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**Interleukin-1 receptor antagonist and interleukin-1 beta levels in equine synovial fluid
of normal and osteoarthritic joints and the influence of two different autologous
conditioned serum treatment intervals on cytokine and cartilage biomarker levels in
equine osteoarthritic joints**

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„Was Hänschen nicht lernt, lernt Hans nimmer mehr.“

Für meine Familie und Fritz Flotow

Contents

1	Introduction	5
2	Scientific publications in peer reviewed journals.....	8
2.1	Interleukin-1 receptor antagonist and interleukin-1 beta levels in equine synovial fluid of normal and osteoarthritic joints: influence of anatomical joint location and repeated arthrocentesis.....	8
2.2	Evaluation of two protocols utilizing autologous conditioned serum for intraarticular therapy of equine osteoarthritis – a pilot study monitoring cytokines and cartilage-specific biomarker.....	15
3	Discussion.....	26
4	Summary.....	29
5	Zusammenfassung	30
6	References for introduction and discussion.....	32
7	Oral presentations	39
8	List of publications	39
9	Acknowledgements/ Danksagung.....	40
10	Declaration of own research activity.....	41

1 Introduction

Osteoarthritis (OA) represents one of the major reasons for premature retirement of human and equine athletes and thus has an enormous socioeconomic impact. Its aetiopathogenesis is multifactorial but still in great parts unknown. Therefore, current treatment options for OA are limited and have primarily symptom modifying effects. Since years research focuses on the elucidation of the biochemical processes in osteoarthritis in order to find treatment methods with not only symptom but also disease modifying ability.

The joint is a complex organ composed of many different specialized tissues producing a wide variety of interleukins, metalloproteinases and growth factors. In the center of OA pathology stands the articular cartilage which is a bradytrophic tissue with very low matrix turnover and the weight-bearing lining of the synovial joint. For this reason, cartilage repair is one of the major goals in OA therapy research and simultaneously the greatest challenge remembering the half-life of collagen type II (over 100 years) and aggrecan (3-24 years), the two major components of articular cartilage [1]. Yet OA is considered as a group of disorders which does not only affect the articular cartilage but also involve the entire joint, including the subchondral bone, ligaments, capsule, synovial membrane and peri-articular tissues. The common end stage of all these disorders is the progressive deterioration of the articular cartilage (fibrillation, fissures, ulceration, full thickness loss of the joint surface) accompanied by changes in the bone and soft tissue of the joint [2].

The two fundamental mechanisms for the development of OA have been classified into either the adverse effects of 'abnormal' stresses on 'normal' cartilage or of 'normal' stresses on 'abnormal' cartilage [3]. Yet the reaction in the above mentioned various joint-associated tissues cannot be considered in isolation and primary damage to all of these tissues (i.e. subchondral sclerosis, synovitis or capsulitis) has been proposed to often lead to secondary damage to the articular cartilage from either loss of support or release of cytokines [2].

Two of the major cytokines in this process are the interleukin 1 beta (IL-1 β) which is mainly produced in OA cartilage but not to a significant extent in synovium and the tumor necrosis factor alpha (TNF α), expressed in synovial membrane and cartilage [4]. While TNF α is thought to be the major cytokine in the acute stages of human OA, IL-1 β remains high throughout all stages [5]. Human and equine studies showed that IL-1 β works similar to a hormone and due to its regulatory character is of vital importance to the joint physiology [6]. However, this regulatory character is failing in OA joints and changes into a progressive tissue destructive process by activating the secretion of prostaglandin E2 (PGE2) [7] in chondrocytes and synovial fibroblasts and increasing the activities of matrix metalloproteinases (collagenase, gelatinase, proteoglycanase and plasminogen activator) [3; 8-10]. Matrix metalloproteinases

