Biological Evaluation of Nutraceuticals Affecting Cartilage Metabolism and Inflammation

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Illustration of the walking pattern of a healthy (brown) and an arthritic (white) subject

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Biological Evaluation of Nutraceuticals Affecting Cartilage Metabolism and Inflammation

Bestudering van de effecten van nutraceuticals op het kraakbeen metabolisme en ontstekingsreacties

(met een samenvatting in het Nederlands)

Proefschrift

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Anita Hartog - te Kortschot
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Contents

Chapter 1

General introduction

"Musculoskeletal disorders, including osteoarthritis, rheumatoid arthritis and osteoporosis, are the most frequent cause of disability in the modern world, and the prevalence of these diseases is rising at an alarming rate [WHO, 1]".

Arthritis

The word arthritis is formed by two Greek words *arthon* (joint) and *itis* (inflammation). It means "joint inflammation" literally. The term arthritis holds no clear boundary; it refers to a group of more than 100 rheumatic diseases and other joint related disease conditions. Degenerative joint changes can be detected in different ancient animals including a case of post-traumatic arthritis in dinosaurs [2]. The first known human arthritis dates back as far as 4500 B.C. [3]. In the fourth century B.C., Hippocrates, the father of western medicine, was the first to recognize and record the clinical signs of a number of these rheumatic conditions including gout and rheumatic fever [4]. Although rheumatic diseases can affect people at all ages, the two most common and important forms, osteoarthritis (OA) and rheumatoid arthritis (RA) have a relative high prevalence in elderly [5]. The prevalence rates of arthritis are expected to increase in the coming years due to the increasing proportion of the elderly in the population.

Osteoarthritis

OA is the most common joint disease and an important cause of physical disability [6]. More than 10% of the adult population is affected [5]. Noteworthy, more than 80% of the people older than 75 do have clinical signs while more than 80% of the people older than only 50 years of age show radiological evidence of OA [7]. Clinical symptoms are frequently associated with a significant functional impairment and signs and symptoms of inflammation, including pain, stiffness and loss of mobility [8]. The etiology of OA is suggested to be multifactorial. Risk factors can be separated into systemic factors as well as local factors. Systemic factors include: genetic susceptibility, dietary intake, race, gender, age, and bone density. Local factors include biomechanical (such as traumatic lesions and abrasion) and inflamma-

tory conditions [9]. The main characteristics of OA comprise involvement of multiple processes and tissues including cartilage loss, bone remodeling, synovial fibrosis, osteophyte formation at the joint margins and synovial inflammation (figure 1).

Rheumatoid arthritis

RA is a chronic, inflammatory, systemic autoimmune disease that affects about 1% of the general population in the western countries [10]. It is two to three times more common in females compared to males and it can start at any age, with a peak incidence between the fourth and sixth decade of life [10, 11]. The joints most commonly involved are those of the hands, feet and knees. However, almost all peripheral joints can be affected. RA is a so-called “multifactor” disease in which both genetic [12] as well as environmental factors are involved. External factors associated with increased risk of RA include smoking, low socio-economic status and a higher birth weight [13]. The inflamed synovium consists of diverse cell populations including B cells, T cells, macrophages and synovial fibroblasts. Synovial fibroblasts are key players in the destruction of the joint by secreting matrix degrading enzymes en pro-inflammatory cytokines [14]. The cartilage and bone destruction are directly linked to the exacerbation of inflammatory processes (Figure 1).

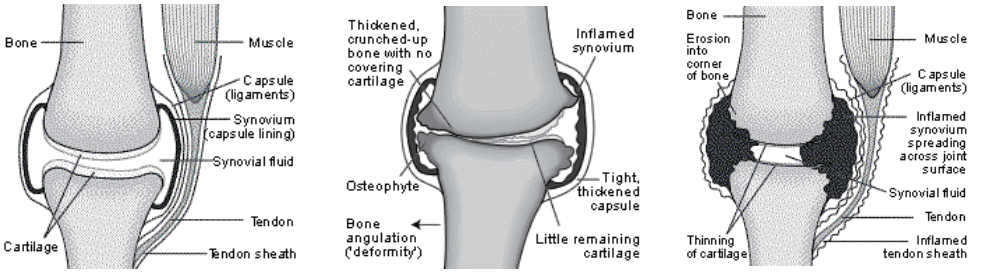

Fig. 1 Schematic representation of osteoarthritis and rheumatoid arthritis in a joint. The left figure represents a normal healthy joint whereas in the middle figure an osteoarthritic joint is depicted. The right figure shows a joint heavily affected by rheumatoid arthritis. Pictures are adapted from the Arthritis Research Campaign.

Tissues involved in the pathogeneses of OA and RA

Both OA and RA are diseases in which not only the articular cartilage of the synovial joint is affected but also the adjacent bone, ligaments, capsule and synovial membrane, and even peri-articular muscles.

Articular cartilage

Articular cartilage is an avascular, aneural, very smooth and highly compressible and resilient connective tissue covering the joint surfaces. It is critical to normal joint function by distributing forces over the joint surface, acting as a shock absorber and aiding in joint lubrication for nearly frictionless articulation. Articular cartilage consists of a highly organized extracellular matrix containing water (60-80%), collagen (10-20%), proteoglycans (PGs) (5-15%), other matrix proteins, lipids and chondrocytes. It can be divided in several different zones according to chondrocyte morphology and/or collagen organization [15-17]. The superficial zone is composed of flattened ellipsoid chondrocytes. The collagen fibers are aligned along the surface. The transitional/middle zone contains predominantly spheroid shaped cells. In this middle zone the large diameter collagen fibers are randomly arranged. The proteoglycan concentrations increases from the superficial zone to the deeper zone. The deep zone contains spherical cells. The cells and collagens in this deep zone have a perpendicular orientation. In the calcified zone a small number of cells are embedded in a calcified matrix (Figure 2). The cells in this zone express a hypertrophic phenotype and show very low metabolic activity. The collagen network defines the form and strength of the articular cartilage, while the highly hydrophilic PGs (a core protein with linked glycosaminoglycans) are responsible for the resilience of the cartilage. The largely negative charged PGs attract anions, and by means of the developed osmotic differences also water, into the cartilage. This results in a large swelling force which is constrained by the tensile stiffness of the collagen network and provides the cartilage its shock absorbing capacity [18]. The matrix shields the ensconced chondrocytes from high stresses and strains generated by joint loading, while not completely isolating the cells from their mechanical environment [19]. In normal cartilage the balance, between cartilage matrix synthesis and degradation is controlled by the chondrocytes.

Both OA and RA display cartilage matrix destruction by enzymatic and mechanical processes. While in OA the proteinases are mainly released by the chondrocytes, in RA these enzymes are mainly derived from cells of the synovial pannus [20]. The matrix metalloproteinases (MMPs) and aggrecenases, MMP-13, MMP-2/MT1-MMP, MMP-3, a disintegrin and metalloproteinase with thrombospondin type 1 motives (ADAMTS)-4 and ADAMTS-5, are indicated to play key roles in cartilage PG and collagen destruction in OA and RA [20, 21]. Disruption of the cartilage structure will have immediate consequences for the function and therefore for the mobility of the joint.

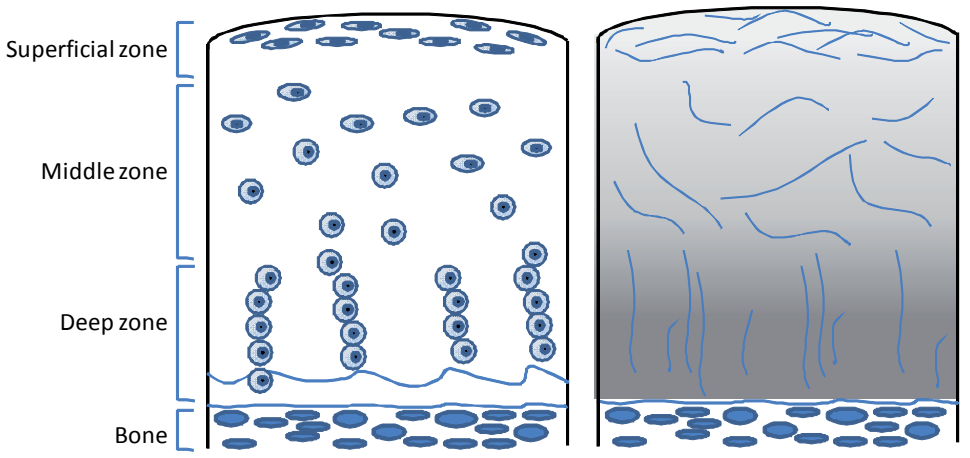

Fig. 2 Structure of articular cartilage. Three distinct zones can be identified. The left schematic diagram shows the cellular organization. In the right diagram is the collagen fiber orientation depicted. The deep zone contains more proteoglycans than the superficial zone (indicated by the background colour in the right figure).

Subchondral bone

Bone remodeling is a natural occurring feature. In physiologic conditions, bone quantity is balanced in such a way that bone mass is maintained. The shape and architecture of the bone may be modified by the so-called basic multicellular units (BMUs), permitting adaptation to mechanical changes and providing a mechanism of repair [22]. Experimental and clinical observations suggest that the structural integrity of articular cartilage is dependent on normal subchondral bone turnover [23, 24].

During the course of OA the organization and functional properties of the periarticular bone change markedly. These changes are detectable already early in the development of OA. Bone changes include appearance of osteophytes at the margins of the joint, subchondral cysts, thinning of the trabeculae and subchondral sclerosis [25]. Osteophytes represent fibrocartilaginous bone outgrowths that are localized at the joint margins and are recognized as a radiographic hallmark for OA. The development of osteophytes is associated with proliferation of periosteal cells. The periosteal cells differentiate into hypertrophic chondrocytes which create, through the process of endochondral ossification, enlarging bone outgrowth. Different growth factors of the transforming growth factor (TGF)-β family appear to play a crucial role in the induction of osteophytes [26].

Subchondral bone thickness is increased in OA affected joints. Although the volume fraction

of the trabecular bone increases, the trabecular structure thinness. Results of bone mineral density measurements are conflicting; decreases as well as increases of bone mineral density have been reported [27-29]. The decrease in mineral density can be explained by the increased rate of remodeling and bone turnover. Bone cysts develop in the focal areas of bone damage and necrosis [30]. Results from preclinical studies evaluating the effect of antiresorptive drugs (inhibiting osteoclast function) show beneficial effects in both bone resorption and cartilage degradation [31, 32]. Whether these results are achieved via a direct effect on osteoclast or via modulation of chondrocyte catabolism has to be evaluated. These future studies will give more insight into the interaction between bone and cartilage changes in OA.

Bone destruction is one of the central features of RA. Pro-inflammatory cytokines promote osteoclastogenesis resulting in local and systemic abnormalities of bone remodeling, including bone erosions and focal and systemic osteoporosis [33, 34]. Tumor necrosis factor (TNF)-α and interleukin (IL)-1 synergize with the receptor activator for nuclear factor-κB ligand (RANKL) to potentiate bone resorption directly by osteoclasts. Under normal circumstances RANKL is derived from osteoblasts. However, during inflammation also inflammatory cells and fibroblasts, found in the inflamed synovium, produce RANKL [35, 36]. Many indirect factors associated with inflammatory arthritis contribute to osteoporosis risk. These include high rates of immobility, weight loss, and use of medications known to promote bone loss such as glucocorticoids [37].

Synovium

The synovium is the soft tissue lining the joint cavity. The synovium is divided in an intima and a subintima. In a normal joint the synovium is mainly populated by two cell types the macrophage like cells (type A) and the fibroblast like cells (type B). The main functions of the synovium is the production of synovial fluid [38], essential for the smooth movement of the joint, removal of waste products from the joint cavity, and immunological defense [39]. For a long time the extent and relevance of inflammatory changes in the synovial membranes from OA patients was controversial. Immunohistochemical studies have demonstrated that synovial tissue from patients with early OA is characterized by infiltration of mononuclear cells [40], which produce pro-inflammatory cytokines and other mediators of joint damage [41]. There are studies pointing to an association between synovitis and the progression of structural changes [42, 43]. Moreover, serum C reactive protein levels are associated with the progression of OA [44]. Synovial inflammation contributes to cartilage damage by the production of catabolic cytokines (IL-1 and TNF-α), inducing the release of MMPs, nitric

oxide (NO) and other cytokines. The most severe inflammatory synovial membrane changes in OA resemble those seen in RA patients at the clinical stage [45]. The present knowledge supports the contribution of the synovial inflammation to OA pathology.

RA synovitis is characterized by a massive inflammatory cell infiltration. The mononuclear cells consisting of monocytes, T cells and B cells and various subsets of dendritic cells accumulate in the perivascular areas [46]. Neutrophils mainly accumulate in the synovial fluid. The inflammatory cells produce a large number of cytokines and enzymes [47], which in turn play a key role in the progressive destruction of both cartilage and bone.

Pain in OA and RA

Arthritic pain is one of the major determinants of functional disability and quality of life [48, 49]. Surprisingly, it is the least well-studied symptom in arthritis. Arthritis pain may reflect active inflammation as well as damage to joint structures [50]. Although the normal joint may respond predictably to painful stimuli, there is a poor correlation between the apparent joint disease and perceived pain during chronic arthritis. Different pain states are recognized including nociceptive pain (a transient response to acute injury) and neuroplastic/ inflammatory pain (increases the excitability of the nociceptive pathway) [51]. Neuroplastic pain which arises as a result of mediators released from damaged tissue or inflammation, is the most common pain state associated with musculoskeletal diseases. Chronic arthritis (OA or RA) results in allodynia* and hyperalgesia**, clinically leading to enhanced pain perception at the site of the injury, as well as to the development of pain and tenderness in the normal tissues both adjunct to and removed from the primary site. One way by which pain is generated in arthritis joints is via the stimulation of so-called 'silent nociceptors'. Nociceptors are located throughout the joint [52]. Following tissue injury and joint swelling, these normally quiescent fibers (silent nociceptors), become active and start sending nociceptive information to the central nervous system [53].

Inflammatory mediators released into the joint act on joint sensory nerves as well, leading to either excitation or sensitization. Inflammatory mediators associated with joint nociception are so-called neuropeptides (substance P, calcitonin, gene-related peptide, and vasoactive intestinal peptide), eicosanoids (prostaglandins, anandamide), ion channel ligands , and several chemical mediators (histamine, adenosine and nitric oxide) [52]. Spinal nociceptive processing in arthritis patients is under influence of inhibitory controls and inputs from oth-

* *Pain in response to a normally innocuous stimulus*

** *Heightened pain intensity in response to a normally painful stimulus*

er somatic structures. Both previous pain episodes and genetic factors are likely to influence activity. Also physiological and social factors have been shown to be important predictors of both presence and severity of pain in OA and RA [54] (Figure 3).
Although current therapies to help alleviate joint pain have limited effectiveness, reducing the amount of inflammatory mediators in the joint might decrease the pain perception [55].

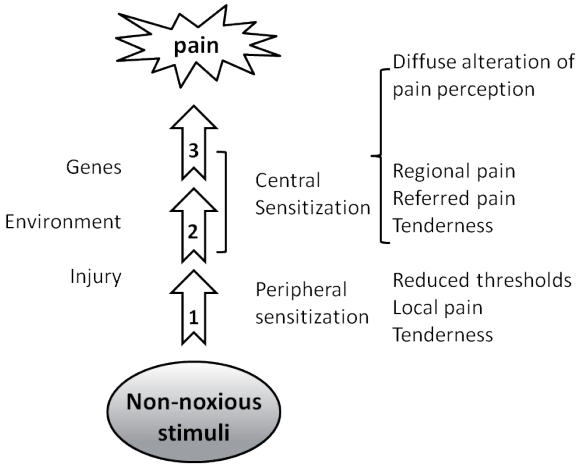

Fig. 3 Tissue injury and inflammation can trigger nociceptor sensitization in peripheral neurons (1), other somatic, psychological and environmental influences are likely to determine the magnitude of any change as a result of activity at spinal (2) or cortical (3) levels [54].

Inflammatory mediators in OA and RA

Inflammation is an important factor in the pathogenesis and clinical presentation of joint diseases. Joint inflammation is known to have destructive effects on the different joint tissues, most importantly the articular cartilage.

Cytokines

Cytokines mediate a wide variety of immunologic actions and are key effectors in the pathogenesis of several chronic diseases. Dysregulation of cytokine activity in cartilage contributes to the disruption of the balance between anabolism and catabolism. In OA the pro-inflammatory cytokines are mainly derived from chondrocytes while in RA the main source of cytokine production is the synovium. The role of pro-inflammatory cytokines, particularly

IL-1 and TNF-α, in cartilage pathology in RA and OA is well established, based on *in vitro* and *in vivo* studies [56, 57]. Both, IL-β and TNF-α suppress the synthesis of PG and type II collagen. They stimulate degradation of cartilage matrix components. This results in chondrocyte dedifferentiation and prevention of cartilage repair. IL-1β and TNF-α stimulate the synthesis of prostaglandins, MMPs, aggrecanases, NO, cytokines, chemokines and adhesion molecules [58]. In inflammation models, the effect of IL-1 and TNF-α is strongly synergistic. TNF-α is indicated to drive acute inflammation whereas IL-1 has a pivotal role in sustaining inflammation and cartilage erosion [59, 60].

Since IL-1 plays a key role in destruction of the cartilage matrix in OA, targeting of IL-1 in OA seems to be an appealing approach for disease modification. The present clinical data, although limited, show that intra-articular injection of IL-1 receptor antagonists in OA might improve pain and WOMAC* global score [61, 62]. The feasibility of such intra-articular injections opens a promising therapeutic perspective for patients suffering from OA. More studies are needed to validate effectiveness and risk-benefit calculations for the postulated therapies.

Strong pre-clinical evidence implicates that TNF-α and IL-6 are critical cytokine effectors in inflammatory synovitis. RA synoviocytes produce TNF-α and other pro-inflammatory cytokines such as IL-1, IL-6 and IL-8. Importantly, if TNF-α bioactivity is blocked in cell cultures of synoviocytes, the spontaneous production of IL-1 bioactivity is neutralized [63, 64]. The wide variety of actions of TNF-α (Figure 4) relevant to the pathogenesis of RA (and to a lesser extend of OA) are recently reviewed by Brennan and McInnes [65].

Another cytokine indicated to play a critical role in the pathogenesis of RA is the pleiotropic cytokine IL-6. IL-6 is over-expressed in RA patients. It mediates maturation and activation of B and T cells, macrophages, osteoclasts, chondrocytes and endothelial cells [66]. Besides TNF-α and IL-6, several types of the IL-1 super-family have been implicated to play a role in RA. IL-1α and IL-1β are expressed in abundance in the synovial membrane. Both induce *in vitro* cytokine production by synovial mononuclear cells, prostanoid and MMP release by fibroblasts, catabolism and cytokine production by chondrocytes, and bone erosion by osteoclasts [67].

