL cells. However, the amount of PGE₂ of the control and of SNP-stimulated cells was significantly lower compared to the amount in human OA chondrocytes measured in parallel under the same conditions (Table 2). Moreover, the increased PGE₂ production could not significantly be withdrawn by the NSAIDs in the concentration range from 0.1 to 10 µg/mL. Therefore, the antiapoptotic effect of the NSAIDs acetylsalicylic acid, carprofen, meloxicam, and robenacoxib seemed not to depend on the inhibition of the PG production. Further, it was not possible to detect COX-2 expression in the same canine CCL and CaCL cells before and after SNP stimulation by western blot analysis (data not shown). This finding supports the view that the mild PGE₂ increase may be caused by an up-regulation of COX-1 expression rather than COX-2. COX-1 seems to be constitutively expressed in many cells and tissues and is considered to generate PGs for physiologic functions. In contrast, COX-2 is almost undetectable under physiologic conditions in most tissue. Its expression is highly induced by proinflammatory agents. In view of the results of this study, it can be speculated that canine cruciate ligaments can undergo apoptosis during the degenerative process while their cellular PG levels are not elevate in contrast to their neighboring cells from the synovial tissue. Consequently, we assume that inflammation is not a prominent hallmark within the ligament during the degenerative process. By contrast, in human patients with inflammatory joint disease, high expression of COX-2 has been observed in the surrounding tissue of the joint including the enveloping synovial membrane, the capsule, as well as the chondrocytes.

In conclusion, these data provide clear evidence of specific molecular mechanisms involved in NO-mediated apoptotic cell loss, and suggest that the therapeutic application of NSAIDs are also useful in protecting against this form of degeneration. If the hypothesis that increased apoptosis in canine cranial cruciate ligament disease has an impact on ligament degeneration proves correct, these findings indicate that these drugs, in addition to their analgesic effects, may confer ligament protection. Further elucidation of the underlying pathways leading to ligamentocytes apoptosis and degradation of extracellular matrix could improve the likelihood of developing effective strategies to preserve stifl joint function.

Abbreviations

CCL    cranial cruciate ligament
CaCL  caudal cruciate ligament
COX    cyclooxygenase
DETA/NONOate (Z)-1-[N-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2-dioate
DMEM  Dulbecco’s Modified Eagle Medium
ELISA Enzyme Linked Immunosorbent Assay
FCS    fetal calf serum
MTT    3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromid
NO     nitric oxide
NSAIDs non-steroidal anti-inflammatory drugs
OA     osteoarthritis
OD     optical density
PGE₂   prostaglandin E₂
SNAP   S-nitroso-N-acetyl-D,L-penicillamine
SNP    sodium nitroprusside

References


a. Sigma-Aldrich, Buchs, Switzerland
b. Abcam, Cambridge, MA, USA
c. Enzo Life Sciences, Lausen, Switzerland.
d. Rimadyl, Pfizer Animal Health, Zurich, Switzerland.
e. Metacam, Boehringer Ingelheim, Basel, Switzerland.
f. Onsior, Novartis, Basel, Switzerland.
g. FACS LRII, BD Biosciences, New York, NY.
h. Cayman Chemical Co, Ann Arbor, MI, i. NCSS 2007 (www.ncss.com).


Final discussion

Ligamentocytes play an important role in producing extracellular matrix, which is essential for functionality and biomechanics of the cruciate ligaments. These cells continue to produce new extracellular matrix throughout their lives, but their response to injury is insufficient. Whereas collateral ligaments spontaneously heal with minimal treatment [35], patients with a partial tear of the anterior cruciate ligament progress to complete ligament deficiency [76,78,106]. Reasons for this incapacity of the CCL to heal are unknown, but this discrepancy offers a unique opportunity to study the reasons for these differences. Early studies favored a constant disruption of the ligament stumps during joint motion, but different suture repair techniques could not solve this problem [66]. Subsequent research compared collateral and cruciate ligaments at their cellular and tissue level. However, ruling out reasons for different healing capacities, no differences in proliferation, migration, and biosynthesis have been found [40]. Other factors may therefore be responsible for degeneration and for the poor healing capabilities of the CCL as compared to other ligamentous structures. Among others, a disregulation of cell death has been considered a central feature in the canine cruciate ligament disease [39,58]. To our knowledge, studies on apoptosis in canine cruciate ligaments have to date only been performed by our research group.

Apoptosis-induction in the CCL and CaCL

The present thesis is a continuation of prior investigations of our research group and is based on preliminary results on the subject.