(MMPs) specifically degrade native collagens and proteoglycans, which lead to a higher synovial fluid (SF) concentration of biomarkers of cartilage turnover in OA joints than in normal joints [7; 11-13]. Collagenase dependent cleavage of type I and type II collagen leads to an increased level of the C1,2 C neoepitope in SF [14], which is seen as a biomarker for cartilage degradation. During incorporation of newly synthesized type II procollagen molecules into collagen fibrils the carboxy-propeptide (CP II) is released by proteinases into SF [15]. Different studies suggested, that its content is directly related to the type II collagen synthesis [16-18]. Another expressive biomarker, proposed to reflect the turnover of novel aggrecan molecules, is the aggrecan chondroitin sulphate 846 epitope (CS 846) [12]. Frisbie *et al.* [13] induced iatrogenically an OA in the mid-carpal joint using an osteochondral model and could show a significant progressive increase of C1,2C, CPII and CS 846 concentrations compared to normal joints during a study period of 90 days. Two other equine studies showed a parallel increase of these three cartilage biomarkers in joints after induction of acute joint inflammation by a lipopolysaccharid injection [7; 11]. All three biomarker levels were positively correlated with the severity of joint inflammation and decreased again with elimination of the inflammatory agent. Thus C12C, CP II and CS 846 have proven capable of signaling changes in cartilage matrix turnover and joint inflammation [7; 11; 18] and are very useful for the evaluation of mechanism and effectiveness of new agents in OA therapy.

This new therapy research tries to find ways to interfere as early as possible with the described deterioration cascade to reduce or even prevent the progression of OA. Consequently, one main focus of research lies on IL-1 β and mechanisms of its inhibition. Granowitz *et al.* [19] could show that IL-1 β induces its effects by binding to the IL-1-receptor located on various human body cells. In the 1990s researchers identified an IL-1-receptor antagonist (IL-1ra) that antagonizes the proinflammatory effect of IL-1 β by competitive inhibition of the IL-1 receptor on human monocytes [19; 20]. Further Arend *et al.* [21] showed in a human in vitro study that IL-1 β has a more potent effect on the IL-1 receptor than IL-1ra, and a maximal biological response can be observed even when less than 5% of available receptors are occupied by IL-1 β [21]. Martel-Pelletier *et al.* [22] found that the number of IL-1 receptors is twofold higher in human chondrocytes from OA joints compared to normal joints and that cells in OA joints need less IL-1 receptor occupancy to induce the release of pro-inflammatory cytokines than in normal joints. Arend [23] revealed from his human in vitro studies that to inhibit IL-1 β activity, the rise in IL-1Ra levels must be 100 to 2000 times the rise in IL-1 β [21; 23-30]. Following human and equine studies assumed that a deficit of IL-1Ra relative to IL-1 β within the OA joint might facilitate the progression of OA [31; 32] and that this IL-1ra/ IL-1 β ratio plays a substantial role for the outcome of regenerative therapies [33-35].

One basic regenerative therapeutic approach in OA therapy research is to counteract the degenerative effects of IL-1 β by substitution of IL-1ra to joint homeostasis, using autologous conditioned serum (ACS) [13; 36], platelet rich plasma (PRP) [37-39], autologous processed plasma (APP) [40] or gene therapy [41-43]. Different in vitro [21; 37-39] and in vivo [40; 41; 44-48] studies have already demonstrated the symptom and disease modifying qualities of IL-1ra and results indicate, that a sustained high IL-1ra concentration might have an appreciable therapeutic effect [41; 42; 44].

Autologous conditioned serum (ACS), is a biological treatment agent which increases the IL-1ra concentration and other anti-inflammatory substances and growth factors by a 24 h incubation of whole blood in a syringe containing chromium sulfate-etched medical-grade glass beads [49]. It is used for intraarticular therapy in humans and horses suffering from OA since several years. Yet opinions on this therapy are highly divided and studies have shown contradictory results [36; 45; 50-52] concerning its usefulness and efficiency. Scientific knowledge about the induced effects in joint homeostasis, the duration of the effect and thus the application interval of intraarticular ACS injections is still sparse.

Common equine practice, based on clinical experience and human applications, is a sequence of three to six intraarticular injections once a week [45; 53]. Following up the scientific work that has been done in this field one notices that not just knowledge about the changes in intraarticular IL-1ra and IL-1 β levels post ACS injection are rare but also about these cytokine levels in untreated OA and normal joints. Consequently, the objective of the first study was to investigate the influence of anatomical variations and closely repeated arthrocentesis on IL-1ra and IL-1 β levels in the SF of normal joints and to determine the IL-1ra, IL-1 β and protein concentrations as well as the white blood cell (WBC) count in the SF of normal and OA equine joints, using equine specific antibody enzyme-linked immunosorbent assays (ELISAs).