* *The WOMAC score is a set of standardized questionnaires used by doctors to evaluate the condition of OA patients. It was developed at Western Ontario and McMaster Universities*

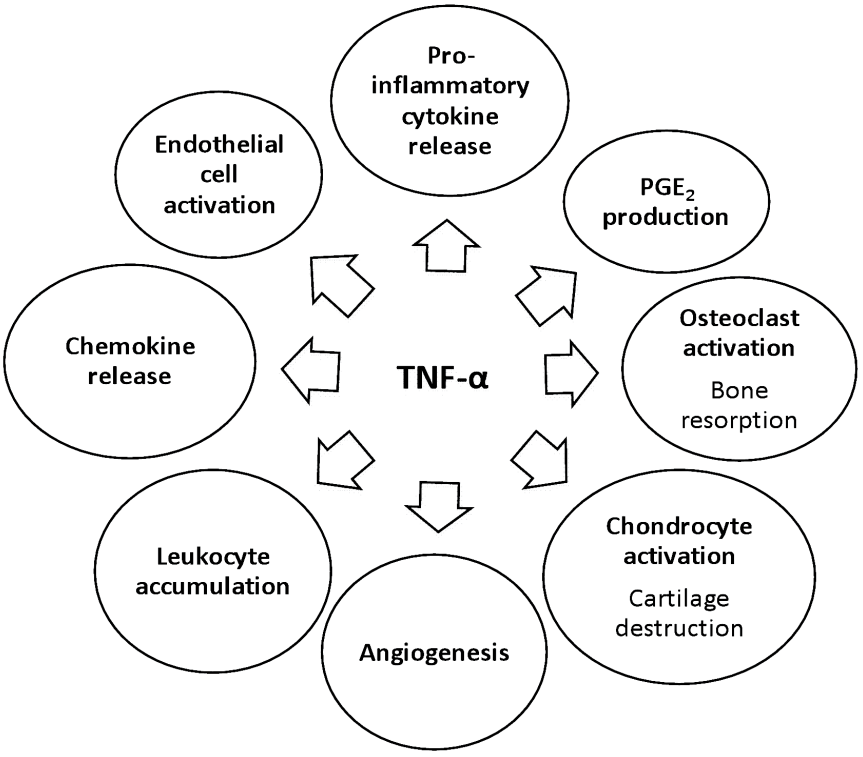

Fig.4 TNF-α is mainly produced by activated macrophages in the inflamed synovial membrane in patients with RA. TNF- α induces the production of other pro-inflammatory cytokines and chemokines. Destruction of cartilage and subchondral bone is initiated by the induction of proteolytic and metalloproteinase enzymes [65].

The pivotal proof of concept for the critical role of TNF-α and IL-6 in the pathogenesis of RA is provided by clinical trials in which TNF- α and IL-6 blocking agents were tested. TNF-α blocking with infliximab [68], a chimeric antibody specific for human TNF-α, etanercept [69], a fusion protein comprising human soluble TNF receptor linked to the Fc component of human immunoglobulin (Ig)G1, or adalimumab [70], a fully human antibody specific for human TNF-α, result in a decrease of RA disease signs and symptoms, improvement of quality of life and prevention of structural joint damage. Moreover, tocilizumab, a humanized monoclonal antibody specific for the IL-6 receptor, has been shown to suppress disease activity and erosive progression in patients with RA [71]. Anakinra, a recombinant, non-glycosylated version of the human IL-1 receptor antagonist, reduces measures of inflammation and suppresses bone erosion but appears however, to be less effective, clinically, than TNF-α blocking agents [72].

Nitric oxide

NO is an important mediator in both health and disease and is fundamental in a wide array of physical processes. NO can be synthesized by many different cell types and because it is uncharged it can freely diffuse across membranes. The enzyme nitric oxide synthase (NOS) facilitates the synthesis of NO. L-arginine, NADPH (reduced Nicotinamide Adenine Dinucleotide Phosphate), and oxygen are converted by NOS into the free radical NO, L-citrulline and NADP (Nicotinamide Adenine Dinucleotide Phosphate) [73]. There are three different isoforms of NOS identified with a 51 -57% homology between the human isoforms: neuronal NOS (nNOS, NOS1), endothelial NOS (eNOS, NOS3) and the inducible NOS (iNOS) [74]. Inducible NOS is induced in cells by trauma, injury, inflammatory mediators or infections [75]. Overproduction of NO can be autotoxic and contributes to tissue damage. Experiments indicated that NO plays a catabolic role in the development of OA, mediates the inflammatory response, is involved in the activation of MMPs, inhibits the synthesis of both collagen and PG synthesis, and helps to mediate apoptosis [76, 77]. However, more recent studies indicate that in cultured chondrocytes the addition of exogenous NO may inhibit pro-inflammatory activation by preventing the nuclear localization of the transcription factor NF-κB [78]. Moreover, inhibition of the NO production with nonspecific NOS inhibitors, targeting all three isoforms, reduces signs and symptoms of erosive arthritis. Whereas, targeting of iNOS resulted in exacerbation of the synovial inflammation and degradation of joint structures. These data might point out that the constitutive isoforms of NOS do contribute to the joint pathology while iNOS may function, at least in part, in a protective way [79].

Prostaglandins

The synthesis of prostaglandin E_2 (PGE_2) is the endpoint of a sequence of enzymatic reactions, including the release of arachidonic acid from membrane phospholipids by soluble phospholipase A2 ($sPLA_2$) and conversion of the free arachidonic acid by cyclooxygenase (COX)-1 and COX-2 into prostaglandin H_2 (PGH_2) and subsequently PGE_2 (Figure 5).

COX-1 is expressed constitutively by many cell types, whereas COX-2 requires specific induction by inflammatory mediators such as lipopolysaccharide (LPS) and cytokines [80, 81]. In articular chondrocytes, IL-1β and TNF-α synergistically induce COX-2 expression, whereas COX-1 expression remains unchanged [82]. COX-2 is able to mediate, via the production of prostaglandins, inflammation, pain, and fever. Nonsteroidal anti-inflammatory drugs (NSAIDs), widely used to alleviate these symptoms, inhibit both COX-1 and COX-2. This inhibition appears to be correlated with gastrointestinal (GI) toxicity. The drugs with a higher COX-1 selectivity have a tendency to cause more gastrointestinal damage [83].

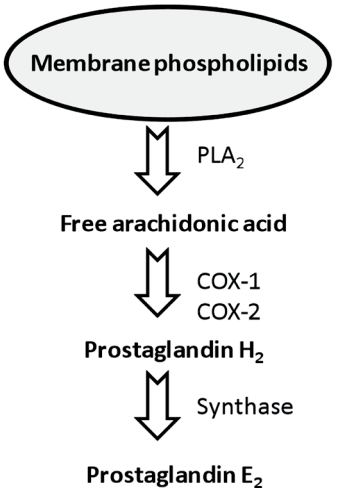

Fig. 5 Schematic representation of the conversion of membrane phospholipids into PGE_2.

A new class of NSAIDs was developed, the coxibs, based on sparing of COX-1 activity. It was demonstrated in different trials that the risk of developing major GI complications was reduced by one half to two third in patients treated with a selective COX-2 inhibitor compared to patients treated with ibuprofen or naproxen, drugs that are slightly more potent in inhibiting COX-1 than COX-2 [84]. In the recent years it was suggested that the highly selective COX-2 agents (coxibs) carry greater risk for cardiovascular side effects. A meta-analysis of approximately 140 randomized trials of five different coxibs revealed the following: (1) coxibs are associated with a moderately increased risk of major vascular events, (2) high-dose regimens of some traditional NSAIDs, such as diclofenac and ibuprofen, are associated with a similarly increased risk and (3) a high dose regimen of naproxen is not associated with a cardio-toxic phenotype [85, 86]. *In vivo* studies on human OA cartilage indicate that NSAIDs with a low COX-2/COX-1 selectivity exhibit adverse effects on OA cartilage proteoglycan turnover, whereas high COX-2/COX1 selective NSAIDs did not show such effects and might have some cartilage protective efficacy [87].

Therapeutic approach of OA and RA

Osteoarthritis

The current therapeutic approach of OA is to alleviate symptoms and preserve function. Some symptomatic relief is provided by attaining and maintaining ideal body weight and

graded exercise [88]. Pharmaceutical treatment is limited by the inability of prescribed medications to alter disease outcome. Moreover, the prescribed NSAIDs and coxibs have limited efficacy and are associated with a number of potentially serious toxicities including gastrointestinal bleeding and increased incidence of ischemic cardiovascular events [89]. As a result, a lot of OA patient seek for alternative therapies in an attempt to modulate both pain and the structural changes that occur within a degenerating joint.

Rheumatoid arthritis

For the treatment of RA there are a number of disease-modifying anti-rheumatic drugs (DMARDs) available. All of these drugs inhibit inflammation. Methotrexate (MTX), with a well documented efficacy including slowing down the progression of radiological deterioration, is commonly used as the first DMARD. TNF-α and IL-1β antagonists are potent anti-inflammatory drugs, with a more rapid onset of effects compared to the traditional DMARDs. It has been indicated that specific combination therapies with DMARDs are more potent that the mono-therapies [90]. Although RA treatment has largely improved in the past years, for many people the therapies are still far from optimal. Most of the RA patients want to do as much as possible to improve their sense of well-being. Therefore the majority of the RA patients will, at least for a certain period during the course of the disease, make use of complementary medicine.

Nutraceuticals

In 1989 Dr. Stephen DeFelice introduced the term “nutraceutical”. The term nutraceutical combines the words “nutrition” and “pharmaceutical”. It is a comprehensive term which includes foods, dietary supplements and medical foods that have a health-medical benefit including risk reduction and or disease management [91]. Nutraceuticals have a defined positive physiological effect and may be incorporated in functional foods and pharmaceutical preparations. They do not easily fall into the legal categories of food or drug and often inhabit a grey area between the two (figure 6).

The development of foods and food components that provide benefits beyond their traditional nutritional value has created tremendous academic, commercial, regulatory and public interest.

The challenges ahead relate to quality, safety and efficacy. The scientific rationale for the use of nutraceuticals has evolved fast in recent history although skepticism remains, due to missing or limited scientific evidence.

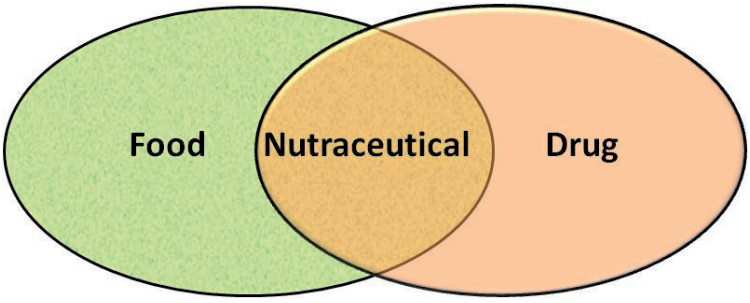

Fig. 6 Nutraceutical occupies position between food and drug.

The fact that, more than 25% of all prescribed medications in the industrialized counties find their origin in natural ingredients in one way or another, supports the untapped potential of natural products for clinical application. Technological advances applied to innovative research, led to a better understanding of the underlying mechanisms of action of some of these substances [92]. The development of aspirin® out of willow bark, pictures an example of a natural product at the birth of the most commonly used and registered drug. The use of *Salix alba* (willow bark) dates back thousands of years, to the time of Hippocrates when patients were advised to chew on the bark to reduce fever and inflammation. The pharmacologically active principle (salicin) was isolated between 1826 and 1829. In 1852 salicylic acid and a year later acetylsalicylic acid were synthesized. The first clinical reports on the clinical use of salicylic acid in rheumatic disorders were published in 1876 [93]. It took roughly 100 years from the initial reports of the beneficial effects of salicylic acid to the discovery of the principal mechanisms of action of the salicylate, being the earliest representative of the NSAIDs. It would take another 20 years until the next major scientific and clinical breakthrough in the NSAID story: the discovery of the COX-1 and COX-2 iso-enzymes and the development of highly COX-2 selective inhibitors.

Complementary and alternative medicine (CAM) is immensely popular for musculoskeletal conditions. Up to 50% of the patients suffering from arthritis report the use of CAM remedies [94, 95]. Use of nutraceuticals is reported as the most frequently used CAM [96]. Setty et al. and Khanna et al. reviewed literature on different preparations commonly used in the treatment of rheumatic indications [97, 98]. These preparations include white willow, stinging nettle, and devils claw, but also onions, thyme and black tea, which are extractions of common food components. Most of the patients using nutraceuticals, believe that natural

remedies with a long history of use are safe. Generally, neither real clinical efficacy, nor the quality of the preparations, nor side effects or interactions with pharmacological medication is questioned by the users.
There are indications (although the amount of research is limited) that natural products might interact with prescription and non prescription (over the counter) drugs. Such interactions may intensify or reduce the effectiveness of a drug or may cause serious side effects. Both patients and physicians should become aware of these risks.

Final remark
Most of the therapies based on natural products are not or only limited studied. Because of this lack of knowledge, these therapies might not be without any risk. It is evident that more preclinical and clinical research is needed to evaluate the way of action of these products, to validate their safety, and to prove there efficacy. An increased scientific knowledge of biological based therapies will allow evidence-based decision making regarding its application. Moreover, elucidation of the mechanisms of action of the various therapies may be instrumental in discovering new molecular targets for the safe treatment of the specific disease.

Aim and outline of this thesis

Management of osteoarthritis is primarily focused on the relief of symptoms, using agents such as analgesics and NSAIDs. This approach however, fails to prevent the continued articular cartilage degeneration. Identification of agents capable of preventing, slowing, or reversing the structural and pathological alterations in osteoarthritis joints is an important research and clinical objective.
Screening of biological based libraries for components with a cartilage protective efficacy is one of the routes which might lead to the identification of new therapeutic agents. These therapies might involve both nutritional as well as pharmaceutical concepts. Therefore a screening assay was developed in which large numbers of plant and mushroom based extracts have been tested regarding their ability to stimulate the production of the cartilage matrix component, glycosaminoglycan (GAG, chapter 2). From one of the “positive stimulatory/stimulating” preparations, an extract of the eatable mushroom *Grifola frondosa*, the mechanism of action was evaluated in more detail.
Additionally, besides the screening of these libraries, literature was screened focusing on ingredients indicated to stimulate matrix production by chondrocyte related cell types. For this, a number of biological agents able to stimulate wound healing (matrix production by

fibroblasts) was selected. These agents were tested on chondrocyte cultures. The most effective agent, able to stimulate GAG production and counteracting cartilage degradation by bovine chondrocytes was an extract of the vegetable *Centella asiatica*. This extract, was studied in more detail in *in vivo* and *in vivo* models (chapter 3).

In addition a multi-plant preparation (SKI306X), described in literature to modulate cartilage metabolism *in vivo* and in animal models, was tested extensively. SKI306X is a mixture of three different plant extracts, *Clematis mandshurica, Trichosanthes kirilowi and Prunella vulgaris.* In placebo controlled clinical studies a beneficial effect of SKI306X was shown in a group of patients suffering from classical OA of the knee [99, 100]. In chapter 4 the biological activity of SKI306X and its individual components was evaluated in more detail using several *in vitro* assays.

All components identified and studied in chapter 2 to 4 were able to affect cartilage matrix synthesis directly or indirectly via modulation of the activity of the pro-inflammatory cytokines IL-1β and TNF-α. IL-1 and TNF-α, key players in OA but also in RA, are able to drive the inflammation by stimulating the production of additional inflammatory mediators such as IL-6, IL-8, IL-18, PGE_2, NO and MMPs [101, 102]. Inhibition of the production or activity of IL-1 and/or TNF-α is indicated to represent an important tool in the treatment of both OA and RA.

Apocynin is a constituent of the herb *Picrorhiza kurrooa* to which a range of biological activities and anti-inflammatory effects are attributed. Apocynin is able to inhibit the superoxide generating enzyme NADPH-oxidase in neutrophils [103], and prevents COX-2 expression in monocytes [104]. For this reason the anti-inflammatory and cartilage protective effects of apocynin were studied *in vivo* in a mouse model of zymosan-induced ear-skin inflammation and a mouse model for acute arthritis, respectively (chapter 5).

Finally, two well known and frequently used food ingredients were tested for their therapeutic potency regarding arthritis. The amino acid glycine and bovine lactoferrin (milk derived protein) have been indicated to modulate innate immune reactions [105, 106]. The *in vivo* anti-inflammatory activity of the two nutritional components was evaluated separately and in combination in a zymosan-induced ear-skin inflammation and a zymosan-induced joint inflammation model (chapter 6). The anti-inflammatory effect of the combination of orally administered glycine and bovine lactoferrin in the zymosan-induced ear swelling was proven to be synergistic. This synergistic decrease in inflammation-induced ear-swelling was accompanied (tested *ex-vivo*) by a decrease in the number of LPS stimulated TNF-α producing spleen cells. These strong anti-inflammatory effects indicated that the glycine bovine lactoferrin mixture might offer a powerful nutritional way of modulating chronic inflamma-

tion including RA [107]. The collagen-induced arthritis (CIA) model was used to evaluate the effectiveness of the glycine and lactoferrin combination in chronic inflammation (chapter7). This mouse model is an extensively studied RA model sharing many immunological and pathological features with human RA. The development and severity of arthritis in the CIA model is mostly detected by a semi-quantitative clinical scoring system based on the severity of arthritis in the peripheral joints. Chapter 8 shows the applicability of locomotion and muscle mass changes as readout parameters in the CIA model and its relevance for intervention studies. In chapter (chapter 9) the results of the different studies are summarized and discussed.

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Chapter 2

Identification of *Grifola frondosa* as a new cartilage supportive lead using a validated functional screening assay

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Abstract

The pro-inflammatory cytokine interleukin (IL)-1β plays a pivotal role in the initiation and the preservation of osteoarthritis (OA). The catabolic activities of IL-1β include a decreased synthesis of one of the main cartilage matrix components, proteoglycan (PG). The aim of the present study was to develop a rapid *in vivo* screening assay for evaluation of the effect of novel components on the IL-1β-mediated PG synthesis by chondrocytes. Concentration dependency and way of action of one of the positive tested herbal extracts, *Grifola frondosa*, was evaluated in more detail.

Primary bovine chondrocytes were cultured in 96-wells plates, in the presence of recombinant human (rh)IL-1β. The amount of PG released into the culture supernatant was detected by a modified 1,9-dimethylmethylene blue (DMB) assay. From the 600 tested nutraceuticals, 22 extracts, including *Grifola frondosa*, were able to stimulate PG synthesis significantly.

Grifola frondosa extract stimulated PG synthesis by bovine chondrocytes cultured in alginate in the absence and presence of rhIL-1β in a concentration dependent manner. The increased PG synthesis was accompanied by an increase in transforming growth factor (TGF)-β2 production. Moreover, the rhIL-1β-induced PG degradation in bovine cartilage explants was counteracted by *Grifola frondosa* extract.

Detection of PG synthesis by bovine chondrocytes in the presence of IL-1β using a modified DMB assay is a quick and valid model system to test novel compounds for potential cartilage protective effects. The *Grifola frondosa* extract was one of the 22 fractions, which tested positive. The cartilage protective effects of *Grifola frondosa* suggest a possible disease-modifying osteoarthritic activity which could be beneficial for OA patients. More research on this promising extract is necessary to explore effectiveness of *Grifola frondosa in vivo*.

Introduction

Osteoarthritis (OA) is a degenerative joint disease leading to chronic pain and disability. It is characterized by breakdown of articular cartilage, joint space narrowing, thickening of the underlying subchondral bone, and osteophyte formation [1, 2].