Using caspase-3 as a marker of apoptosis, significantly more apoptotic cells were seen in biopsies of ruptured canine CCLs compared to intact CCLs [39]. The finding that no differences in the amount of apoptotic cells existed between the intact area of partially ruptured CCLs, the torn portion of partially ruptured CCLs, and the completely ruptured CCLs suggests that apoptosis may be an intrinsic etiopathogenic factor leading to CCL disease rather than a consequence of acute rupture of the ligament [58].

However, the activator and the mechanism by which the programmed cell death occurs are presently unclear. Mechanical microinjury, excessive shear stress, or biological factors such as loss of growth factors, excessive levels of reactive oxygen species and inflammatory cytokines may elicit apoptosis. Apoptosis is a physiological process and is a highly selective way to eliminate aged and injured cells. In addition to internal pathways that trigger apoptosis mainly in response to cytotoxic stress, apoptosis can be induced through cell surface death receptors that contain molecular structures called death domains [68]. Fas and TNFRI are well-known examples of such receptors.

Results from the first in vitro study of this thesis demonstrated that cruciate ligament cells respond with apoptosis to different natural and experimental stressors in two different pathways. In particular, ligamentocytes die either by stimulating the cell surface receptor through Fas Ligand or by activation of the mitochondrial apoptotic pathway through doxorubicin, cisplatin, and NO donors.

As a very interesting aspect, this study revealed that canine fibroblasts from the cranial and caudal cruciate ligament are intrinsically different despite being present within the same joint [34]. Ligamentocytes from the CCL have a higher susceptibility to apoptosis, a phenomenon likely related to the most common type of CCL rupture. By contrast, stimulation with TNFα, as a known marker of joint inflammation in dogs and humans [37,44,99], could induce apoptosis neither in CCL nor in CaCL cells, although they express TNFRI and TNFRII receptors on their surface.
Caspase inhibition in the CCL

One goal of the first study was to investigate the potential of a pancaspase inhibitor (z-VAD.fmk) in attenuating the effects of the different apoptosis inducers. Apoptosis triggered by Fas-FasLigand binding was reduced by a caspase inhibitor, indicating the value of an anti-apoptotic in vivo intervention in dogs with CCL rupture. However, zVAD.fmk exhibited no significant protection against other apoptosis inducer such as NO or peroxynitrite. NO and peroxynitrite are two molecules which are thought to play a role in CCL disease and OA [43,50,56].

These observations raise the following questions:

1) Was the caspase inhibition simply inefficient in this case? In general, zVAD.fmk does not inhibit all caspases to the same extent. For example, 1,000-fold more zVAD.fmk is required to inhibit caspase-2 compared with caspase-1 in vitro [9].

2) Is the participation of a caspase independent cell death pathway (CICD) possible? An involvement of CICD has often been noticed in the presence of the broad-spectrum caspase inhibitor [9] and is further supported by our observation that western blotting revealed no detectable active caspase-3 in ligamentocytes after stimulation with the NO generator SNP (see study 2). Meanwhile, the absolute requirement for caspase activation in apoptosis is no longer considered dogma. CICD occurs when a signal that normally engages apoptosis fails to activate caspases, but the cell, nevertheless, dies. Typically, mitochondrial functions decline during CICD, although this is generally a slower process than is seen in apoptosis [14]. In the second study, we observed this delay of cell death in SNP-treated canine ligamentocytes in the presence of zVAD.fmk as well.

NO-signaling in the CCL

Our first study highlights the limitation of one-sided caspase-inhibitory therapies for degenerative disorders involving canine ligaments. Based on these results, we focused on the characterization of signaling during NO-induced cell death in canine ligamentocytes. In the literature, there is no consensus as to the exact function of endogenous NO production in chondrocytes [8,36,97] as well as in ligamentocytes. Several studies have documented that ligamentocytes are not only the sites of NO production [62,88] but also themselves targets for NO and undergo apoptosis upon exposure of NO [34,74]. NO has been identified to cause phosphorylation of various targets, including the mitogen activated protein (MAP) kinase family [60,84]. MAP kinases, and in particular p38, have been implicated in the upregulation of iNOS gene expression and NO production in OA chondrocytes [59]. In our second study, we interrupted signaling by using the MAP kinase MEK1/2 inhibitor PD98059 and the p38 kinase inhibitor SB202190 to elucidate the role of the extracellular signal-regulated protein kinases during NO-mediated cell death. Using PD98059 and SB202190, it was not possible to significantly reduce cell death induced by SNP in our ligamentocytes. We performed the same experiments on human OA chondrocytes which served as positive controls and could verify the protective effect of these inhibitors as reported for these cells [80]. These findings support the view that MAP kinases regulates very specific biological responses that differ from cell type to cell type and do not act as mediators of the NO-mediated cell death in canine ligamentocytes.