The second study compared two different ACS treatment protocols by the quantitative assessment of cytokines (IL-1ra, IL-1 β) and cartilage biomarkers (C12C, CS 846, CP II) using commercially available ELISA kits. We hypothesised that an intraarticular ACS injection increases the SF concentration of IL-1ra, but that the half-life of the supplemented IL-1ra would be shorter than 1 week. We further presumed that a two-day treatment interval would maintain a better anti-inflammatory effect in the joint than a weekly treatment interval.

2 Scientific publications in peer reviewed journals

2.1 Interleukin-1 receptor antagonist and interleukin-1 beta levels in equine synovial fluid of normal and osteoarthritic joints: influence of anatomical joint location and repeated arthrocentesis

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2.2 Evaluation of two protocols utilizing autologous conditioned serum for intraarticular therapy of equine osteoarthritis – a pilot study monitoring cytokines and cartilage-specific biomarker

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3 Discussion

The main objective of the present study was to examine the effect of intraarticular ACS injections on selected cytokines in the synovial fluid (SF) of osteoarthritic (OA) joints in order to get more information about the optimal treatment interval of intraarticular ACS injection.

Equine studies on OA pathology and therapy have been performed to a large extent on an 'Equine Osteochondral Fragment Exercise Model' (EOFEM) [54-57] or joints of different anatomical locations with natural occurring OA [53; 58]. To the authors knowledge there is no data available about the difference of synovial parameters in joints of different anatomical locations. The study did not identify a significant difference between SF concentrations of IL-1 β and IL-1ra in high motion joints in three different anatomical locations in normal horses. The data suggests a similar concentration of SF IL-1 β and IL-1ra in metacarpo/metatarso-phalangeal, radiocarpal and talocrural joints. It can therefore be concluded that values from high motion joints in different anatomical locations are most likely comparable.

Studies have suggested very short half-life times (i.e. 4 to 5 hours) for IL-1ra and IL-1 β [59-61] in general. IL-1 β in particular is known to be one of the major cytokines that increases rapidly during inflammatory insults [62]. Thus, a timely more frequent observation of the IL-1ra and IL-1 β concentration in SF after ACS injection was intended in the present ACS treatment study. It is generally assumed that micro lesions caused by a needle puncture have the potential to promote increases in SF IL-1ra and IL-1 β levels and thus falsify the evaluation of biomarker concentration changes due to intraarticular treatment. Previous studies showed that increases in several other biomarkers were significantly associated with repeated arthrocentesis [63-65], yet for IL-1ra and IL-1 β such information was lacking. The present study revealed that SF IL-1ra and IL-1 β concentrations were not affected by repeated arthrocentesis within 60 minutes. This does not exclude the possibility of increasing IL-1ra and IL-1 β levels due to an inflammatory reaction induced by the needle puncture, but shows that if this increase exists, it is not detectable within the first hour after arthrocentesis. This finding suggests that a closer SF sampling after arthrocentesis is possible.

In the past, the evaluation of therapeutic agents in human and equine studies were often assessed by clinical parameters (lameness, joint effusion etc.). However, this method includes many bias and by itself is insufficient to produce reliable and objective study results. Therefore, recent studies focused on the measurement of endogenous biomarker or have chosen a combination of both [4; 32; 45; 66; 67]. Yet studying the literature one notices that it is quite difficult to find reliable biomarker which allow the evaluation of symptom- and disease modifying qualities of a therapy agent, especially because a great part of the pathologic