In healthy mature cartilage a balance exists between different cytokines and growth factors that maintain tissue homeostasis. The chondrocytes show little metabolic activity resulting in a low turnover of matrix components [3, 4]. The loss of cartilage associated with OA implicates a disturbance in the regulation of anabolic and catabolic activities of these chondrocytes. Pro-inflammatory mediators, including nitric oxide (NO), IL-1β and tumor necrosis factor (TNF)-α, play a pivotal role in the initiation and development of OA [5]. Cytokines originating from the inflamed synovial membranes as well as from the chondrocytes inhibit cartilage anabolism and promote catabolism [6, 7]. IL-1β is an important pro-inflammatory cytokine which, during the pathogenesis of arthritis, plays a crucial role in cartilage matrix destruction. It mediates amongst others, inhibition of extracellular matrix de novo synthesis and proliferation of chondrocytes. Additionally, it induces the production of a number of matrix metalloproteinases (MMP's), aggrecanases, and induces chondrocyte cell death [8, 9]. Both IL-1β and TNF-α are able to stimulate NO production by chondrocytes [10]. NO exerts, in an autocrine manner, detrimental effects on chondrocyte functions, including the inhibition of collagen and PG synthesis [10-12]. Since the number of possible disease-modifying osteoarthritic drugs (DMOADs) is limited, and their effectiveness is still under debate [13], there is a need for new components slowing down or stabilizing the pathologic changes in OA patients. Factors modulating the catabolic effect of IL-1β are recognized as a serious target for disease intervention. The present study depicts and validates a screening assay (MTS) in which the effect of 600 nutraceuticals was tested on the IL-1β-modulated PG synthesis by bovine chondrocytes. The way and possible mechanism of action of one of the positive tested herbal extracts, *Grifola frondosa*, was evaluated in more detail.

Materials and Methods

Preparation of the test agents

Extracts of plants and mushrooms used in traditional and/or contemporary medicine were gathered and stored in a sample library. Stock solutions of the extracts, obtained from different suppliers, were prepared by dissolving 30 mg/ml of the extracts in 20% dimethyl sulfoxide (DMSO, Sigma-Aldrich Chemie, Zwijndrecht, The Netherlands) in water. The samples were vortexed for 30 seconds followed by sonication for 10 minutes and

centrifugation for 2 minutes at 3.000 rpm in an Eppendorf centrifuge. The supernatant was divided in 96 well plates and stored at -20°C. The described *Grifola frondosa* extract was derived from Maruzen (Maruzen Pharmaceuticals Co., Ltd., Hiroshima, Japan).

Isolation of bovine chondrocytes

Full thickness bovine articular cartilage slices were aseptically dissected from the metacarpophalangeal joint of young bulls (1-2 years). The slices were minced and digested overnight at 37°C in 1.5% (w/v) collagenase B (Roche Applied Science, Almere, The Netherlands) in DMEM/F12^{+} (DMEM/F12 + penicillin 100 U/ml / streptomycin 100 µg/ml, Life Technologies Merelbeke, Belgium). After removing the undigested cartilage pieces by filtration through a 40 µm nylon cell strainer (Becton Dickinson BV, Breda, The Netherlands) the cells were washed in DEMEM/F12^{+}, counted and suspended in culture medium (DEMEM/F12^{+}, 10% heat-inactivated fetal calf serum (FCS^{hi}, Sigma-Aldrich Chemie) and 10 µg/ml ascorbic acid).

MTS: chondrocyte culture

The chondrocytes, 2.5.10^{4} cells/100 ml/well, were plated into flat bottom 96-wells culture plates (day 1) and incubated at 37°C in a humidified atmosphere containing 5% CO_2 in air. The culture medium was refreshed three times a week. After 7 days, when the cultures reached confluence, the FCS^{hi} concentration in the medium was diminished to 1%. At day 9, 15 ng/ml rhIL-1β (Tebu-Bio, Heerhugowaard, The Netherlands) with or without 50 µg/ml of the different test components was added to the culture (600 different nutraceuticals were tested) in a final volume of 150 µl. All tests were performed in triplicate. RhTGF-β1, which is reported to strongly counteract the IL-1β effects [14, 15], was included in each plate, at a concentration of 2 ng/ml as a positive control (TGF-β1, R&D Systems, Abingdon, United Kingdom). The DMSO concentration (derived from the stock solution of the extracts) in the final incubation medium was 0.03% in all tested conditions. At day 11, the culture medium, including the different additives, was refreshed. After 3 days of incubation, at day 14, 50 µl of the culture supernatant was collected for glycosaminoglycan (GAG) measurement. Cellular toxicity of the extracts was tested by detection of chondrocyte metabolic activity.

Glycosaminoglycan measurement

PGs are supramolecular complexes consiting of proteins and glycosaminoglycan (GAG). The amount of GAG released into the medium was detected by a modified 1,9-dimethylmethylene blue (DMB) assay (Sigma-Aldrich Chemie) according to Farndale [16]. Briefly, 50 µl of culture

supernatant was mixed with 100 µl DMB reagents (48 mg/l DMB, 40 mM glycine, 40 mM NaCl, 10 mM HCl, pH 3.0). Absorbance was measured at 595 nm within 5 minutes after addition of the dye.

MTS: validation of the assay

The stability of the DMB reagent was tested by repeated measurements of a "standard curve" (range 0-35 µg/ml) of chondroitin 6-sulfate from shark cartilage (Sigma-Aldrich Chemie) at different time points after preparation of the DMB reagent. The specificity of the GAG measurement in culture medium was tested in a calibration curve, as mentioned above, in the presence of: 1) different concentrations of protein (1-10% FCS^{hi}), 2) different DNA concentrations (0-250 ng/ml λ DNA), 3) different pH values (pH 3-pH 8) and 4) 0.03 % DMSO. The repeatability was tested by performing the assay (control and positive control) with chondrocytes derived from three different donors at three independent days.

MTS: chondrocyte metabolic activity

WST-1 (4-{3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio}-1, 3-benzene disulfonate, (WST, Roche Diagnostics) is a substrate for the enzyme succinate dehydrogenase. The conversion product formazan can be measured at 450 nm with a reference filter at 655 nm. The activity of succinate dehydrogenase reflects mitochondrial activity and is therefore indicative for metabolic activity.

At day 14, after collection of 50 µl of culture supernatant for DMB analysis, WST-1 solution was added to the wells (10 µl/well). Absorbance was measured directly after WST-1 addition and at 5 hours after start of the WST-1 incubation period.

***Grifola frondosa*: PG synthesis in alginate culture**

Bovine articular chondrocytes were isolated by digestion as described above. After removing undigested cartilage pieces by filtration through a 40 µm gauze, cells were washed twice with a physiological salt (PS) solution after which cell entrapment was performed. Briefly, cells were suspended in 1.2% (w/v) alginate (Keltone ® LV, ISP alginates, Sint-Niklaas, Belgium) in PS at a density of 8.10^6 cells/ml and passed drip-wise through a 22-gauge needle into a 102 mM $CaCl_2$ solution. After 10 minutes of polymerization, beads were rinsed twice with PS. Four beads per well were cultured in a 24-wells plate for 2 weeks in culture medium (without ascorbic acid) at 37°C in a humidified atmosphere containing 5% CO_2 in air; the medium was refreshed twice weekly. Following the stabilization period of 2 weeks, the beads were cultured in the presence or absence of 6.3, 12.5, 25 and 50 µg/ml of the *Grifola frondosa*

extract. After one hour pre-incubation with the different concentrations of the extract, rhIL-1β was added to the beads. The culture medium with the different ingredients was replaced after 3 days. Following the 5 days of incubation with the *Grifola frondosa* extract, half of the culture medium was collected and frozen for TGF-β measurement. As a measure of PG synthesis, the rate of sulphate incorporation was determined. A two-hour pulse with 5 µCi/ml $^{35}SO_4^{2-}$ ($Na_2{}^{35}SO_4$, PerkinElmer life sciences, Zaventem, Belgium) was executed on the beads. Afterwards the beads were rinsed 5 times in PS and each bead was dissolved in 500 µl Lumasolve® (PerkinElmer life sciences) during 16 hours at 60°C. 10 ml Lipoluma plus® (PerkinElmer life sciences) was added to the samples and incorporated radioactivity was counted (LKB Wallac liquid scintillation counter, PerkinElmer life sciences).

***Grifola frondosa*: TGF-β detection**

TGF-β1, TGF-β2 and TGF-β3 levels were measured in the supernatant of bovine chondrocytes, cultured in alginate, using the corresponding human TGF-β ELISA kits (R&D systems, Abingdon, United Kingdom). The ELISAs were performed according to the manufacturer's protocol.

***Grifola frondosa*: GAG degradation detected in cartilage explants**

Full thickness bovine articular cartilage slices were aseptically dissected from the metacarpophalangeal joint of young bulls (1-2 years of age) and placed in PBS (Life Technologies). Cartilage explants were isolated using a 3 mm biopsy punch (Stiefel Laboratories, Coral Gables, Florida, USA) and transferred to 24-wells plates, each well contained 3 randomly picked explants. Explants were incubated in 1 ml culture medium (without ascorbic acid) at 37°C in a humidified atmosphere containing 5% CO_2 in air. After a stabilization period of 24 hours, explants were cultured for 7 days in 1 ml culture medium in the presence or absence of *Grifola frondosa* extract at different concentrations. After one hour pre-incubation with the extracts, rhIL-1β was added at a final concentration of 10 ng/ml. At day 4, the culture medium and components were refreshed. At day 7, supernatants were collected and stored at -80°C until GAG analysis.

Results

Validation of the MTS assay

The stability of the DMB reagents was tested by repeated measurement (three weeks in a row, one measurement each week) of a concentration curve (range 0-35 μg/ml) of chondroitin 6-sulfate from shark cartilage (Sigma-Aldrich Chemie). After one week the reactivity of the reagents decreased significantly, followed by a further decrease in the second week (data not shown). It was concluded that the DMB reagent should be prepared fresh before use.

Specificity of the assay was tested by evaluating the effect of a protein (FCS^{hi}) and DNA concentration changes in the culture medium, which might be brought about by cell death, on a chondroitin 6-sulfate concentration curve. Effects of a changed pH in the culture medium, resulting in a change of the colour, and addition of 0.03% DMSO were tested as well. None of the tested conditions did change the course of the concentration curve significantly (data not shown).

Repeatability was tested by performing the control (rhIL-1β condition) and the positive control (rhIL-1β with TGF-β1) on three different chondrocyte isolations each performed on three different plates. From each of these assays/plates the Z-factor was calculated (Fig. 1). The Z-factor predicts the suitability of the assay for testing in a high throughput setting. A Z-factor of 1 is ideal, a Z-factor between 0.5 and 1 indicates an excellent assay, and a Z-factor between 0 and 0.5 indicates a marginal assay. The following formula: Z-factor = $1- (3*(\sigma_p+\sigma_n))/|\mu_p-\mu_n|$, in which p=positive control, n=negative control, σ=stdev and μ=average [17] was used. The average Z-factor of the present assay is 0.82±0.17 (n=3, ±stdev). Addition of TGF-β1 to chondrocytes cultured in the presence of rhIL-1β increased the PG concentration in the culture supernatant significantly from 4.2 μg/ml to 16.3 μg/ml ($P<0.001$). The rhIL-1β (control) condition was set to 100%. In figure 2 the rhTGF-β1 effect is depicted as % of control.

MTS

In the MTS, 600 nutraceuticals were tested on their ability to stimulate PG synthesis by bovine chondrocytes cultured on 96-wells tissue culture plates, in the presence of rhIL-1β. Herbal extracts able to improve the PG synthesis by more than 75% of the rhTGF-β1 effect were selected as positive. All positive components with a WST below 75% of the control are indicated to be toxic and were excluded from further evaluation. 22 Out of the 600 extracts were tested as positive without affecting the WST significantly. *Grifola frondosa*, one of the extracts tested positive, was further evaluated.

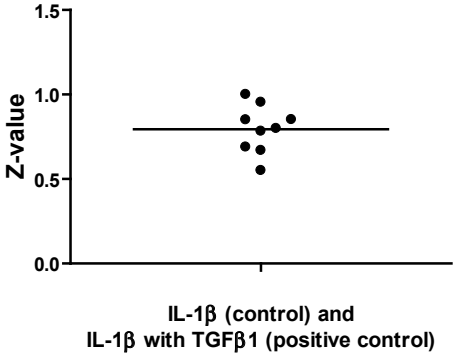

Fig. 1. Bovine chondrocytes, isolated from 3 different animals at 3 different days, were cultured on 3 different 96 wells tissue culture plates/animal. After reaching confluence, the cells were incubated for 5 days with IL-1β, with and without TGF-β1. At day 5 GAG levels in the culture supernatant were evaluated. From each culture plate the Z-value was calculated and depicted.

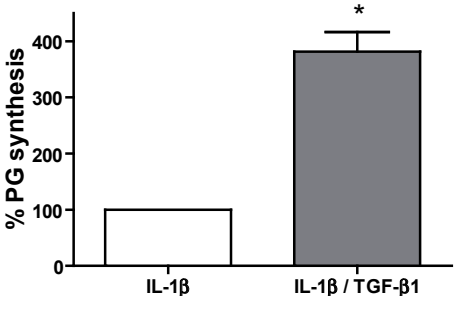

Fig. 2 Bovine chondrocytes were cultured in 96 wells tissue culture plates. After reaching confluence, the cells were incubated for 5 days with rhIL-1β, with and without rhTGF-β1. At day 5 GAG levels in the culture supernatant were evaluated and expressed as % of the rhIL-1β- stimulated control. Values are expressed as the mean ± SEM. Significant differences vs. the rhIL-1β stimulated condition (100%) are indicated with *P<0.001, n=3.

Grifola frondosa: PG synthesis

The concentration dependency of the effect of the *Grifola frondosa* extract on PG synthesis by chondrocytes cultured in alginate was detected by sulphate incorporation. After a stabilisation period of 14 days, the chondrocytes in alginate were cultured for 5 days in the presence of *Grifola frondosa* with or without rhIL-1β (10 ng/ml). Sulphate incorporation

in the absence of IL-1β (control) was set at 100%. All values were expressed as percentage of the control. RhIL-1β decreased the PG synthesis from 100% to 75% (P<0.001). In both the presence and absence of rhIL-1β, *Grifola frondosa* was able to increase the synthesis (incorporation) of PG significantly in a concentration dependant manner (Fig. 3A and 3B respectively).

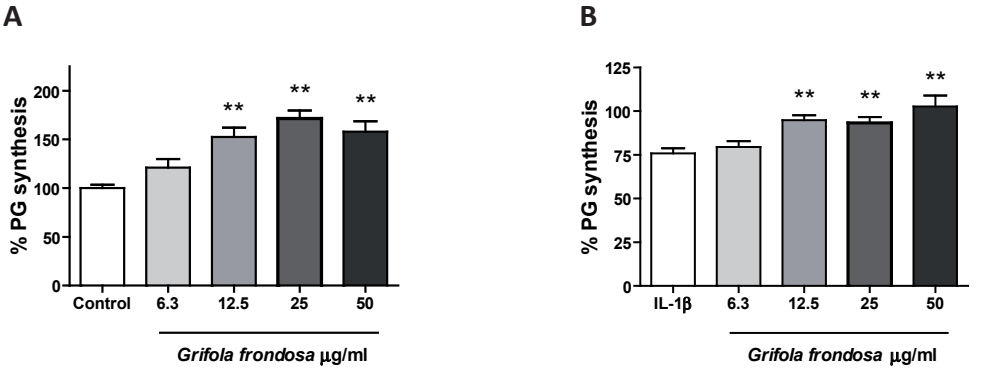

Fig. 3 Bovine chondrocytes cultured in alginate were incubated without (A) or with (B) rhIL-1β in the presence or absence of different concentrations (6.3 µg/ml, 12.5 µg/ml, 25 µg/ml, and 50 µg/ml) *Grifola frondosa* extract. GAG synthesis, detected 5 days after start of the stimulations, was depicted as mean ± SEM. Significant differences vs. the non-stimulated control (100%) are indicated with *P<0.05 and **P<0.01, n=3.

Grifola frondosa: TGF-β detection

TGF-β1, TGF-β2 and TGF-β3 levels were measured in the supernatant of bovine chondrocytes cultured in alginate. No changes were detected in the production of TGF-β1 and TGF-β3 (data not shown). RhIL-1β incubation decreased the TGF-β2 production significantly from 1085 pg/ml to 768 pg/ml (P<0.01). The TGF-β2 production in the absence of rhIL-1β was set to 100%; all values were expressed as percentage of this control. *Grifola frondosa* was able to increase the TGF-β2 production in the presence and absence of IL-1β, in a concentration dependant manner (Fig. 4A and 4B, respectively). At a concentration of 50 mg/ml, *Grifola frondosa* was able to counteract the rhIL-1β-reduced TGF-β2 production completely.

Grifola frondosa: glycosaminoglycan degradation

To investigate the effect of *Grifola frondosa* on PG degradation in the absence and presence of rhIL-1β, bovine cartilage explants were cultured for 7 days with or without rhIL-1β (10 ng/

ml), with or without different concentrations of *Grifola frondosa*. At day 7, the GAG content was detected in the culture supernatants. RhIL-1β increased the PG release from 26 µg/ml up to 59 µg/ml (P<0.05). The PG release in the absence of rhIL-1β and test agents was set to 100%. All values were expressed as percentage of this basal release. *Grifola frondosa* showed no effect on the control condition (Fig. 5A) whereas it inhibited the rhIL-1β-mediated PG degradation in a concentration dependant manner (Fig. 5B). At a concentration of 50 mg/ml, *Grifola frondosa* was able to counteract the rhIL-1β induced PG degradation completely.

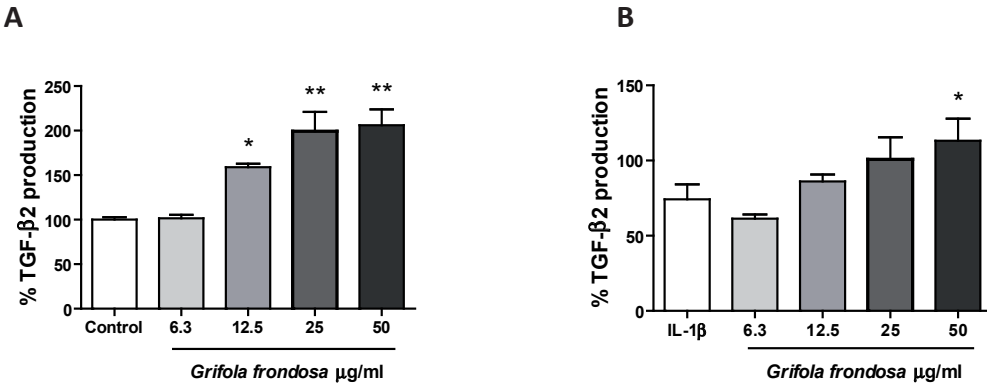

Fig. 4 Bovine chondrocytes cultured in alginate were incubated without (A) or with (B) rhIL-1β in the presence and absence of different concentrations (6.3 µg/ml, 12.5 µg/ml, 25 µg/ml, and 50 µg/ml) *Grifola frondosa* extract. TGF-β2 production, detected 5 days after start of the stimulations, is depicted as mean ± SEM. Significant differences vs. the non-stimulated control (100%) are indicated with *P<0.05 and **P<0.01, n=3.

Discussion

Natural products provide the foundation for a great number of therapeutic agents. They contribute to approximately 25% of the currently used crude drugs and another 25% is derived from chemically altered products [18]. The present study describes a functional assay in which nutraceutical extracts can be tested on cartilage-supporting properties.