Using the broad tyrosine kinase inhibitor, genistein, we found that tyrosine kinase is a major transducing pathway in regulating cellular susceptibility to NO in canine ligamentocytes, a finding similar to that reported in synoviocytes [54]. The tyrosine kinase inhibitor genistein markedly and dose-dependently preserved ligamentocyte’s viability in the presence of SNP associated with a complete inhibiton of PGE2 secretion.

Several possible systems have been considered to explain the mechanisms of NO-
mediated cytotoxicity [59,96]. SNP-generated NO is able to induce cell death due to a direct cytotoxic effect on the one hand and/or due to a reaction to different free radicals on the other hand [24]. In the present study, reactive oxygen species (ROS) were found to be a major activation pathway in SNP-induced ligamentocyte cytotoxicity. Scavengers for ROS were able to attenuate apoptosis significantly. Thus, uric acid totally abolished NO-mediated cell death in CCL as well as in CaCL cells. Accordingly, ROS formation appears to be one of the key regulators of NO-induced cell death in canine ligamentocytes. In human OA, the uncontrolled production of free radicals is considered an important factor in the pathogenesis of osteoarthritis [50,105].

**COX and prostaglandin in the CCL**

The NO synthesis and the prostaglandin (PG) synthesis pathways both involve enzymes that have constitutive and inducible isoforms (cNOS and iNOS for NO, COX-1 and COX-2 for PG). Several stimuli (IL-1, T NFα and lipopolysaccharide) induce both iNOS and COX-2. In chondrocytes and synoviocytes of human patients with OA, NO-mediated cell death was dependent on the induction of PG synthesis and COX-2 expression [54,80]. In canine ligamentocytes, stimulation with the NO donor SNP led to a significant PGE2 release. However, the amount of the PGE2 by ligamentocytes was significantly lower compared to the amount of PGE2 by human OA chondrocytes measured in parallel (see study 2). The lower concentrations of PGE2 can be a hint for the lower metabolic activity of ligamentocytes compared to highly active synoviocytes or chondrocytes. There are likely differences in the cellular response to PGE2 reflecting the tissue and/or cell-specific role of COX-2 in apoptosis. In addition, interventions with COX-2 inhibitor NS-398 reduced apoptosis in cruciate ligament cells but less clearly than in human chondrocytes or synoviocytes [54,80].

Based on the potential crosstalk between the two systems in OA chondrocytes and synoviocytes and the clinical usage of COX inhibitors in orthopedic diseases in dogs, we evaluated the efficacy of anti-inflammatory drugs with regard to ligamentocytes apoptosis in a third study.

The protective effect of the tested NSAIDs was dependent on the cytotoxic degree of SNP. The strongest protection was present at a mean cytotoxicity of SNP. Acetylsalicylic acid, carprofen, meloxicam, and robenacoxib significantly prevented CCL and CaCL cells to undergo apoptosis when they were exposed to NO. Cell viability rose by 15 to 45% depending on the drug. A similar effect was reported for chondrocytes [73]. Different COX-2 inhibitors protected the chondrocytes against cell death in a concentration-dependent manner. Moreover, they were able to inhibit the increase of PGE2 and of NO levels in osteoarthritic chondrocytes [64,73]. In our study, high concentrations of SNP induced a high amount of apoptotic ligamentocytes (80%). This strong effect was not possible to reduce by a pretreatment with selective COX-2 inhibitors. Such high apoptotic levels are normally not present in the CCL in vivo independently from its degenerative situation [39]. Results from our study suggest that the therapeutic application of the tested NSAIDs is useful to protect the joint against CCL degeneration.

In conclusion, comparison of canine cruciate ligament cells with other cells originating from joint tissue clearly showed cell-type-specific responses. These specific responses depend critically on the presence of individual components of the different signaling pathways in a particular cell. NO-induced ligament cell death seemed to be mediated mainly by ROS as well as an upstream signaling pathway of tyrosine kinases.
Outlook and future directions

At first and as a precondition, detection of early partial CCL rupture in dogs will be necessary for the development of disease-modifying therapy aimed at preventing further deterioration in the biomechanical properties of the CCL. Certainly, a loss of fibroblasts in the ligament is problematic, because they are needed for tissue maintenance and not easily replaced after they have died.