mechanism of OA is still not enlightened. For many synovial biomarkers and cytokines, reliable information about the concentration within normal equine joints is lacking. Obviously, this knowledge is a prerequisite to understand the pathogenesis of OA and to evaluate biomarker concentration changes due to treatment of OA joints. In the present study, IL-1ra levels in SF were significantly higher in OA joints compared to normal joints. In vivo experiments have revealed that to inhibit IL-1 β activity, the rise in IL-1ra levels must be 100 to 2000 times the rise in IL-1 β [23]. The necessity for a large excess of IL-1ra over IL-1 β is likely to explain the finding that, even though a high level of IL-1ra is found in SF of horses with OA, there could be a deficit of IL-1ra relative to IL-1 β within the joint which facilitates the progression of OA [32]. The body seems to counteract the harmful increase of IL-1 β with a parallel increase of its antagonist IL-1ra to uphold the physiologic IL-1ra / IL-1 β ratio. Consequently an endogenously induced simultaneous increase of IL-1 β and IL-1ra seems to be a sign for inflammatory processes [32]. This increase has to be differentiated from an induced high SF IL-1ra concentration without a parallel increase in the SF IL-1 β concentration. The latter is assumed to have an anti-inflammatory as well as cartilage protective effect, due to competitive inhibition of the IL-1 receptor [41; 42].

These findings enabled us to design and evaluate the second study which aimed to compare a weekly- and a two-day-treatment interval of three intraarticular ACS injections due to evaluation of changes in SF cytokine and cartilage biomarker concentrations.

We could demonstrate, that an intraarticular ACS injection led to an increase in the SF IL-1ra concentration of OA joints 1 hr (group 1) and 4 hrs (group 2) post injection and that this increase was positive correlated with the concentration of IL-1ra in the injected ACS. Further we could demonstrate that these increased IL-1ra concentrations were decreased back to baseline concentrations within 48 h (group 2) and 7 days (group 1) post injection. This leads to the assumption that the half-life of IL-1ra lies between 4 hrs and 48 hrs post ACS injection and that its anti-inflammatory effect might be annulled within less than two days. An assumption which is supported by comparing the SF IL-1ra concentration trend after ACS treatment between the weekly (group 1) and two-day treatment interval (group 2). Interestingly the latter has shown an approximation of the SF IL-1ra and IL-1 β as well as C1,2C, CP II and CS 846 levels on levels in normal joints [7; 11; 13]. An explanation for these findings could be an anti-inflammatory effect of ACS of less than 48 hours. ACS might induce this anti-inflammatory effect by increasing the intraarticular IL-1ra concentration for less than 48 hrs. This effect attenuates with decreasing IL-1ra concentration by giving IL-1 β more possibilities to bind to the IL-1 receptor. From there it might again induce tissue destructive processes and a pathologic parallel increase of IL-1ra and IL-1 β levels, which could be an explanation for the controversial findings in group one of increasing and decreasing concentration trends during ACS treatment. Based on these assumptions the above-mentioned decrease of SF cytokine

and cartilage biomarker levels after ACS treatment in group 2 might display a reduction of inflammatory and cartilage degrading processes in the joint.

Nevertheless, the data about the IL-1ra half-life in this study is not satisfying. The half-life of IL-1ra might be much shorter than 48 hrs and an even closer treatment interval than the two-day interval might be expedient. Yet more frequent joint injections include a higher risk of infection and still might not be able to ensure a continuous high SF IL-ra level in the OA joint. This was long since presumed by some researcher and lead to further research in treatment possibilities which might be able to ensure this continuous high SF IL-ra level. According to their research work of the last years gene therapy seems to be a promising method to reach this aim and need to be further investigated.

In conclusion, the present study results suggest that the long-term effect of an ACS treatment given at two-day intervals is characterized by decreased SF IL-1ra, IL-1 β , C12C, CP II and CS 846 concentrations, which might indicate a reduction in joint inflammation and cartilage degrading processes. The treatment protocol at two-day-intervals could be preferable to the widely-used treatment protocol of weekly intraarticular ACS injections. Yet these findings are of limited significance and have to be proven in future studies with larger sample size and uniform OA pathologies.