In healthy cartilage there is a balance between cytokines and growth factors that maintains the tissue homeostasis [1]. The pro-inflammatory cytokine IL-1β plays a central role in OA by promoting matrix degradation and down-regulating cartilage repair [8, 9]. Counteracting the IL-1β-induced responses might offer an opportunity to modulate the misbalance in cartilage homeostasis.

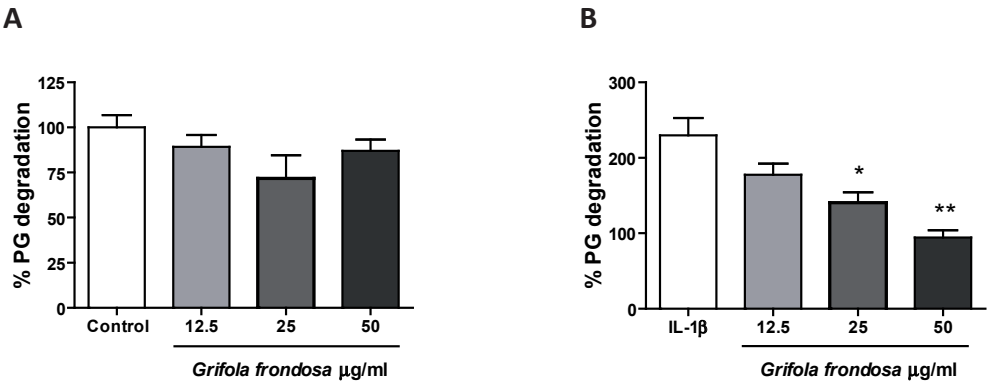

Fig. 5 Bovine cartilage explants were incubated without (A) or with (B) rhIL-1β in the presence and absence of different concentrations (12.5 µg/ml, 25 µg/ml, and 50 µg/ml) *Grifola frondosa* extract. PG degradation, detected 7 days after start of the stimulations, is depicted as mean ± SEM. Significant differences vs. the non-stimulated control (100%) are indicated with *P<0.05 and **P<0.01, n=3.

It is demonstrated that GAGs produced by chondrocytes, cultured in monolayer, are largely (80% to 90%) secreted into the culture supernatant [19, 20]. This property enables the easy detection of GAG synthesis modulation. For the detection of GAG levels in solutions, the DMB assay has extensively been used [16, 21, 22]. In the present study this assay was adapted and used for medium-throughput screening purposes. The implemented validation studies demonstrate that the assay was not influenced by DNA, protein or pH changes. The average Z-factor of 0.82 indicates that the assay is well suitable for medium-throughput screening purposes. Out of the 600 tested extracts, 22 nutraceuticals, including *Grifola frondosa*, were found to stimulate PG synthesis.

The edible fungus *Grifola frondosa* (Maitake) is known as a culinary as well as a medicinal mushroom. Maitake can be found in parts of north-eastern Japan, the northern temperate forests of Asia, Europe and eastern North America. In the past decades, Maitake cultivation has been developed mainly for food production and for use as a dietary supplement. A wide range of therapeutic effects have been described for the different extracts of the mushroom. Numerous studies indicate that a standardized beta-glucan fraction (fraction D) has prominent beneficial effects on immune function and shows anti-tumor capabilities [23-26]. Anti-diabetic and cholesterol lowering effects have been suggested of the total Maitake fraction [27-29]. Cyclooygenase inhibitory and antioxidant activities are reported of a fatty acid fraction of Maitake [30].

The present *in vivo* study shows a concentration-dependent stimulatory effect of *Grifola frondosa* extract on PG synthesis by bovine chondrocytes cultured in alginate, both in the presence and absence of rhIL-1β. This stimulation of PG synthesis was accompanied by a concentration-dependent increase in TGF-β2 production by the chondrocytes. No effects on the production of TGF- β1 and TGF- β3 were detected (data not shown). Literature indicates, in accordance with the presented results, that TGF-β is able to stimulate chondrocyte matrix synthesis [15, 31]. Moreover, during OA, chondrocytes undergo changes comparable to alterations that take place in terminal differentiation. TGF-β has been shown to inhibit terminal differentiation of chondrocytes [32]. However, different studies suggest that the resident chondrocytes in OA cartilage have acquired an abnormal phenotype that is not conductive of appropriate tissue repair. A rabbit study indicated that OA chondrocytes become progressively unresponsive to the action of TGF-β1 due to the loss of expression of the TGF-β type II receptor [31]. Moreover, a study in mice indicated a decrease in TGF-β1 receptors and in the underlying signaling molecules, resulting in a reduced ability of TGF-β to counteract IL-1 effects [33]. These latter data indicate that at least a part of the presented *Grifola frondosa* effects on cartilage might be hampered in the OA condition, by a modulated responsiveness to TGF-β.

Besides the stimulation of PG synthesis, *Grifola frondosa* inhibited the rhIL-1β-induced PG degradation in cartilage explants. This effect of *Grifola frondosa* can, at least partially, be explained by the increased TGF-β2 production by chondrocytes. Literature indicates that TGF-β not only stimulates the production of the extracellular matrix, but it also counteracts the IL-1β-induced reduction of the PG content in cartilage [34-36]. Moreover, preliminary data indicate that addition of latency associated peptide (binds to, and inactivates TGF-β) counteracts the protective effects of *Grifola frondosa* on rhIL-1β-induced PG degradation (data not shown).

In vivo data of avocado soybean unsaponifiables (ASU) on chondrocytes indicate a possible mode of action via stimulation of TGF-β production [37, 38]. Treatment with ASU (Piascledine®) has symptomatic effects on knee and hip OA and slows down joint space narrowing in severe hip OA [39-42]. These ASU results might be indicative for the potential of TGF-β stimulating components.

In conclusion, detection of GAG production by rhIL-1β-stimulated bovine chondrocytes cultures in monolayer is a quick and valid model system to test compounds on their potential cartilage protective effects. The selected *Grifola frondosa* extract was able to stimulate GAG synthesis and inhibit PG degradation by bovine chondrocytes. These cartilage protective effects indicate a possible disease-modifying osteoarthritic activity which might be

beneficial for OA patients. More research on this promising extract is necessary to explore effectiveness of *Grifola frondosa in vivo*.

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Chapter 3

In vitro and *in vivo* modulation of cartilage degradation by a standardized *Centella asiatica* fraction

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Abstract

Osteoarthritis (OA) is a degenerative joint disease in which focal cartilage destructions is one of the primary features. The present study aims to evaluate the effect of a *Centella asiatica* fraction on *in vitro* and *in vivo* cartilage degradation.

Bovine cartilage explants and bovine chondrocytes cultured in alginate were stimulated with IL-1β in the presence or absence of different concentrations (2, 5 and 10 µg/ml) of a standardized *Centella asiatica* triterpenes (CAT) fraction. The CAT fraction inhibited the IL-1β-induced proteoglycan (PG) release and nitric oxide (NO) production by cartilage explants in a dose-dependent manner. The IL-1β-induced reduction in PG synthesis and proliferaration of chondrocytes cultured in alginate was counteracted by the CAT fraction at a concentration of 10 µg/ml. In a zymosan-induced acute arthritis model the CAT fraction inhibited PG depletion without modulating joint swelling and inflammatory cell infiltration. In conclusion, the present study demonstrated for the first time that the tested *Centella asiatica* fraction was able to inhibit the zymosan-induced cartilage degradation *in vivo* without affecting the zymosan-induced inflammatory cell infiltration and joint swelling. The *in vitro* data indicate that the cartilage protective activity might, at least partially, be induced by the inhibition of NO production. The overall results indicate a possible disease modifying osteoarthritic activity of the *Centella asiatica* fraction.

Introduction

Osteoarthritis (OA) is one of the most common joint diseases and an important cause of physical disability in elderly [1]. It is characterized by degeneration of articular cartilage, limited intra-articular inflammation with synovitis, and changes in periarticular and subchondral bone [2]. OA is considered to be caused by an imbalance between anabolic and catabolic processes in the joint leading to a progressive destruction of the tissue, resulting in extensive cartilage damage [3]. The biological changes that occur in OA cartilage affect both major matrix components: proteoglycans (PG) and type II collagen.

Pro-inflammatory cytokines, such as IL-1 and TNF-α play a pivotal role in the initiation and development of OA. Cytokines originating from the inflamed synovial membranes as well as from the chondrocytes inhibit cartilage PG synthesis and promote catabolism [4, 5]. Especially important in this regard is IL-1β, a cytokine initiating a number of events leading to cartilage destruction. IL-1β promotes cartilage destruction by inducing the production of matrix metalloproteinases [6, 7] and oxygen derived free radicals including nitric oxide (NO) [8, 9], which initiate matrix degradation and causes chondrocyte apoptosis. Sabotaging the IL-1β-induced processes is thought to be protective for the cartilage and could serve as a target for the development of new disease modifying osteoarthritic drugs (DMOADs) [10].

Pharmacological management of OA is primarily focused on the relief of clinical signs and symptoms. Analgesics and non-steroidal anti-inflammatory drugs (NSAIDs) represent the mainstay of this treatment [11]. NSAIDs bring about a decrease of pain and stiffness. However, alterations of the underlying cartilage changes by the use of these traditional drugs are under debate [12]. Identification of DMOADs, which reverse slowdown or stabilize the pathologic changes in OA, is an important research object. A number of such new agents has been developed [13] and are shown to have a cartilage protective potential. These drugs are expected to provide a more long-term symptomatic relief compared to the traditional treatment.

In OA cartilage chondrocytes display phenotypic changes. These cells have been indicated to display a dedifferentiated phenotype by the production of type I and III collagen [14, 15]. *In vitro* culturing of chondrocytes can result in comparable changes including a transition to a fibroblastic cell shape [16]. *Centella asiatica* (L.) Urb. (Apiaceae) (CA) has been traditionally used for the treatment of different types of diseases in various parts of the world including Eastern Asia, China and India [17]. More recent experimental investigations showed stimulatory effects of extracts, triterpenes fractions or isolated active components of CA, on

collagen, glycosaminoglycan and fibronectin production by fibroblasts (*in vitro*). Whereas positive effects on wound healing were shown *in vivo* [18-22]. In view of the biological properties of the CA preparations in combination with the fibroblast-like phenotype of at least a part of the OA chondrocytes, the effect of a CA fraction on articular chondrocytes and arthritis was studied.

In the present study the *in vitro* effects of a standardized *Centella asiatica* triterpenes (CAT) fraction were studied on PG synthesis and degradation, NO production and chondrocyte proliferation using bovine explants or bovine chondrocytes cultured in alginate. The *in vivo* effect of the standardized CAT fraction on cartilage degradation and joint inflammation was histologically evaluated in a mouse zymosan-induced acute arthritis model.

Materials and Methods

All experimental procedures using laboratory animals were approved by an independent animal experiments committee (DEC Consult, Bilthoven, The Netherlands). The standardized *Centella asiatica* triterpenes (CAT) fraction, containing 42% asiaticoside (w/w) and 55% genins (w/w), was obtained from Cognis (Barcelona, Spain). The microbiological analysis of the CAT fraction revealed that the total count of aerobes (bacteria, yeast, and mould) was very low (< 1000 cfu/gram). No *Staphylococcus aureus, Pseudomonas aeruginosa, Candida albicans or Escherichia coli* were detectable in 1 gram of the CAT fraction.

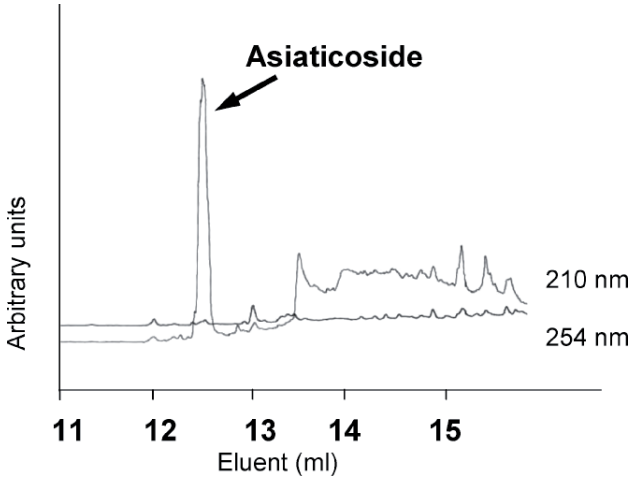

Fig. 1 HPLC fingerprints of the ethanolic extract of CAT at the wavelength of 210 and 254 nm.

Preparation of test agents

The CAT fraction was dissolved in 70% ethanol at a concentration of 150 mg/ml. The stock solution was sonicated for 10 minutes and subsequently centrifuged at 13.000 rpm in an Eppendorf centrifuge. The supernatant was further diluted in culture medium to the appropriate test concentrations. Solvent concentrations used in the various assays were included in the experiments as a negative control.

A HPLC fingerprint analysis was recorded from the supernatant of the CAT solution in 70% ethanol (150 mg/ml), processed as described above. A 50 µl aliquot of the extract was injected on a Superspher 100 RP18 column, 125x3mm (Bischoff YC17841230, Leonberg, Germany), with a precolumn 10x3mm (Bischoff YC17840130). Mobile phases were 0.01% TFA (v/v) in demiwater (A) and 0.01% TFA (v/v) in Acetonitril (B). The gradient elution program started with 2.3 column volumes (CV) A, followed by a linear gradient to 25% B in 9CV's and after that a linear gradient to 100% B in 3.5CV's. The column effluent was monitored at UV 210 and 254 nm (Fig. 1)

Bovine cartilage explant isolation

Full thickness bovine articular cartilage slices were aseptically dissected from the metacarpophalangeal joint of young bulls (1-2 years) and placed in PBS (Life Technologies, Merelbeke, Belgium). Cartilage explants were prepared using a 3 mm biopsy punch (Stiefel Laboratories, Coral Gables, Florida, USA) and transferred to 24-wells plates, each well contained 3 randomly picked explants. Explants were incubated in 1 ml culture medium (DMEM/F12 and penicillin 100 U/ml / streptomycin 100 µg/ml, Life Technologies, Merelbeke, Belgium) containing 1% heat-inactivated fetal calf serum (FCS^{hi}) at 37°C in a humidified atmosphere containing 5% CO_2 in air. After a stabilization period of 24 hours, explants were cultured for 7 days in 1 ml culture medium in the presence or absence of the CAT fraction at 2, 5 and 10 µg/ml. After one hour pre-incubation with the different concentrations of the CAT fraction, recombinant human (rh) IL-1β (Tebu-Bio, Heerhugowaard, The Netherlands) was added to a final concentration of 10 ng/ml. At day 4, the culture medium and components were refreshed. Culture supernatant obtained on day 7, was collected for PG and NO detection and stored at -20°C until analysis.

Bovine chondrocyte isolation

Bovine articular chondrocytes were isolated by collagenase digestion; cartilage slices were prepared as described above. The slices were minced and digested overnight at 37^0C in 1.5% (w/v) collagenase B (Roche Applied Science, Almere, The Netherlands), in DMEM/F12

(DMEM/F12, containing penicillin 100 U/ml / streptomycin 100 mg/µl (Life Technologies)). After removing undigested cartilage pieces by filtration through a 40 µm gauze (Becton Dickinson BV, Breda, The Netherlands), cells were washed twice with a physiological salt (PS) solution after which cell entrapment was performed. Briefly, cells were suspended in 1.2% (w/v) alginate (Keltone ® LV, ISP alginates, Sint-Niklaas, Belgium) in PS at a density of 8.10^6 cells/ml and passed drip wise through a 22-gauge needle into a 102 mM $CaCl_2$ solution. After 10 minutes of polymerization, beads were rinsed twice in PS. The beads were cultured, four beads per well in a 24 well plate, for 2 weeks in culture medium with 10% FCS^{hi} , at 37°C in a humidified atmosphere containing 5% CO_2 in air; the medium was refreshed twice weekly. After the stabilization period of 2 weeks the beads were cultured in the presence or absence of 2, 5 and 10 µg/ml of the CAT fraction. After one hour pre-incubation with the different concentrations of the CAT fraction, rhIL-1β was added to the beads. The culture medium with the different components was replaced after 3 days. Four and 5 days after start of the incubation with the CAT fraction the cell proliferation and PG synthesis assays were executed respectively.

Proteoglycan measurement

The amount of PG released by the explants into the medium was detected by a modified 1,9-dimethylmethylene blue (DMB) assay (Sigma, Zwijndrecht, The Netherlands) according to Farndale [23]. Briefly, 50 µl of 5 times in culture medium diluted culture supernatant was mixed with 100 µl DMB reagents (48 mg/l DMB, 40 mM glycine, 40 mM NaCl, 10 mM HCl, pH 3.0). Absorbance was measured at 595 nm within 5 minutes after addition of the dye. A standard curve of chondroitin 6-sulfate from shark cartilage (Sigma) in the range of 0-35 µg/ml was used for quantification.

Nitric oxide assay

NO production was determined by measurement of nitrite released in the culture supernatants of the explants using the Griess reaction [24]. Briefly, 100 µl conditioned culture medium or sodium nitrite ($NaNO_2$) standard dilution were mixed with 100 µl Griess reagent (1% sulphanilamide (Sigma), 0.1% naphtylethylenediamide dihydrochloride (Sigma), 10.2% H_3PO_4). Absorbance was measured within 15 minutes at 550 nm.

Proliferation

Proliferation of chondrocytes cultured in alginate was detected by thymidine incorporation. After the cells were stimulated with rhIL-1β and incubated with various concentrations of

the CAT fraction for 4 days, ^{3}H-Thymidine (PerkinElmer life sciences, Zaventem, Belgium) was added to the culture medium to a final concentration of 5 µCi/ml. After 16 hours the beads were rinsed 5 times in PBS and each bead was dissolved in 500 µl Lumasolve® (PerkinElmer life sciences) at 60°C, during 16 hours. 10 ml Lipoluma plus® (PerkinElmer life sciences) was added and the incorporated radioactivity was counted (LKB Wallac liquid scintillation counter, PerkinElmer life sciences).

Proteoglycan synthesis

PG synthesis by chondrocytes cultured in alginate, was detected by sulphate incorporation. After 5 days of incubation with the different concentrations of the CAT fraction in the presence of rhIL-1β, a two-hour pulse with $^{35}SO_4^{2-}$ ($NA_2{}^{35}SO_4$, 5 µCi/ml PerkinElmer life sciences) was executed. Afterwards the beads were rinsed 5 times in PBS, each bead was dissolved in 500 µl Lumasolve® during 16 hours at 60°C. 10 ml Lipoluma plus® was added to each dissolved sample and incorporated radioactivity was counted (LKB Wallac liquid scintillation counter, PerkinElmer life sciences).

Prompting zymosan-induced arthritis

Female C57Bl/6 mice (Charles River, Maastricht, The Netherlands), aged 14 weeks at the start of the experiment were acclimatized to the animal housing one week prior to the start of the experiment. All animals had free access to a standard rodent diet and tap water. CAT (0.3 mg/mouse/day) was administered daily for 11 days through gavage. Tap water (vehicle) was applied by gavage in the same volume, 200 µl, to control mice. At day 7, 6 µl zymosan (3% suspension in PBS) was injected into the joint cavity of the right knee to induce arthritis. The left knee-joint served as an internal control. The injections were performed by highly trained personal. At the training stage the intra-articular location of the injection was confirmed by the injection of dye. A “butterfly” distribution of dye in the knee joint confirms evenly intra-articular distribution. Only personal 100% successful with this procedure performed experimental intra-articular injections.