As discussed before, cell death in canine ligamentocytes can proceed by more than one mechanism depending on the death stimulus. Therefore, apoptosis of ligamentocytes seems to be a potential target for therapeutic interventions. Our results indicate that a general caspase inhibition alone does not completely protect canine CCL cells from apoptosis. Molecules with the capacity to modulate pro-apoptotic stimuli (NO, prostaglandins, cytokines, ROS) could be further excellent targets to modulate CCL cells not to undergo apoptosis and to continue producing matrix. Therefore, investigations focused on the amount and formation of NO or ROS in both ligaments would be helpful in order to define internal differences and to find successful inhibitors or, better, a combination of different apoptosis inhibitors. Moreover, the regulation of the natural inhibitors of apoptosis (for examples c-FLIP, BAR, ARC and HC-gp39) could complement other strategies to reduce ligament degeneration. We have started first interventions, but further studies are necessary regarding the microenvironment of the ligament cells which is as important as the cell death stimulus itself.

This thesis provides new insights into different susceptibilities of CCL and CaCL cells to apoptosis. Specimens of the cranial and caudal cruciate ligament from neonatal beagle dogs served as source for our primary cell cultures.

Further investigations with ligament fibroblasts from adult dogs and especially from dogs which are predisposed to CCL rupture will be helpful in finding out if differences between cranial and caudal ligamentocytes still subsist.

Employing the broad caspase inhibitor zVAD.fmk, we were able to distinguish between a caspase dependent or independent pathway but could not define the exact initiators and executioner caspases involved. In this context, further investigation using inhibitors against a single caspase might give more details on the exact pathway and might subsequently reveal accurate targets to block apoptosis in ligaments.

In conclusion, the therapeutic application of NSAIDs is suggested to have a benefit in protecting against CCL apoptosis. If the hypothesis that increased apoptosis in canine cranial cruciate ligament disease has an impact on ligament degeneration proves correct, these findings indicate that these drugs, in addition to their analgesic effects, may confer ligament protection. Further elucidation of the underlying pathways leading to ligamentocyte apoptosis and degradation of extracellular matrix could improve the likelihood of developing effective strategies to preserve stifle joint function. Therefore, it would be of great interest to analyze the impact of apoptotic ligamentocytes on the quality of the extracellular matrix. Different studies already showed that NO is able to modulate the synthesis of extracellular matrix proteins. However, further studies are necessary to clarify if the inhibition of apoptosis in ligamentocytes induced by NO or by other stressors would be effective in preventing enhanced matrix metalloproteinase production.

This thesis provides a rational framework for future therapeutic studies. Until now the standard of care for a partially ruptured CCL is surgical. With this study in perspective, we open the discussion to the use of the novel concept of regenerative medicine as a less invasive, yet still effective procedure in the treatment of CCL disease.
References


References


101. Wilke VL, Conzemius MG, Besancon MF, et al. Comparison of tibial plateau angle between clinically normal Greyhounds and Labrador Retrievers with and without rupture of the


List of Publications

Selective iNOS-inhibition does not influence apoptosis in ruptured canine cranial cruciate ligaments

In vitro effect of different mediators of apoptosis on canine cranial and caudal cruciate ligament fibroblasts and its reversibility by pancaspase inhibitor zVAD.fmk
Forterre S, Zurbriggen A, Spreng D
Vet Immunol Immunopathol 2011; 139:264-270

NSAIDs protect canine cruciate ligament cells against nitric oxide induced programmed cell death
Waldherr K, Zurbriggen A, Spreng D, Forterre S

Evaluation of apoptotic cell death in normal and chondrodystrophic canine intervertebral discs
Klauser M, Forterre F, Doherr M, Zurbriggen A, Spreng D, Forterre S

Nitric oxide induces cell death in canine cruciate ligament cells by activation of tyrosine kinase and reactive oxygen species
Forterre S, Zurbriggen A, Spreng D
BMC Veterinary Research 2012, 8:40 doi:10.1186/1746-6148-8-40
Published: 29 March 2012
Declaration of Originality

Last name, first name: Forterre, Simone
Matriculation number: 07-126-493

I hereby declare that this thesis represents my original work and that I have used no other sources except as noted by citations.
All data, tables, figures and text citations which have been reproduced from any other source, including the internet, have been explicitly acknowledged as such.
I am aware that in case of non-compliance, the Senate is entitled to divest me of the doctorate degree awarded to me on the basis of the present thesis, in accordance with the “Statuten der Universität Bern (Universitätsstatut; UniSt)”, Art. 20, of 17 December 1997.

Place, date Signature