4 Summary

A great deal of research is done for decades to elucidate the complexity of osteoarthritis pathology and to develop therapeutic strategies with symptom and diseases modifying ability. In the recent years, basic knowledge of the synthesis and mechanism of IL-1 β and IL-1ra was determined, which are assumed to play a key role in the pathogenesis of osteoarthritis. Consequently, different therapy approaches (i.e.: ACS, PRP, gene therapy) try to influence the ratio of these two interleukins by substitution of IL-1ra. Nevertheless, there still exist great gaps in the knowledge of IL-1 β and IL-1ra concentrations in sound and OA joints and the effect of intraarticular IL-1ra substitution.

The first study aimed to evaluate the concentrations of Interleukin-1 receptor antagonist (IL-1ra) and interleukin-1 beta (IL-1 β) in normal and osteoarthritic (OA) joints as well as the influence of joint location and arthrocentesis on these concentrations. It could show that the IL-1ra and IL-1 β concentration in metacarpo-/metatarsophalangeal, radiocarpal and talocrural joints did not differ significantly and that arthrocentesis did not increase these cytokine concentrations within 60 minutes after joint puncture. Synovial fluid (SF) IL-1ra and IL-1 β concentrations were significantly higher in OA than in normal joints. Thus, a parallel increase of both cytokines seems to be an indicator of joint inflammation. Yet on their own these cytokines are not able to differentiate between healthy joints and different OA stages due to great value ranges and value overlap. Yet it has to be further investigated if in combination with other biomarkers a clearer differentiation of pathologic processes in the joint can be made. The second study hypothesised that shorter treatment intervals of intraarticular autologous conditioned serum (ACS) injections would more beneficially affect the SF concentrations of IL-1ra, IL-1 β and cartilage biomarkers, compared with the traditional weekly treatment intervals in joints suffering from natural OA.

The results indicate that the long-time effect of an ACS treatment given at two-day intervals is characterized by decreased SF IL-1ra, IL-1 β , C12C, CP II and CS 846 concentrations and thus an approximation to concentrations in normal joints. This might indicate an improvement in joint inflammation and cartilage degrading processes, which lead to the assumption, that a two-day treatment interval is preferable to the commonly used weekly interval. Yet these findings are of limited significance and have to be proven in future studies with greater sample size and uniform OA pathologies.

5 Zusammenfassung

Interleukin-1 Rezeptor Antagonist und Interleukin-1 beta Konzentrationen in equiner Synovia von gesunden und osteoarthritischen Gelenken und der Einfluss zweier unterschiedlicher Behandlungsintervalle mit autolog konditioniertem Serum auf die Konzentration von Zytokinen und knorpelspezifischen Biomarkern in equinen osteoarthritischen Gelenken

Seit Jahrzehnten konzentriert sich die wissenschaftliche Forschung auf die Aufklärung der komplexen Pathologie der Osteoarthritis (OA) und der Entwicklung von Therapieformen, die nicht nur Symptombefreiheit schaffen, sondern auch regenerative Wirkung besitzen. In den letzten Jahren konnte grundlegendes Wissen über die Synthese und den Wirkungsmechanismus von Interleukin 1 beta (IL-1 β) und den Interleukin-1 Rezeptor Antagonist (IL-1ra), welche eine Schlüsselrolle in der Pathogenese von Osteoarthritis einnehmen, erlangt werden. Daraus resultierend entwickelten sich verschiedene Therapieansätze (z.B. IRAP, PRP, Gentherapie), die durch intraartikuläre Substitution von IL-1ra versuchen das Verhältnis von IL-1 β und IL-1ra im Gelenk zu beeinflussen. Dennoch ist das Wissen bezüglich der IL-1 β und IL-1ra Konzentrationen in gesunden und OA Gelenken und deren Beeinflussung durch intraartikuläre IL-1ra Substitution immer noch lückenhaft und auch über die Zusammensetzung und Wirkung von autolog konditioniertem Serum ist bisher wenig bekannt. Die vorliegende Studie hatte daher das Ziel diese Fragestellungen genauer zu untersuchen.

Das Ziel des ersten Teils dieser Arbeit konzentrierte sich daher auf die Bestimmung und den Vergleich der IL-1 β und IL-1ra Konzentrationen in gesunden und OA Gelenken sowie den Einfluss von anatomischer Lage des Gelenks sowie wiederholter Arthrozentese auf die Konzentrationen beider Zytokine.