Joint inflammation was detected 24 hours after zymosan injection. At day 11, 4 days after zymosan injection, mice were bled under general anesthesia and sacrificed. The knees were processed for histological analysis.

Assessment of joint inflammation

Joint inflammation (swelling) was measured 1 day after zymosan injection by 99mTc-pertechnetate (99mTc) uptake in the knee joints [25, 26]. Briefly, 10 µCi 99mTc in 0.2 ml saline

was injected subcutaneously in the scruff of the neck. After 15 minutes the accumulation of the isotope in the knee due to increased blood flow and edema was determined by external gamma counting. The severity of inflammation was expressed as the ratio of the 99mTc uptake in the inflamed knee over its non-inflamed counterpart.

Histological processing and analysis of knee joint

Knee joints were dissected, fixed, decalcified, dehydrated and embedded in paraffin [27]. Standard frontal sections of 7 μm were prepared and semi-serial sections were stained with Safranin O and counter stained with Fast Green for cartilage PG measurements. Detection of cartilage PG depletion was carried out on the patella. The mean score of the sections of each animal was calculated. Cartilage PG depletion was visualized by a diminished staining of the matrix and quantified by Zeiss image analysis software (KS300 version 3.0, Carl Zeiss, Sliedrecht, The Netherlands) as described previously [28]. The average value of the non-injected knees was calculated and subtracted from the average of each zymosan injected knee, resulting in a change of PG content. A corresponding group of sections was stained with heamatoxylin and eosin (HE) for the detection and evaluation of inflammation. Inflammation was scored by influx of inflammatory cells in the joint cavity and synovium. A score of 0 indicated no cell influx, 1 to 4 was scored according to the degree of cell infiltration.

Statistical analysis

NO production, proliferation and PG synthesis and degradation, of explants or chondrocytes cultured in alginate were compared to the rhIL-1β-stimulated condition using one-way ANOVA. The overall significance of differences for all calculations was tested using the post hoc Dunnett's test. The joint swelling, PG content and cell infiltration of the zymosan injected, CAT supplemented animals was compared to the zymosan injected sham supplemented condition by T-Test. P- values <0.05 were considered statistically significant.

Results

The *in vitro* effect of CAT on rhIL-1β-induced NO production and PG degradation was detected on bovine cartilage explants. The *in vitro* effect of CAT on rhIL-1β-reduced proliferation and PG synthesis was detected in bovine chondrocytes cultured in alginate beads. The zymosan-induced arthritis model was used to study the *in vivo* effect of CAT on the zymosan-induced joint inflammation (99mTc-pertechnetate uptake and zymosan-induced inflammatory cell infiltration) and cartilage PG depletion.

Nitric oxide production by cartilage explants

To determine whether CAT alters the rhIL-1β-induced NO release by bovine cartilage explants, the nitrite concentration in the culture supernatant at day 7 was analyzed. RhIL-1β in the absence of test agents increased the NO production from 0.92 µM to 16.92 µM ($P<0.01$). CAT dose-dependently inhibited the NO production (Fig. 2A). At a concentration of 10 µg/ml, CAT inhibited the rhIL-1β-induced NO production significantly to 4.0 µM ($P<0.001$).

Proteoglycan degradation by cartilage explants

The effects of increasing doses of CAT on rhIL-1β-induced PG release by bovine cartilage explants was established by analising the PG concentration in the supernatant at day 7 was analyzed. RhIL-1β in the absence of test agents increased the PG release from 19 µg/ml to 56 mg/ml ($P<0.05$). The different doses of CAT, 2, 5 and 10 µg/ml, inhibited the PG release dose-dependently to 37, 29 and 22 µg/ml respectively (Fig. 2B).

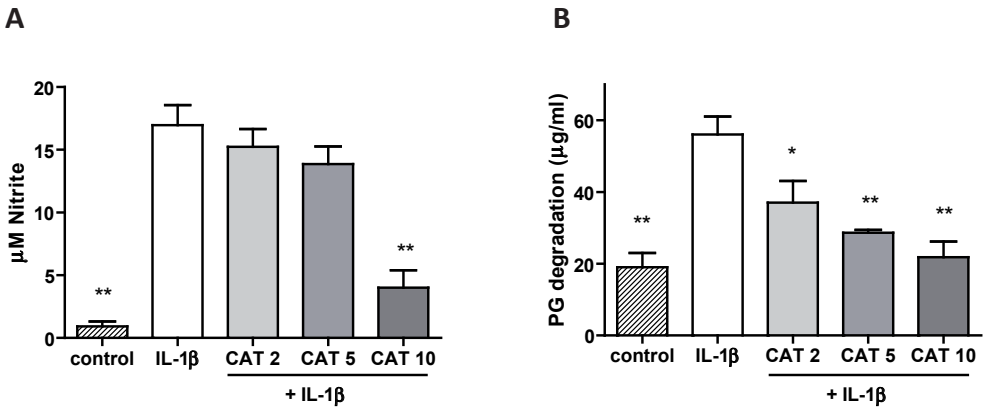

Fig. 2 Concentration dependent effects of CAT (µg/ml) on rhIL-1β (10 ng/ml)-induced NO_2 production as a measure for NO production (A) and PG release (B) by bovine cartilage explants. Values are expressed as the mean ± SD of at least three independent experiments. Significant differences to the IL-1β group were indicated by * $P<0.05$ and ** $P<0.01$.

Proliferation of chondrocytes in alginate beads

The effect of different doses of CAT on proliferation was detected by ^{3}H-Thymidine incorporation in chondrocytes cultured in alginate in the presence of rhIL-1β. The ^{3}H-Thymidine incorporation in the absence of rhIL-1β (control) was set at 100%. All values were expressed as % of the control. RhIL-1β decreased the proliferation from 100.0% to

45.1%. Only the highest concentration of the CAT fraction was able to influence the rhIL-1β-reduced proliferation significantly from 45.1% to 204.8% (P<0.01) (Fig. 3A).

Proteoglycan synthesis by chondrocytes in alginate beads

The effect of different doses of the CAT fraction on the PG synthesis by chondrocytes cultured in alginate was detected by sulphate incorporation. The sulfate incorporation in the absence of rhIL-1β (control) was set at 100%. All values were expressed as % of the control. RhIL-1β decreased the PG synthesis from 100.0% to 70.5%. Only the highest tested concentration of the CAT fraction (10 µg/ml) was able to stimulate the rhIL-1β-reduced PG synthesis significantly from 70.5% to 83.0% (P<0.05) (Fig. 3B).

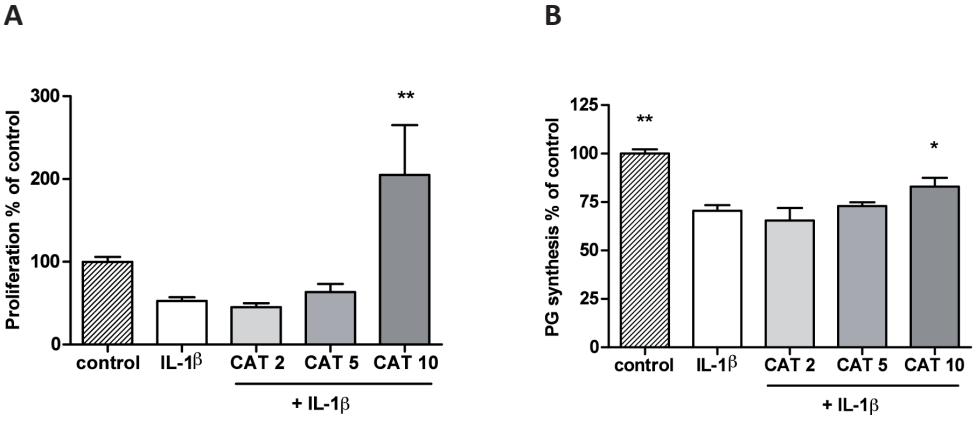

Fig. 3 Effects of CAT (µg/ml) on the proliferation (A) and PG synthesise (B) by bovine chondrocytes cultured in alginate, in the presence of rhIL-1β (10 ng/ml). Values are expressed as the mean of the % of the control ± SD of three independent experiments. Significant differences to the IL-1β group were indicated by * P<0.05 and ** P<0.01.

Joint inflammation

To investigate the effect of CAT on zymosan-induced joint swelling in mice, the CAT fraction was administered orally for 11 days. At day 7, zymosan was injected into the right knee joint. Knee swelling was measured after 24 hours by 99mTc-pertechnetate uptake. CAT 0.3 mg/mouse/day did not influence the zymosan-induced joint swelling (Fig. 4A).

Proteoglycan depletion*, in vivo*

To investigate the effect of the CAT fraction on zymosan-induced proteoglycan depletion in mice, CAT was administered orally for 11 days. At day 7, zymosan was injected into the right knee joint. At day 11 the joints were processed for histology and semi-serial sections were stained with Safranin O and counter stained with Fast Green. The average of the PG content in the patella of the non injected knee, 49.9 ± 6.1, was subtracted from the zymosan-injected sham and the CAT fraction (0.3 mg/mouse/day) treated values. No differences were indicated between the proteoglycan content in the not injected knees of the sham and the CAT treated animals (data not shown). The CAT fraction was able to inhibit the zymosan-induced decrease in PG content in a significant manner from 73.6 ± 6.03 to 51.9 ± 5.6 (Fig. 4B).

Inflammatory cell infiltration, *in vivo*

To investigate the effect of the CAT fraction on zymosan-induced inflammaotry cell infiltration in mice, The CAT fraction was administered orally for 11 days. At day 7, zymosan was injected into the right knee joint. At day 11 the joints were processed for histology and semi-serial sections were stained with HE. CAT supplementation had no effect on the zymosan-induced infiltration of inflammatory cells into the joint (Fig. 4C).

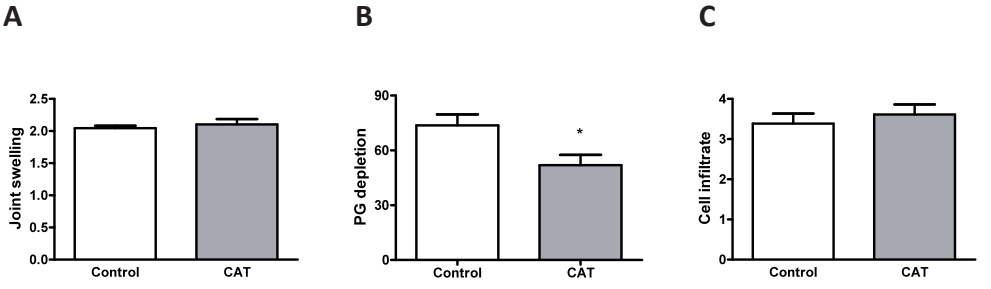

Fig. 4 Vehicle (Control) and CAT (0.3 mg/mouse/day) were orally administered for 11 days, at day 7 the right joint was injected with zymosan. Joint swelling was detected by 99mTc-pertechnetate (99mTc) uptake measurements at 24 h after the injection and was depicted as the ratio of the inflamed knee above the not injected knee (A). At day 11 the knee joints were isolated and processed for histology. PG depletion was quantified by computer analysis. The results were expressed in arbitrary units expressing the change in inversed color intensity (B). Cell infiltration was scored at a scale from 0 (no cells) to 4 (severe cell influx in joint cavity and synovium) (C). Results are shown as means ± SEM, n=6. Significant differences to the corresponding zymosan-injected control group were indicated by * $P<0.05$.

Discussion

OA is in the context of the aging western population a serious and highly significant medical problem. It has a major impact on the quality of life for a rapidly increasing number of patients. Therefore identification of new DMOADs, reversing, slowing down or stabilizing the pathologic changes is an important target in OA research.

The present study demonstrated an inhibitory effect of the *Centella asiatica* triterpenes fraction on the zymosan-induced PG degradation in mouse knee-joints (*in vivo*). *In vitro* the CAT fraction was able to inhibit the rhIL-1β-induced decrease in cartilage synthesis and cartilage degradation, indicating a cartilage protective effect. Furthermore, a strong inhibition of the rhIL-1β-induced NO production by bovine chondrocytes was demonstrated. NO production is known to be elevated in synovial fluid of OA patients [29]. Immunostaining of biopsy samples of OA patients showed a high expression of iNOS in cartilage as compared to a low or absent expression of iNOS in the synovial membrane [30]. These data suggest that the main source of iNOS-derived NO in the joint is the cartilage. The role of NO in cartilage degradation in OA is comprehensive as reviewed by Vuolteenaho et al. [31]. NO seems to be a pro-inflammatory and destructive mediator in cartilage, involved in processes leading to chondrocyte death. In addition, NO has been found to regulate various mediators and processes related to the pathogenesis of cartilage degradation. It has been reported to activate metalloproteinases and cyclo-oxygenase, to inhibit collagen and proteoglycan synthesis, and to increase susceptibility to injury by other oxidants. As a consequence of the aforementioned, it is believed that reducing the NO production will reduce the symptoms and also slow the disease progression of OA. This hypothesis is supported by *in vivo* findings in which van de Loo et al. demonstrated that in iNOS gene knock out mice the zymosan induced PG loss was markedly ameliorated compared to the wild-type mice [32]. Moreover, Pelletier and collaborators demonstrated that L-NIL, a specific inhibitor of iNOS is able to prevent cartilage degradation in a dog model of OA [9]. Therefore, the inhibition of NO, as observed in the present *in vitro* study might contribute to the cartilage protective effect of the CAT fraction *in vitro* as well as *in vivo*.

The finding that the CAT fraction did not influence the joint swelling in the zymosan-injected joint is in line with a possible activity of the CAT fraction via inhibition of NO production. Van de Loo et al. demonstrated that the joint swelling of the zymosan-injected joint of the iNOS knock out mice was equal to the wild-type mice, indicating that NO plays a minor role in oedema [32].

Inhibition of NO production by triterpenes or genins derived from *Centella asiatica* was indicated in different other studies, *in vitro* in macrophages (RAW 264.7) and *in vivo* during

gastric ulcer healing in rats [33, 34]. A study of Punturee et al. showed a condition-dependent increase or decrease of NO production by a *Centella asiatica* extract on mouse macrophages (J774.2) [35]. The present data however are the first indicating a CA-induced inhibition of the NO production by chondrocytes. These studies all show a decrease in iNOS expression or activity. Yun et al. showed in the RAW 264.7 cells an inhibition of the lipopolysaccharide (LPS)-induced NF-kappaB via suppression of IKK and MAP kinase phosphorylation by asiatic acid [34].

Literature indicates that one of the main constituent of the CAT extract, asiaticoside, by it self is able to modulate wound healing and fibroblast activity [18, 19, 21]. The *in vitro* effect of asiaticoside (Apin Chemicals Ltd, Abingdon, Oxon, England) on rhIL-1β-induced NO production and cartilage degradation by bovine cartilage explants was tested using asiaticoside concentrations comparable to the concentrations present in the CAT extract. The asiaticoside however, was not able to bring about any effect on chondrocytes (data not shown). These results indicate that another constituent or a specific combination of constituents of the extract give rise to the cartilage protective effects seen in this study.

Articular cartilage is exposed to continuous mechanical wear there is however, a surprisingly low turnover in cells and extracellular matrix [36, 37]. This low turnover could be a reason for the inability of adult cartilage to respond to injuries and subsequently repair lesions. It has been shown that there is an increased, although still very low, proliferative activity in osteoarthritic chondrocytes. Translating the *in vitro* induction of chondrocyte proliferation, as indicated in the present study, to the *in vivo* situation may lead to an increased cartilage repair in patients suffering from OA. However, more research is needed to reveal the *in vivo* effect of the CAT fraction on chondrocyte proliferation and to picture the consequences of increased cell proliferation on cartilage repair.

In conclusion, the present study demonstrated that the tested *Centella asiatica* fraction was able to inhibit the zymosan-induced cartilage degradation *in vivo* without affecting the zymosan-induced inflammatory cell infiltration and joint swelling. The *in vitro* data indicate that this cartilage protective activity might at least partially be brought about by an inhibition of the NO production. The tested *Centella asiatica* fraction pictures to be cartilage protective, indicating a possible disease modifying osteoarthritic activity which could be beneficial for OA patients.

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Chapter 4

The multicomponent phytopharmaceutical, SKI306X, inhibits *in vitro* cartilage degradation and the production of inflammatory mediators

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Abstract

Clinical studies have demonstrated that SKI306X, a purified preparation of three medicinal plants, relieves joint pain and improves functionality in osteoarthritis patients. To study the biological action of SKI306X, bovine cartilage explants and human peripheral blood mononuclear cells (PBMC) were stimulated with IL-1β and lipopolysaccharide (LPS) respectively, in the presence or absence of SKI306X and its individual composites. All tested compounds inhibited dose-dependently IL-1β-induced proteoglycan release and nitric oxide production by cartilage, indicating cartilage protective activity. SKI306X and two of its compounds inhibited PGE_2, TNF-α and IL-1β production by LPS-stimulated PBMC, indicating anti-inflammatory activity. These results demonstrate that the biological effect of SKI306X is at least bipartite: (1) cartilage protective and (2) anti-inflammatory. The observed anti-inflammatory effects may provide an explanation for the outcome of the clinical studies. Long-term clinical trails are necessary to elucidate whether the *in vitro* cartilage protective activity results in disease-modifying effects.

Introduction

Osteoarthritis (OA) is the most common joint disease and an important cause of physical disability in elderly. It is characterised by cartilage loss, bone remodelling and in the majority of cases synovial inflammation. OA is considered to be caused by an imbalance between anabolic and catabolic processes in the joint.

Pro-inflammatory cytokines, such as IL-1 and TNF-α play a pivotal role in the initiation and development of OA [1-4]. These cytokines can be produced by mononuclear cells, infiltrating the synovium during inflammation [5] but also by synoviocytes and chondrocytes [6]. IL-1 and TNF-α are able to drive the inflammation by stimulating the production of additional inflammatory mediators such as IL-6, IL-8, IL-18, PGE_2, nitric oxide (NO) and matrix metalloproteinases (MMPs) produced by cells in the joint [7]. These inflammatory mediators inhibit cartilage proteoglycan (PG) synthesis and stimulate cartilage degradation [1, 8]. Therefore, inhibition of inflammatory mediators represents an important tool in the treatment of osteoarthritis.

So far, pharmacological management in OA has mainly targeted the symptoms of the disease rather than the underlying cause. Analgesics and nonsteroidal anti-inflammatory drugs (NSAIDs) represent the mainstay of treatment. In search for a safe and effective treatment of osteoarthritis, traditionally used preparations of medicinal plants could provide new and challenging opportunities.

SKI306X (Cararthron®, SK Pharma Co. Ltd, Korea) is an extract from dried root of *Clematis mandshurica*, dried root of *Trichosanthes kirilowii*, and dried flower and stem of *Prunella vulgaris*, mixed in the weight ratio 1:2:1. The individual ingredients from SKI306X are traditionally used for treatment of inflammatory conditions including arthritis [9]. In a rabbit OA model prophylactic administration of SKI306X significantly inhibited the progression of collagenase-induced OA-like changes [10]. The same authors show *in vitro* protection of rabbit cartilage explants against IL-1-induced PG degradation and protection of a rat chondrocyte cell line against staurosporin-induced apoptosis [11]. Moreover, placebo-controlled clinical studies showed beneficial effects of SKI306X on a group of patients with classical OA of the knee [12, 13]. A significant improvement of the 100mm visual analogue scale (VAS), the Lequesne index and both the patients' and investigators' opinion was shown. SKI306X demonstrated efficacy statistically comparable to that of diclofenac. Interestingly, the SKI306X treated patients experienced less side-effects compared to the patients treated with diclofenac.

In the present *in vitro* study the biological activity of SKI306X and its individual components was evaluated. SKI306X and its individual components inhibited pro-inflammatory cytokine

production in LPS-stimulated human PBMC as well as in IL-1β-stimulated bovine cartilage explants. Incubation of bovine explants with SKI306X or its individual components also decreased IL-1β-induced PG degradation and NO production.

Materials and Methods

Preparation of test agents

The SKI306X homogenate was prepared by grinding the commercially available Cararthron® (Australia) or Joins Tab.® (Korea) tablets, after removal of the coating. The SKI306X tablets were prepared by extracting a mixture of 3 crude herbal components (dried root of *Clematis mandshurica*, dried flowers and stem of *Prunella vulgaris* and dried root of *Trichosanthes kirilowii*, 1:1:2 (w/w), respectively) in 7 volumes of 30% (v/v) ethanol-water, at 85°C for 6 hours. The extracted solution was filtered and dried under vacuum. The residue was portioned in a mixture of butanol and water. The butanol layer was separated and dried under vacuum until complete removal of the solvent. Typical active ingredients in SKI306X are oleanolic acid from *Clematis mandshurica*, rosmarinic acid and ursolic acid from *Prunella vulgaris*, and 4-hydroxybenzoic acid, 4-hydroxy-3-methoxybenzoic acid and trans-cinnamic acid from *Trichosanthes kirilowii*, which should be present in more than 4.00, 0.20, 0.50, 0.03, 0.03 and 0.05% (w/w), respectively.

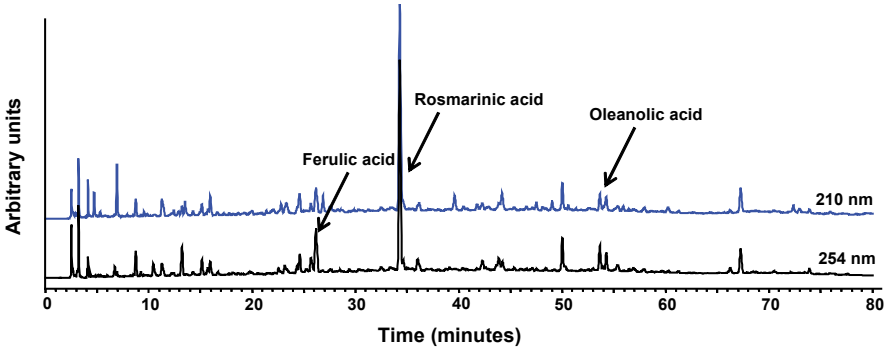

Fig. 1 HPLC fingerprints of the ethanolic extract of SKI306X tablets recorded at the wavelengths of 210 nm and 254 nm.

The individual medicinal plant extracts, *Clematis mandshurica* (*Clematis*), *Prunella vulgaris* (*Prunella*) and *Trichosanthes kirilowii* (*Trichosanthes*) a gift from SK Chemicals Co., Ltd (Suwon, Korea) were isolated according to Park et al. [9]. SKI306X and the individual ingredients

were dissolved in 70% ethanol to stock solutions of 150 mg/ml. They were sonificated for 10 minutes and subsequently centrifuged at 13.000 rpm in an Eppendorf centrifuge. The supernatants were further diluted in culture medium to the appropriate concentrations. Solvent concentrations used in the various assays were tested and did not show any effect.
A HPLC fingerprint analysis was recorded from the supernatant of the SKI306X tablet solution (150 mg/ml in 70% ethanol, processed as described above). A 10 µl aliquot of the extract was injected on an YMC J'sphere ODS-H80 (4.6 X 250 mm I.D., 5 µm) column (YMC separation technology, Kyoto, Japan). A mobile phase program was used starting with 90% 0.1% phosphoric acid and 10% MeCN. The percentage of MeCN increased till 50% after 70 minutes at a flow rate of 1.0 ml/minute (maintain 10 minutes). The column eluent was monitored at UV 210 nm and 254 nm (Fig. 1).

Bovine cartilage explant assay

Full thickness bovine articular cartilage slices were aseptically dissected from the metacarpophalangeal joint of young bulls (1-2 years) and placed in PBS (Life Technologies, Merelbeke, Belgium). Cartilage explants were made with a 3 mm biopsy punch (Stiefel Laboratories, Coral Gables, Florida, USA) and transferred to 24-wells plates, each well contained 3 randomly picked explants. Explants were incubated in 1 ml culture medium (DMEM/F12 containing 1% heat-inactivated fetal calf serum (FCS^{hi}) and penicillin 100 U/ml / streptomycin 100 µg/ml (Life Technologies)) at 37°C in a humidified atmosphere containing 5% CO_2 in air. After a stabilization period of 24 hours, explants were cultured for 7 days in 1 ml culture medium in the presence or absence of the different test agents at 30, 100 and 300 µg/ml. After one hour pre-incubation with SKI306X or the individual ingredients, recombinant human IL-1β (Tebu-Bio, Heerhugowaard, The Netherlands) was added to a final concentration of 10 ng/ml. Recombinant human transforming growth factor-β1 (TGF-β1), reported to strongly counteract IL-1 effects [14, 15], was included as a reference at a concentration of 2 ng/ml (TGF-β1, R&D Systems, Abingdon, United Kingdom). At day 4, the culture medium and components were refreshed. Lactate dehydrogenase (LDH) levels were assayed directly after collection of the culture medium at day 4 and 7. Supernatant obtained for glycosaminoglycan (GAG), NO and PGE_2 detection was stored at -80°C until analysis.

Glycosaminoglycan measurement

The amount of glycosaminoglycan (GAG) released into the medium was detected by a modified 1,9-dimethylmethylene blue (DMB) assay (Sigma, Zwijndrecht, The Netherlands)

according to Farndale [16]. Briefly, 50 µl of 5 times diluted culture supernatant was mixed with 100 µl DMB reagents (48 mg/l DMB, 40 mM glycine, 40 mM NaCl, 10 mM HCl, pH 3.0). Absorbance was measured at 595 nm within 5 minutes after addition of the dye. Quantification was performed using a standard curve of chondroitin 6-sulfate from shark cartilage (Sigma) in the range of 0-35 µg/ml.

Nitric oxide assay

NO production was determined by measurement of nitrite released in the culture supernatants of the chondrocytes using the Griess reaction [17]. Briefly, 100 µl conditioned culture medium or sodium nitrite ($NaNO_2$) standard dilution were mixed with 100 µl Griess reagent (1% sulphanilamide (Sigma), 0.1% naphtylethylenediamide dihydrochloride (Sigma), 10.2% H_3PO_4). Absorbance was measured within 15 minutes at 550 nm.

Cell toxicity assay

Quantification of cell death and cell lysis of chondrocytes in the treated cartilage explants was performed using a Cytotoxicity Detection Kit (Roche Diagnostics, Almere, The Netherlands). This colorimetric assay is based on the measurement of LDH released from the cytosol of damaged cells into the supernatant. Tissue culture supernatant (50 µl) was mixed with 50 µl LDH reagent and incubated for 30 minutes protected from light. Absorbance was measured at 490 nm.

PBMC assay

PBMC from healthy donors were obtained from buffy coats supplied by the Sanquin Bloodbank of Nijmegen and prepared by Ficoll gradient centrifugation. In short, 10 ml of Ficoll-Paque (Amersham Pharmacia Biotech) was stratified under 20 ml of peripheral blood and centrifugation was performed at 400g for 20 minutes at room temperature (RT). Recovered PBMC were washed three times with PBS (Life Technologies) containing 2% FCS^{hi}. Cells were cultured in RPMI-1640 culture medium containing 25 mM HEPES and 2 mM L-glutamine enriched with 100 U/ml penicillin/ 100 µg/ml streptomycin, 1.0 mM sodium-pyruvate and 10% FCS^{hi} and were counted using a Coulter Counter®. Cell viability was checked with trypan blue staining using a microscope. The cells were diluted to a concentration of $1x10^6$ cells/ml, in culture medium.

SKI306X and its individual ingredients were tested at different concentrations (31.3, 62.5, 125 and 250 µg/ml) in LPS-stimulated PBMC to investigate their effect on the production of pro-inflammatory cytokines and PGE_2. Concentration ranges of test agents were pipetted

into a flat bottom 96-wells culture plate, 20 µl per well after which 150 µl PBMC were added (1.10^6 cells/ml). Test agents and PBMC were pre-incubated for 1-hour at 37°C in a humidified environment containing 5% CO_2 in air. LPS (E.Coli, O55:B5, Sigma) was added (30 µl/well), to a final concentration of 10 ng/ml and the cells were subsequently incubated for another 20 hours. Supernatants were harvested and stored at -80°C until analysis.

Cytokine (IL-1β and TNF-α) determination

TNF-α and IL-1β levels were measured in supernatants of PBMC using ELISA antibody pair kits from Biosource (Cytoset, Biosource, Camarillo, California, USA). ELISAs were performed according to the manufacturer's protocol.

PGE_2 determination

PGE_2 levels were measured in supernatants of PBMC and bovine explants using the Prostaglandin E_2 enzyme-immunoassay (EIA) system. EIAs were performed on ice according to the manufacturer's protocol number 2 (Biotrak, Amersham Pharmacia Biotech).

PBMC metabolic activity

WST-1 (4-{3-(4-Iodophenyl) -2-(4-nitrophenyl)-2H-5-tetrazolio} -1,3-benzene disulfonate), (WST, Roche Diagnostics) is a substrate for the enzyme succinate dehydrogenase. The conversion product formazan can be measured at 450 nm with a reference filter at 655 nm. The activity of succinate dehydrogenase reflects mitochondrial activity and may therefore be indicative for metabolic activity.

After 20 hours incubation of the different test agents on LPS-stimulated PBMC, WST-1 was added undiluted (10 µl/well). Absorbance was measured directly after WST-1 addition and after another 5 hours incubation period of the cells.

Statistics

IL-1β-induced GAG release; NO production and PGE_2 production by bovine explants was set to 100%. Concentration-dependent effects of SKI306X and its individual ingredients were calculated and expressed as mean ± SEM. Statistical differences between the IL-1β group and the treated groups were analysed by analysis of variance (ANOVA). LPS-stimulated PGE_2, IL-1β and TNF-α production by human PBMC was set to 100%. Concentration-dependent effects of SKI306X and its individual ingredients were calculated and expressed as mean ± SEM. Statistical differences between the LPS-stimulated controls and the different dose-response effects was tested using Dunnett's T-test.

Results

Glycosaminoglycan degradation

To investigate the effect of SKI306X and its individual ingredients on IL-1β-induced PG degradation, bovine cartilage explants were cultured for 7 days with or without IL-1β (10 ng/ml) in the absence or presence of test agents. In the supernatants from day 7 the GAG content was detected. IL-1β-induced the GAG release from 13.5 µg/ml up to 80.7 µg/ml ($P<0.0001$). The IL-1β-increased GAG release in the absence of test agents was set at 100%. All values were expressed as % of induced release. TGF-β1 (2 ng/ml) decreased the IL-1β-induced GAG release to 45% ($P<0.0001$). SKI306X as well as the individual components showed a concentration dependent inhibition of the IL-1β-mediated proteoglycan degradation (Fig. 2A). At a concentration of 100 µg/ml, SKI306X, *Clematis*, *Prunella* and *Trichosanthes* inhibited the IL-1β stimulated release significantly to 50, 65, 34 and 73% respectively. At 300 µg/ml *Clematis* completely reversed the IL-1β-induced GAG release to control levels.

Nitric oxide production by cartilage explants

To determine whether SKI306X and its individual ingredients alter the IL-1β-induced NO production by bovine cartilage explants, the nitrite concentration in the culture supernatant at day 7 was analyzed. IL-1β in the absence of test agents increased the NO production 5-fold from 5.1 µM to 26.4 µM ($P<0.0001$). IL-1β-induced NO production was set at 100%. TGF-β1 inhibited the rhIL-1β-induced NO production to 55% ($p<0.0001$). SKI306X as well as its ingredients dose-dependently inhibited NO production (Fig. 2B). At a concentration of 100 µg/ml, SKI306X, *Clematis*, *Prunella* and *Trichosanthes* inhibited the IL-1β-stimulated release of NO significantly to 22, 47, 21 and 52% respectively. At 300 µg/ml, SKI306X and *Clematis* reversed the NO release even beyond the non-IL-1β-stimulated values.

PGE_2 production by cartilage explants and PBMC

PGE_2 production was determined in the supernatant of cartilage explants and cultured PBMC stimulated with IL-1β or LPS respectively. The PGE_2 concentration in the absence of test agents was set at 100%.

In cartilage explants IL-1β stimulated the PGE_2 production from 63 to 487 pg/ml. TGF-β1 did not affect IL-1β-induced PGE_2 production significantly. SKI306X, *Clematis* and *Trichosanthes* inhibited the PGE_2 production in a concentration dependent manner. At a concentration of 100 µg/ml they inhibited the IL-1β-stimulated PGE_2 production significantly to 35, 58 and 44% respectively. In contrast, *Prunella* counteracted the stimulated PGE_2 production best at 30 µg /ml, whereas 100 and 300 µg/ml showed less pronounced effects (Fig. 2C).

In human PBMC, LPS-stimulated PGE_2 production was inhibited in a concentration-dependent manner by SKI306X, *Clematis* and *Prunella* whereas *Trichosanthes* showed no significant inhibitory effect on PGE_2 production (Fig. 3A).

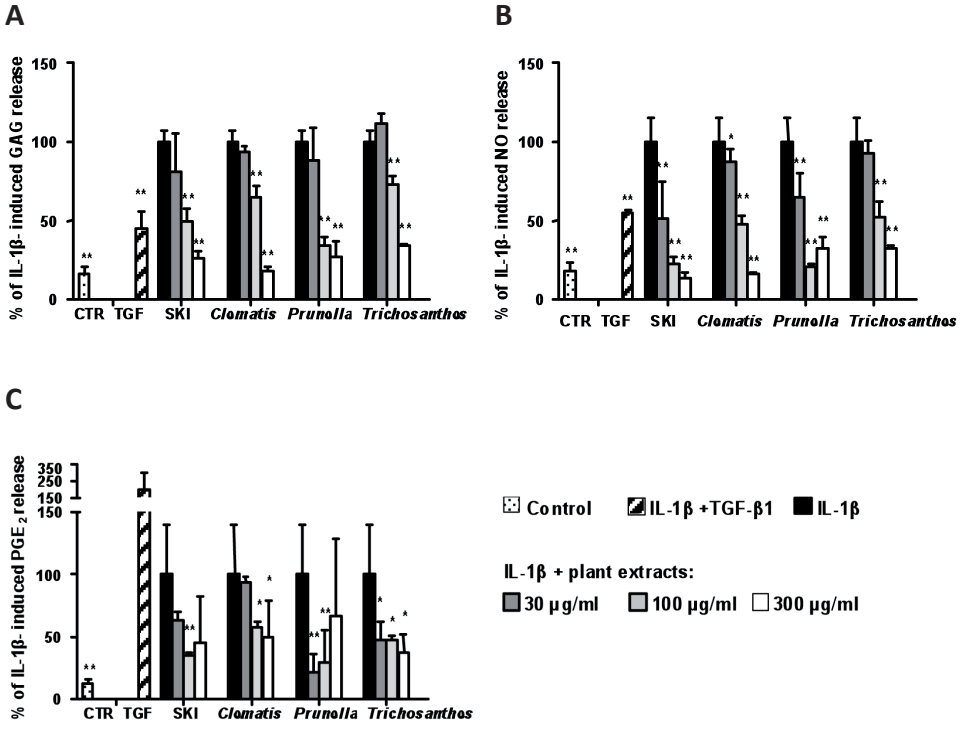

Fig.2. Concentration-dependent effects of SKI306X and its individual ingredients on IL-1β (10 ng/ml) induced GAG release (A), NO_2 production as a measure for NO production (B) and PGE_2 production (C) of bovine cartilage explants. Values are expressed as the mean ± SEM. Significant differences vs. the IL-1β-stimulated condition (100%) are indicated with * P<0.05 and ** indicates P<0.001 vs. IL-1β-stimulated control (100%).

Cytokine production by PBMC

IL-1β and TNF-α production were determined in the supernatant of PBMC cultures. The cytokine concentration in culture supernatants of LPS-stimulated PBMC in the absence of test agents was set at 100%.

SKI306X, *Clematis* and *Prunella* inhibited IL-1β production in a concentration dependent manner. *Trichosanthes* showed an increase in IL-1β production at 31 µg /ml, whereas at the higher concentrations no significant effects were observed (Fig. 3B).

The dose-response studies showed an inhibition in TNF-α production in a concentration dependent manner for all the tested agents (Fig. 3c)

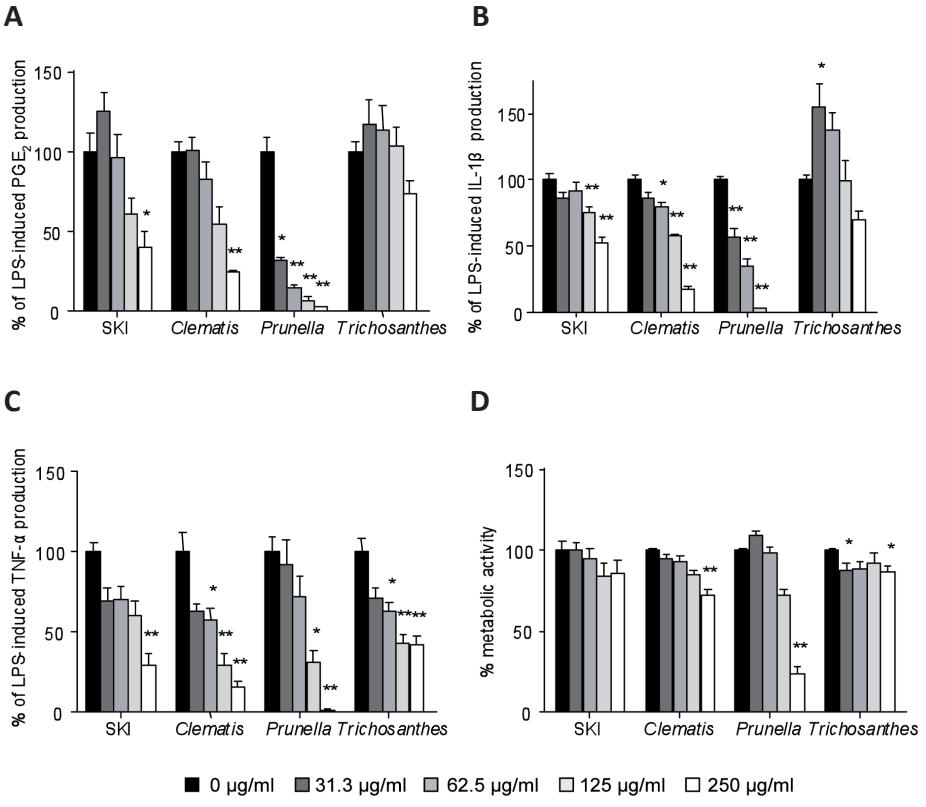

Fig.3. Concentration-dependent effects of SKI306X and its individual ingredient on LPS (10 ng/ml) induced PGE_2 (A), IL-1β (B) and TNF-α production (C) of human PBMC. D shows the concentration dependent effects of SKI306X and its individual ingredients on metabolic cell activity. Values are expressed as the mean ± SEM of three experiments. Significant differences vs. the LPS-stimulated control (100% condition) are indicated with * P<0.05and ** P<0.001.