Die Studie konnte zeigen, dass die anatomische Lage von Fessel- Radiokarpal- und Talokruralgelenken keinen Einfluss auf die synovialen IL-1ra und IL-1 β Konzentrationen hat. Weiterhin bewirkte eine Arthrozentese keine Erhöhung beider Konzentrationen innerhalb der ersten 60 Minuten nach Gelenkpunktion. Die IL-1ra und IL-1 β Konzentrationen in OA Gelenken waren signifikant höher als die in normalen Gelenken.

Somit scheint ein paralleler Anstieg von beiden Zytokinen ein Indikator für eine Gelenkentzündung zu sein. Aufgrund der großen Konzentrationsspanne und – überlappung dieser beiden Zytokine sind sie alleine jedoch nicht geeignet, um eine eindeutige Differenzierung zwischen gesunden Gelenken und verschiedenen OA Stadien vorzunehmen. Weitere Untersuchungen in Kombination mit anderen Biomarkern sind notwendig, um eine eindeutigere Differenzierung von pathologischen Prozessen in Gelenken zu ermöglichen.

Der zweite Teil dieser Arbeit überprüfte die Hypothese, dass durch kürzere ACS Behandlungsintervalle eine grössere Reduktion der synovialen Zytokin- und Knorpelbiomarker- Konzentrationen erreicht wird als durch die allgemein üblichen 1- bis 2-Wochenintervalle.

Die Ergebnisse lassen vermuten, dass der Langzeiteffekt einer ACS-Behandlung im Zwei-Tages-Intervall zu einer Senkung der synovialen Konzentrationen von IL-1ra, IL-1 β , C12C, CP II und CS 846 und somit zu einer Annäherung an die Konzentrationen in gesunden Gelenken führt. Dies könnte eine Verminderung der Gelenkentzündung sowie der knorpelabbauenden Prozesse signalisieren, was zu der Annahme führt, dass ein Zwei-Tages-Intervall den ACS Behandlungen im 1- bis 2-Wochen-Intervall vorzuziehen ist. Es sind jedoch weitere Studien mit größerer Probandenzahl und einheitlicheren OA-Äthiologien notwendig, um diese Annahme zu überprüfen.

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7 Oral presentations

Lasarzik et al.: 'Evaluation of two protocols utilizing autologous conditioned serum for intra-articular therapy of equine osteoarthritis – a pilot study monitoring cartilage-specific biomarkers' German Equine Veterinary Association (GEVA) congress, 6.-9. November 2014, Munich

Lasarzik et al.: 'Evaluation of cytokine and growth factor levels in serum and autologous conditioned serum of sound horses and horses suffering from natural osteoarthritis' European College of Veterinary Surgeons (ECVS) congress, 2.-4. July 2015, Berlin

8 List of publications

Lasarzik, J.; Lischer, C.J.; Ehrle, A.; Estrada, R.; Rettig, M.; Klaus, C.; Einspanier, R.; Bondzio, A. (2016) 'Interleukin-1 receptor antagonist and interleukin-1 beta levels in equine synovial fluid of normal and osteoarthritic joints: influence of anatomical joint location and repeated arthrocentesis' *Journal of Equine Veterinary Science* 42; pp. 67-72.

Lasarzik, J.; Bondzio, A.; Rettig, M.; Estrada, R.; Klaus, C.; Ehrle, A.; Einspanier, R.; Lischer, C.J. (2016) 'Evaluation of two protocols utilizing autologous conditioned serum for intraarticular therapy of equine osteoarthritis – a pilot study monitoring cytokines and cartilage-specific biomarkers' *Journal of Equine Veterinary Science*, *in press*

Ehrle, A., Lischer, C.J., Lasarzik, J., Einspanier, R. and Bondzio, A. (2015) Synovial fluid and serum concentrations of interleukin-1receptor antagonist and interleukin-1 β in naturally occurring equine osteoarthritis and septic arthritis. *Journal of Equine Veterinary Science* **35**, 815-822.

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10 Declaration of own research activity

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbstständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

Berlin den 05.07.2017 Juliane Lasarzik