Cartilage toxicity and PBMC metabolic activity

Cell death of the chondrocytes in cartilage explants was studied by measuring the LDH released into the culture medium at day 4 and 7 after starting the cultures. Neither TGF-β1, SKI306X, nor the three individual ingredients, showed a significant effect on LDH release (data not shown).

PBMC metabolic activity decreased in the individual herbs at the highest test concentration,

indicating a diminished metabolic activity (Fig. 3D). The absorbance of the culture medium of LPS-stimulated PBMC in the absence of test agents was set at 100%. *Prunella* showed a strong inhibition of metabolic activity at 250 µg/ml to 24%, suggesting a possible cytotoxic effect at this concentration.

Discussion

For thousands of years medicinal plants have been used to treat a wide variety of diseases. The individual ingredients of SKI306X are traditionally used for treatment of inflammatory conditions including arthritis. Clinical studies in OA patients revealed that SKI306X, besides an analgesic efficacy and a good safety profile, showed functional improvements of the affected joints [12, 13]. However, little is known about the biological effects of the components that could explain the outcome of the clinical studies.

The present *in vitro* study showed an inhibitory effect of SKI306X and its individual ingredients on IL-1β-induced cartilage degradation, indicating that SKI306X and its individual compounds protect cartilage against damage.

SKI306X and its individual ingredients exhibit a strong inhibition of IL-1β-induced NO production by bovine chondrocytes. To note, these effects are more pronounced for SKI306X and its ingredients than the results obtained with TGF-β1 (2 ng/ml). NO is known to be an important second mediator of IL-1 induced cartilage damage [18, 19]. Therefore, the inhibition of NO, as observed in the present study, might represent an important mechanism by which SKI306X exerts cartilage protective activities in experimental OA in rabbits [10]. Moreover, the inhibition of NO production has been proposed as a therapeutic strategy for treatment of OA. Pelletier and collaborators demonstrated that L-NIL, a specific inhibitor of inducible NO synthase prevents cartilage degradation in a dog model of OA [20], which could be in support of SKI306X as potential (complementary) treatment during OA.

Besides the effects on cartilage degradation and NO production, SKI306X significantly inhibited IL-1β-induced PGE_2 production of bovine cartilage explants. Catabolic effects of PGE_2 on cartilage have been described by induction of apoptosis in articular chondrocytes and the stimulation of proteoglycan degradation [21, 22]. According to these studies the inhibition of PGE_2, as observed in the present study, might represent another mechanisms by which SKI306X could exert cartilage protective activities. However literature describing PGE_2 effects on cartilage metabolism is inconclusive, indicating that the inhibition of PGE_2 could give rise to anabolic as well as catabolic effects. Protective effects of PGE_2 on cartilage described in literature include the up-regulation of type II collagen gene expression [23].

In the present study anti-inflammatory effects of SKI306X and its individual ingredients were shown. They dose-dependently counteract the LPS-induced IL-1β, TNF-α and PGE_2 production by human PBMC. *Prunella* displayed the strongest inhibitory activity (Fig. 3A, B, and C). However at the highest concentration (250 µg/ml) the result is largely influenced by the decrease in metabolic activity, suggesting a cytotoxic effect. The other components tested showed only a slight decrease in the metabolic activity at the similar concentration. SKI306X and its ingredients, as tested by LDH release, did not affect chondrocyte cell death. The inhibition of PGE_2 production by chondrocytes and PBMC might at least be partly responsible for the analgesic effect described for SKI306X by Lung et al. [13], since PGE_2 is a potent mediator of inflammation-induced hyperalgesia [24, 25].

The potential relevance of modulating IL-1 activity is described in a study showing a reduction in the progression of structural changes in experimental OA after intraarticular injection of receptor antagonists [26]. TNF-α is thought to be an important driving force of IL-1 synthesis [27]. Together with the fact that NO is an important second mediator of IL-1 and its role in cartilage destruction [28], a decreased IL-1β production may result in a reduction of cartilage degradation. Consequently, the anti-inflammatory properties of SKI306X, which include the inhibition of IL-1β production by mononuclear cells, as described in the present study, might have cartilage protective effects.

The data from the present study show that there are several mechanisms observed that could underlie the analgesic effect and/or the functional improvements of the effected joints, as observed in the clinical studies [12, 13].

The inhibitory effect of SKI306X and its individual ingredients on IL-1β-induced PG degradation, NO production and PGE_2 production of bovine cartilage explants indicate a cartilage protective effect. However, clinical trials of longer duration are required to determine whether SKI306X is able to slow down the structural disease progression as measured by joint space narrowing. SKI306X also inhibits LPS-stimulated TNF-α, IL-1β and PGE_2 production by PBMC, demonstrating an anti-inflammatory and a probable analgesic capacity.

In conclusion, SKI306X could be beneficial during pain management in OA patients; via the inhibition of PGE_2 and cytokine production. There are indications that SKI306X is able to modulate cartilage metabolism although longer clinical trails are required to demonstrate its cartilage protective activity *in vivo*.

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Chapter 5

Oral administration of the NADPH-oxidase inhibitor Apocynin partially restores diminished cartilage proteoglycan synthesis and reduces inflammation in mice

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Abstract

Apocynin, an inhibitor of NADPH-oxidase, is known to partially reverse the inflammation-mediated cartilage proteoglycan synthesis in chondrocytes. More recently, it was reported that apocynin prevents cyclooxygenase (COX)-2 expression in monocytes. The present study aimed to investigate whether these *in vitro* features of apocynin could be confirmed *in vivo*. In a mouse model of zymosan-induced acute arthritis apocynin was administered orally (0, 3.2, 16 and 80 µg/ml in the drinking water) and the effects on cartilage proteoglycan synthesis were monitored. In a mouse model of zymosan-induced inflammation of the ears apocynin was administered orally (14 mg/kg/day by gavage) and the effects on ear swelling and *ex vivo* produced prostaglandin E_2 (PGE_2) from lipopolysaccharide (LPS)-stimulated blood cells were measured. In this study, ibuprofen was used as a positive control (50 mg/kg/day by gavage) and animals received vehicle as a negative control.

Apocynin dose-dependently reversed the inhibition of proteoglycan synthesis in articular cartilage of the arthritic joint. A statistically significant increase in proteoglycan synthesis was found at a dose of 80 µg/ml Apocynin. Apocynin did not affect the proteoglycan synthesis of the control knee joints. Apocynin significantly decreased the zymosan-induced ear swelling at 1, 2 and 4 h (hours) after zymosan injection versus the vehicle treated group at 14 mg/kg/day. The *ex vivo* production of PGE_2 by LPS-stimulated blood cells was significantly decreased after *in vivo* apocynin treatment. Ibuprofen decreased ear swelling at the same time-points as apocynin and inhibited the *ex vivo* produced PGE_2.

In conclusion, the present study confirmed two important features of apocynin *in vivo*: (1) oral administration of apocynin can partially reverse the inflammation-induced inhibition of cartilage proteoglycan synthesis, and (2) oral administration of apocynin has COX inhibitory effects similar to the nonsteroidal anti-inflammatory drug (NSAID) ibuprofen. Therefore,

apocynin might be of potential use during the treatment of chronic inflammatory joint diseases like osteoarthritis or rheumatoid arthritis.

Introduction

Apocynin (4-hydroxy-3-methoxy-acetophenone) is a constituent of the Himalayan herb *Picrorhiza kurrooa* Royle (Scrophulariaceae) that is well known in traditional Indian medicine (Ayurveda). It is an acetophenone (Fig. 1) to which a range of biological activities is attributed. The *in vitro* anti-inflammatory effects of apocynin include the reduction of neutrophil oxidative burst and neutrophil-mediated oxidative damage [1, 2], the decreased adhesion of the monocytic cell line U937 to tumor necrosis factor alpha (TNF-α)-treated human umbilical vein endothelial cells [3], a reduction of polymorphonuclear granulocyte chemotaxis [4], the inhibition of peroxynitrite [5] and the inhibition of inflammation-mediated cartilage destruction [6]. More recently, apocynin was shown to prevent cyclooxygenase (COX)-2 expression in human monocytes [7].

The underlying mechanism of action for these biological effects of apocynin involve the inhibition of the superoxide ($O_2^{\cdot-}$) generating enzyme NADPH-oxidase [1, 8]. However, the recent finding that apocynin is capable of preventing COX-2 expression might provide an additional explanation for the anti-inflammatory effects of apocynin that have been observed *in vivo*. These *in vivo* effects of apocynin include a reduction of arthritis incidence [9] and a decreased joint-swelling in collagen-induced arthritis in mice [2, 10] and the reduction of ulcerative skin lesions in inflamed skin [11] in rats. Apocynin also prevented airway hyperresponsiveness during allergic reactions in mice [12] and reduced airway hyperreactivity to metacholine when inhaled by humans suffering from mild atopic asthma [13].

The COX-2 enzyme and its major metabolite prostaglandin E_2 (PGE_2) play an important role during inflammatory diseases. For example, in osteoarthritis and rheumatoid arthritis COX inhibitors such as non steroidal anti-inflammatory drugs (NSAIDs) are used to treat joint swelling and pain [14-17]. Osteoarthritis and rheumatoid arthritis are both chronic inflammatory joint diseases, and share the characteristics of an inflamed synovium during certain stages of the disease. Infiltrated mononuclear cells are present in the synovium [18], and produce inflammatory mediators. In chondrocytes, these inflammatory mediators diminish the cartilage proteoglycan synthesis and enhance cartilage matrix breakdown by inducing matrix metallo proteases (MMPs) [19].

The enzyme inducible nitric oxide synthase (iNOS) and its product nitric oxide (NO) are also involved in inflammatory diseases, such as osteoarthritis and rheumatoid arthritis. They

have been implicated in the inflammation-mediated reduction of cartilage proteoglycan synthesis [20, 21]. This is illustrated by the observation that if NO production is blocked, the proteoglycan synthesis is restored, at least partially [22]. However, NO reacts with superoxide to form peroxynitrite at a rate which is diffusion-limited [23, 24]. Interestingly, Oh et al. [25] showed that the concurrent generation of NO and superoxide inhibited (bovine) proteoglycan synthesis and suggested that peroxynitrite is a candidate for this mechanism, rather than NO itself. The formation of peroxynitrite can be inhibited by reducing the amount of NO or superoxide and it has been shown that apocynin is capable of inhibiting peroxynitrite in murine macrophages [5]. By inhibiting the formation of superoxide with apocynin, and hence reducing the amount of peroxynitrite, the inflammation-mediated reduction of proteoglycan synthesis might be restored. *In vitro* this has been demonstrated in human cartilage [6].

In this present study, it is hypothesized that apocynin when administered *in vivo* by the oral route, results in improved cartilage proteoglycan synthesis in mice suffering from zymosan-induced acute arthritis. In addition, the *in vivo* anti-inflammatory capacity of apocynin was investigated in a mouse model for zymosan-induced inflammation of the ear. Finally, the production of PGE_2 in *ex vivo* lipopolysaccharide (LPS)-stimulated whole blood was evaluated. Ibuprofen, a well-known frequently used NSAID for patients suffering from arthritic pain, was used as a positive control.

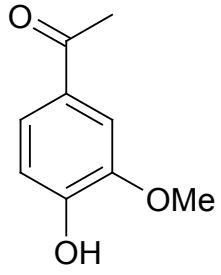

Fig. 1 Structural formula of apocynin.

Materials and Methods

Approval for the animal study protocols was obtained from the animal ethics committee before the start of the studies.

Zymosan-induced acute arthritis in mice

Female C57Bl/6 mice (14 weeks old, Charles River, Maastricht, The Netherlands) were acclimatized to the animal housing building two weeks prior to the start of the experiment and were randomly assigned to a control group or to test groups (n = 7 in the first experiment and n = 6 in the second experiment). Animals were fed standard rodent diet and drinking water, supplemented with apocynin (test groups) or vehicle (control group), was available ad lib. Apocynin (Sigma-Aldrich, Zwijndrecht, The Netherlands) was dissolved in ethanol (absolute, Merck, Rhoden, The Netherlands) and diluted in the drinking water (2.10^3 times). The concentration apocynin in the drinking water was 0, 3.2, 16 or 80 µg/ml and the concentration of ethanol (vehicle) in the drinking water was 0.05% in all groups. Drinking water with apocynin or vehicle was refreshed daily and protected from light by wrapping the drinking water container with aluminum-foil. The daily drinking water consumption was measured by weighing the drinking water containers. At day 5, 6 µl 30 mg/ml zymosan (Sigma-Aldrich) in phosphate buffered saline (PBS) was injected intra-articularly into the right knee-joint. The left knee-joint served as an internal control. At day 7 the mice were sacrificed (2 days after zymosan injection).

$^{35}SO_4$ incorporation as a measure for cartilage proteoglycan synthesis

The patellae with a standard amount of surrounding tissue were removed and incubated for 2 h in RPMI-1640 (Invitrogen, Merelbeke, Belgium) containing 30 µCi/ml $^{35}SO_4$ ($Na^{35}SO_4$, PerkinElmer, Boston, MA, USA). To remove the non-incorporated $^{35}SO_4$, the patellae were rinsed three times with 0.9% NaCl. After overnight fixation in 10% formaldehyde followed by 4 h decalcification in 5% formic acid, the patellae were removed from their tendons and incubated overnight at 60°C with 0.5 ml tissue solubilizer (Lumasolve®, Lumac-LSC, Groningen, The Netherlands). Subsequently, scintillation fluid (Lipoluma®, Lumac-LSC) was added and the $^{35}SO_4$ content of the patellae, which is a reliable measure of cartilage proteoglycan production [26], was measured by liquid scintillation analysis.

Zymosan-induced ear inflammation in mice

Male BALB/c mice (14 weeks old, Charles River, Maastricht, The Netherlands) were acclimatized to the animal housing building two weeks prior to the start of the experiment

and were randomly assigned to a control group or to test groups (n = 6). Animals received standard rodent diet and tap water ad lib. Apocynin was administered through oral gavage in a homogeneous suspension in tap water at a concentration of 14 mg/kg/day. This concentration apocynin was based on the highest concentration apocynin that was given in the drinking water in the acute arthritis study, in which the animals ingested on average 0.35 mg apocynin per day. Ibuprofen was used as a positive control for inhibition of ear swelling and *ex vivo* PGE_2 production in LPS-stimulated whole blood cells. Ibuprofen in a homogeneous suspension in tap water was administered through oral gavage at a concentration of 50 mg/kg/day. Tap water (vehicle) was given through oral gavage in the same volumes to the control mice. Daily supplementation started at day 1 and continued until the animals were sacrificed (day 7). At day 6, basal ear thickness was measured using a micrometer (Mitutoyo Digimatic, Veenendaal, The Netherlands). Subsequently, 25 µl zymosan (1% in PBS) was injected subcutaneously into both ears in the ear pinnea to induce an acute inflammatory reaction resulting in ear swelling [27]. Ear thickness was measured 1, 2, 4 and 24 h after zymosan injection to monitor the ear swelling. Ear thickness of each ear was measured in duplicate . The mice were anesthetized under isoflurane N_2O/O_2 during ear thickness measurements and zymosan injection. After the last ear thickness measurement the mice were bled under isoflurane and N_2O/O_2. Blood was collected into heparin collection tubes (85 IU heparin, Becton Dickinson, Alphen a/d Rijn, The Netherlands), after which the animals were sacrificed.

***Ex vivo* LPS-stimulated whole blood assay**

Heparinized murine blood was pipetted into a 96-well plate in a volume of 100 µl per well. Another volume of 100 µl LPS in RPMI-1640 (1 µg/ml) was added to the wells and incubated for 20 h at 37°C under 5% CO_2 in a humidified environment. Plates were subsequently centrifuged and supernatants were harvested and stored at -80°C until PGE_2 analysis.

PGE_2 measurement

PGE_2 was measured in the thawed supernatants using a commercial anti-PGE_2 rabbit polyclonal antibody-based direct enzyme immunoassay (Oxford Biomedical Research, Oxford, MI, USA) according to the manufacturer's protocol.

Statistical and data analysis

The percentages of the $^{35}SO_4$ incorporation in patellae from the zymosan-injected knees and from the non-injected control knees were averaged per group (total n=13 per group) from

two independent experiments.

Dunnett's T-test was performed to investigate whether there was a statistical significant difference between the groups which received different concentrations of apocynin in the drinking water compared to the control group (no apocynin in the drinking water).

Basal ear thickness was subtracted from the ear thickness at the different time-points after zymosan injection hence representing ear swelling. At each time-point, it was investigated whether apocynin treatment or ibuprofen treatment affected the zymosan-induced ear swelling compared to vehicle treated animals using one-way ANOVA and post-hoc the Dunnett's T-test.

Based upon literature reporting inhibitory activity of apocynin on COX-2 [7] it was hypothesized that *in vivo* apocynin treatment might decrease the PGE_2 production in an *ex vivo* LPS-stimulated whole blood assay. Ibuprofen served as a positive control. To test whether apocynin or ibuprofen significantly decreased *ex vivo* produced PGE_2 the least significant different multiple comparison test (one-tailed) was used. Data were considered statistically significant when $P < 0.05$.

Results

Apocynin administration via drinking water partially prevents a decrease in proteoglycan synthesis during zymosan-induced arthritis in mice

Two days after i.a. zymosan injection the proteoglycan synthesis of the zymosan-treated knee joints was severely decreased. This is reflected in lowered $^{35}SO_4$ incorporation into articular cartilage of patellae from arthritic knee joints compared to the $^{35}SO_4$ incorporation from control knee joints. The percentage of the patellar proteoglycan synthesis of arthritic versus control knee joint is shown in Fig. 2A. Increasing concentrations of apocynin in the drinking water gradually increased the percentage of proteoglycan synthesis of arthritic mice from 32% at 0 μg/ml apocynin to 37% at 3.2 μg/ml apocynin, to 40% at 16 μg/ml and further increased to 45 % at 80 μg/ml (Fig. 2A). This dose-dependent enhancement of proteoglycan synthesis is a steady trend towards the statistically significant enhancement of proteoglycan synthesis observed at 80 μg/ml apocynin in the drinking water. The effect of apocynin on proteoglycan synthesis in normal healthy joints is shown in Fig. 2B. Apocynin did not affect cartilage proteoglycan synthesis in control knee joints that were not injected with zymosan, indicating that the positive effect on proteoglycan synthesis of apocynin occurs specifically in the arthritic knee joint.

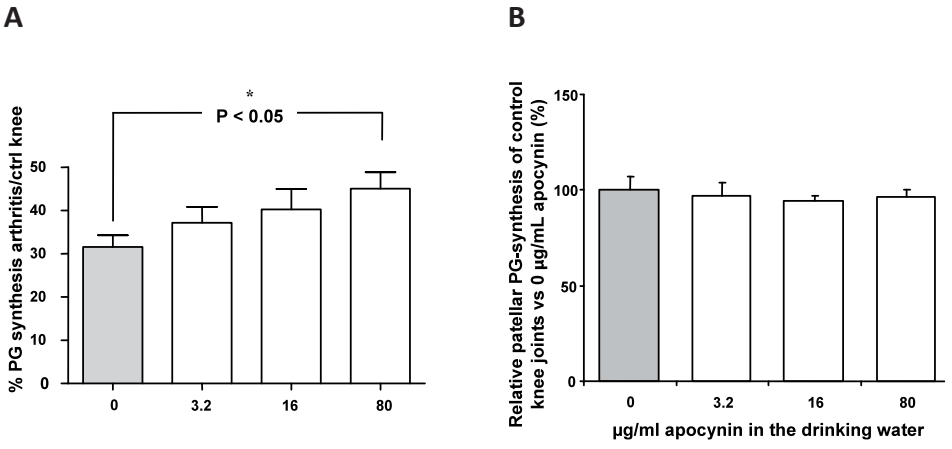

Fig. 2 (A) Patellar proteoglycan synthesis of mice treated with increasing concentrations of apocynin in the drinking water. Bars represent the average of the percentage between the $35SO_4$ incorporation of the patellae from zymosan-injected knees and from the non-injected control knees ± SEM The average proteoglycan synthesis of the control knee of the mice receiving no apocynin (vehicle) is set to 100% and all individual $35SO_4$ incorporation values are expressed as a percentage from the 100% value. Data consist of two experiments (n = 13 total). There is a statistically significant effect of oral administration of 80 µg/ml apocynin versus vehicle-treated animals indicated with *. (B) Relative patellar proteoglycan synthesis from control knee joints of mice treated with increasing concentrations of apocynin in the drinking water. The average proteoglycan synthesis of the mice receiving no apocynin (vehicle) is set to 100% and all individual $35SO_4$ incorporation values are expressed as a percentage from the 100% value. Bars represent the average $35SO_4$ incorporation of the patellae from the non-injected control knees ± SEM. Data consist of two experiments (n = 13 total). There is no effect of apocynin on basal PG-synthesis.

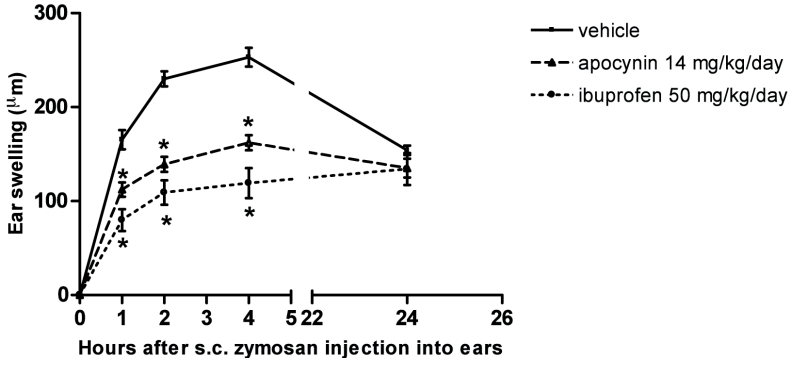

Fig. 3 Ear swelling after s.c. injection of zymosan in the ear pinnea. Data points represent average increases in ear thickness ± SEM (n = 6). Statistical significant differences of either apocynin or ibuprofen treatment versus vehicle treatment are indicated with * (P <0.01).

Oral administration reduced zymosan-induced ear swelling and ex vivo LPS-stimulated PGE_2 production of murine blood cells

Oral administration of apocynin reduced zymosan-induced inflammation at 1, 2 and 4 h after zymosan injection into the ear pinnea when compared to the vehicle treated group. As a positive control ibuprofen was used which reduced the ear swelling at the same time-points compared to apocynin (Fig. 3).

After the last measurement of ear thickness these mice were bled and blood cells were stimulated with LPS *ex vivo*. Blood cells from both apocynin-treated mice as well as from ibuprofen-treated mice produced significantly less PGE_2 compared to the vehicle group. The average amount of PGE_2 produced by the blood cells was 25.7 ng/ml for the apocynin-treated mice, 21.9 ng/ml for the ibuprofen-treated mice and 35.0 ng/ml for the vehicle-treated mice. This is a reduction of more than 25% by apocynin and more than 35% by ibuprofen, and indicates that both apocynin and ibuprofen are capable of reducing the production of PGE_2 systemically (Fig. 4).

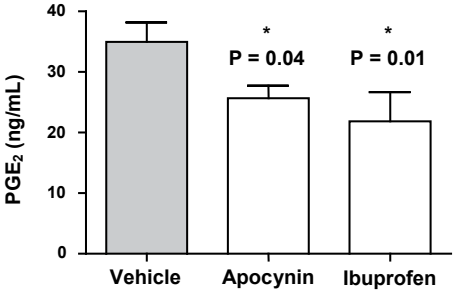

Fig. 4 Average PGE_2 production ± SEM (n = 6) by *ex vivo* LPS-stimulated murine whole blood cells of apocynin-treated mice (14 mg/kg bodyweight/day) versus vehicle-treated mice. Ibuprofen (50 mg/kg bodyweight/day) was used as a positive control. Statistical significance of the effects of apocynin and ibuprofen versus the vehicle-treated group is indicated with *.

Discussion

This present study demonstrates that oral administration of apocynin partially reversed the inhibition of articular cartilage proteoglycan synthesis in a zymosan-induced acute arthritis model in mice. In addition, the anti-inflammatory capacity of oral administration of apocynin is shown using the zymosan-induced inflammation model in mice by a significantly reduced

ear swelling compared to vehicle control. Apocynin also inhibited the *ex vivo* release of PGE_2 after LPS stimulation of whole blood. Ibuprofen, as positive control, reduced ear swelling and PGE_2 production as expected.

The results presented in the present study can be explained by the proposed pharmacological actions of apocynin that have been previously demonstrated by *in vitro* studies. Apocynin has been reported to inhibit the superoxide generating enzyme NADPH-oxidase [1, 8] that is present in chondrocytes [28, 29]. Peroxynitrite is the highly reactive coupling product of superoxide and nitric oxide and has been suggested to play a role in the inflammation-mediated inhibition of cartilage proteoglycan synthesis [25]. By inhibiting either NO or superoxide production the amount of concurrently generated peroxynitrite would be decreased. Hence this would result in prevention of reduced cartilage proteoglycan synthesis under inflammatory conditions. Improved proteoglycan synthesis by NO inhibitors has been shown *in vitro* and *in vivo* in the presence of the cartilage destructive agent interleukin-1 [30, 31]. Also, improved proteoglycan synthesis has been observed using the superoxide inhibitor apocynin *in vitro* [6]. In the present study apocynin was administered *in vivo* orally and was able to partially prevent zymosan-induced inhibition of cartilage proteoglycan synthesis in the mouse patella, which corroborates the findings of the above-mentioned *in vitro* study. Interestingly, it has been reported that peroxynitrite can activate COX-1 and COX-2 in the mouse macrophage cell line RAW264.7 [32]. Therefore, the inhibition of peroxynitrite by apocynin [5] might provide potential NSAID-like activity.

Moreover, another recent study showed that apocynin prevented COX-2 expression in stimulated human monocytes [7]. The mechanism of action involved the inhibition of the NADPH-oxidase-dependent superoxide production, the reduction of the intracellular GSH/GSSG ratio and prevention of the activation of the nuclear transcription factor NF-KB, which is an important mediator of inflammation [33, 34]. By inhibiting the activation of NF-KB, the production of joint destructive inflammatory mediators may be reduced as well. Besides the supposed inhibition of peroxynitrite and hence the reduction of proteoglycan synthesis inhibition by apocynin, the reduction of inflammatory mediators under the control of NF-KB may provide an additional explanation for the diminished inhibition of the cartilage proteoglycan synthesis as observed in this study.

Next, the present study hypothesized the COX-inhibitory effect of apocynin when given orally in a mouse model of zymosan-induced inflammation of the ear. This present study confirmed the anti-inflammatory effect of apocynin *in vivo* since both ear swelling and the *ex vivo* PGE_2 production were inhibited by apocynin (Fig. 3 and Fig. 4). The NSAID ibuprofen served as a positive control and inhibited these inflammatory parameters as expected. The

results from the *in vivo* studies described here are in accordance with the earlier reported *in vitro* findings of apocynin and strengthen the support for the potential use of apocynin especially in inflammatory disorders such as osteoarthritis or rheumatoid arthritis.

However, with respect to cartilage metabolism, not only cartilage proteoglycan synthesis is important. The total amount of cartilage that is present in the arthritic joint is the result of a balance between cartilage synthesis and cartilage degradation. A previous *in vitro* study of apocynin on cartilage proteoglycan degradation revealed a cartilage protective effect of apocynin at a concentration of 30 μg/ml [6]. At present, no data is available regarding the *in vivo* effects of apocynin on total cartilage content. When blocking the formation of peroxynitrite by inhibiting the generation of superoxide from NADPH-oxidase with apocynin, the amount of NO radicals, that normally would react with superoxide to peroxynitrite, might be elevated. Elevated NO levels may represent a potential threat by inducing tissue damage. Consequently, further research should aim at clarifying the effects of unilaterally inhibiting the route to peroxynitrite formation in chondrocytes. In addition, the effects of apocynin (preferably at dosages around 14 mg/kg apocynin per day) on total cartilage content *in vivo* remain to be investigated in animal models of arthritis.

In conclusion, the present study confirmed two important features of apocynin *in vivo*; (1) oral administration of apocynin can partially reverse zymosan-induced inhibition of cartilage proteoglycan synthesis, and (2) oral administration of apocynin has COX inhibitory effects similar to the NSAID ibuprofen. Therefore, apocynin is an interesting candidate for the development of anti-inflammatory agents for treatment of chronic inflammatory joint diseases such as osteoarthritis and rheumatoid arthritis.

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Chapter 6

Anti-inflammatory effects of orally ingested lactoferrin and glycine in different zymosan-induced inflammation models: evidence for synergistic activity

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Abstract

There is a growing awareness of the interaction of food constituents with the immune system. The present study aims to evaluate the anti-inflammatory effects of two of these nutritional components, i.e. glycine and bovine-lactoferrin (b-LF) using two different mouse models.

In a zymosan-induced ear-skin inflammation model both components decreased the inflammatory response locally (ear swelling and inflammatory cytokine concentration in the ears) as well as systemically (number of TNF-α producing spleen cells). Glycine effects (20, 50 or 100 mg/mouse/day) were concentration dependent. B-LF (0.1 or 1 mg/mouse/day) inhibited the inflammatory response although higher doses (5 and 25 mg/mouse/day) were not effective. A combination of b-LF 0.1 mg/mouse/day and glycine 20 or 50 mg/mouse/day counteracted the zymosan-induced ear swelling synergistically and enhanced the decrease in the number of TNF-α producing spleen cells of the individual components.

In a zymosan-induced acute arthritis model glycine (50 mg/mouse/day) inhibited joint swelling, inflammatory cell infiltration and cartilage proteoglycan depletion. A b-LF dose of 5 mg/mouse/day, reduced the zymosan-induced joint swelling, without modulating inflammatory cell infiltration and cartilage proteoglycan depletion.

The present study indicates that the anti-inflammatory effects of glycine are independent of the used models. B-LF displays a reversed concentration dependency and the activity is model dependent. A combination of glycine and lactoferrin demonstrated a synergistic anti-inflammatory effect on zymosan-induced skin inflammation and an enhanced decrease in the number of TNF-α producing spleen cells, compared to the effect of the single components. Therefore, this nutritional concept might be a new option for the treatment of chronic inflammatory diseases.

Introduction

There is a growing awareness of the interaction of food constituents with the immune system [1]. The present study aims to evaluate the anti-inflammatory effect of two of these nutritional components, i.e. the amino acid glycine and the iron binding protein lactoferrin [2, 3].

The simple nonessential amino acid glycine is an inhibitory neurotransmitter in the central nervous system that acts via a glycine-gated chloride channel (GlyR) [4]. Apart from the nervous tissue, glycine has been presumed to be biological neutral for a long time. In the past years however, evidence accumulated indicating that glycine comprises anti-inflammatory and immunomodulatory activities, at least in part, via activation of the GlyR. The existence of a GlyR has been demonstrated on a wide variety of cells including different cell types involved in immune responses, such as macrophages, monocytes, neutrophils and T lymphocytes [3, 5, 6]. Activation of the GlyR blunts calcium ion influxes in these cells via a chloride-induced hyperpolarization of the membrane [7]. Counteracting the calcium ion influxes, which could be induced by many different stimuli, sabotages various downstream events including the production of cytokines and other inflammatory mediators. It has been demonstrated that glycine largely prevents the endotoxin-induced TNF-α production by Kupffer cells and alveolar macrophages [5, 8]. In addition, glycine reduces the LPS-induced TNF-α and IL-1β expression while it stimulates the IL-10 expression in monocytes [9].

Lactoferrin (LF) is a widespread iron-binding protein and member of the transferrin family. It is produced by exocrine glands and might be released by degranulating neutrophils at the side of infection and inflammation. Iron binding is, without any doubt, a key property of LF that accounts for many of its biological roles in host defense such as bacteriostasis and protection against oxygen radicals catalyzed by free iron. Other direct effects of LF on host defense include the binding to bacteria, fungi and parasites. In addition, it has been demonstrated that LF plays a role in modulating immune responses by its ability to interact with target molecules and cells. Anti-inflammatory effects of LF have been shown by the inhibition of the pro-inflammatory cytokine production [10-12] and the up-regulation of anti-inflammatory cytokines [13]. On the other hand, LF may enhance directly or indirectly the immune response (*in vitro* and *in vivo*) by regulating the proliferation, differentiation and activation of both T and B cells [14, 15].

Both glycine as well as LF can modulate innate immune reactions which might offer a new opportunity for the treatment of chronic inflammatory diseases. The present study was designed to evaluate the anti-inflammatory properties in more detail. The objective was bipartite. 1) Literature describes an immune stimulatory as well as an immune inhibitory

potential for LF. The first goal of the study was to evaluate the immunomodulatory activities of orally ingested glycine and b-LF in different models for inflammation. The models used are the zymosan-induced ear-skin inflammation model and the zymosan–induced acute arthritis model. 2) The interaction between different pharmaceutical immunomodulatory medications has been studied extensively. However, the possibility that different food components might boost or counteract their individual effect on the immune system is rarely examined. The second part of the study addresses the question whether the immunomodulatory effects of glycine and LF interact with each other. For this latter goal the ear-skin inflammation model was used. The results indicated that both glycine as well as b-LF were able to significantly inhibit inflammatory responses. Furthermore, the combination of the two nutritional components showed a synergistic anti-inflammatory effect which opens new avenues for the treatment of chronic inflammatory diseases.

Materials and methods

All experimental procedures using laboratory animals were approved by an independent animal experiments committee (DEC Consult, Bilthoven, The Netherlands). The b-LF, with an iron saturation of 16%, was obtained from DMV (DMV International, Veghel, The Netherlands).

Induction of ear-skin inflammation

Male Balb/C mice (Charles River, Maastricht, The Netherlands), aged 14 weeks at the start of the experiment were acclimatized to the animal housing starting one-week prior to the start of the experiment. All animals had free access to a standard rodent diet and tap water. Different amounts of glycine (0, 20, 50 or 100 mg/day/mouse), b-LF (0, 0.1, 1, 5 or 25 mg/day/mouse), or a combination of glycine and b-LF (0.1 mg b-LF combined with 20 or 50 mg glycine/mouse/day) were administered daily for three constitutive days (day 1 till 3) by gavage. Tap water (vehicle) was applied in the same volume, 200 µl, to control mice. Inflammation was induced at day two, three hours after administration of the supplements, by injecting 25 µl zymosan (0.5% suspended in PBS) or PBS (sham), intradermally in both ears [16, 17]. Ear thickness was measured prior to and 3, 6 and 24 hours after zymosan injection using an engineers micrometer (Mitutoyo Dinamic, Veenendaal, The Netherlands). After the last ear thickness measurement, mice were bled under terminal anesthesia (isoflurane/N_2O/O_2) and sacrificed. Both ears and the spleen were collected for histology, inflammatory cytokine detection and spleen cell isolation, respectively.

Histological processing and analysis of ears

Ears were dissected, fixed in 4% formaldehyde in PBS for 16 hours at 4°C, dehydrated and embedded in paraffin. Semi-serial cross sections of 7 µm were prepared and stained with heamatoxylin and eosin (HE). From different sections, originating from the middle of the swollen region, the inflamed area was measured by computer analysis (Leica QWin Image Analysis System; Leica Microsystems BV, Rijswijk, the Netherlands), the mean score was calculated.

Cytokine detection in the ears

Ears were dissected and stored at -80°C until homogenization. Frozen ears were pulverized in liquid nitrogen and dissolved in 250 µl lysis buffer (1% Triton X-100, Sigma, Zwijndrecht, The Netherlands) and enzyme inhibitor-mix (Roche Diagnostics, Almere, The Netherlands) in PBS. The homogenates were centrifuged at 13500 rpm in an Eppendorf centrifuge (Eppendorf, 5810-R, VWR, Roden, The Netherlands). The supernatant was collected and stored at -80°C until cytokine analysis. Cytokine detection was performed using a commercial Multiplex Bead Immunoassay (Biosource, Etten-Leur, The Netherlands), including TNF-α, IL-1β and IL-6, according to the manufacturers protocol. Cytokine levels were detected using the Bio-Plex system (Bio-Rad, Veenendaal, The Netherlands). Results were calculated using Bio-Plex Manager Software 3.1 (Bio-Rad).

Serum isolation

Blood was collected in Eppendorf tubes and incubated for 1 hour at room temperature followed by 1 hour at 4°C. The serum was collected after centrifugation (5 minutes at 6000 rpm in an Eppendorf centrifuge), and stored on ice until dilution into the ELISPOT culture medium (ELISPOT, TNF-α Mouse, R&D Systems, Abingdon, United Kingdom).

Spleen cell isolation

Splenocytes were isolated by pushing the spleens through a cell strainer (40 µm nylon, BD Falcon, Erembodegem Aalst, Belgium). Cells were flushed out with 5 ml of cold lysisbuffer (0.16 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA dihydrate at pH 7.3) and incubated for 5 minutes on ice. Subsequently, 15 ml of cold culture medium (RPMI-1640 containing 25 mM HEPES and 2 mM L-glutamine, Life-Technologies, Merelbeke, Belgium, enriched with 100 U/ml penicillin/streptomycin, 1.0 mM sodium pyruvate) was added to stop cell lysis. Cells were centrifuged for 5 minutes at 1200 rpm and 4°C (Sorvall RT7, Thermo Electron Corporation, Breda, The Netherlands) and subsequently re-suspended in culture medium and counted

using a Coulter Counter (Beckman Coulter, Mijdrecht, The Netherlands). The cells were diluted in culture medium, to a concentration of 1.10^6 cells/ml.

ELISPOT assay

TNF-α producing splenocytes were detected by ELISPOT assay (ELISPOT, TNF-α Mouse, R&D Systems), according to the manufacturers protocol. Briefly, Immunospot plates (Multiscreen HTS-IP, Millipore, Amsterdam, The Netherlands) were coated overnight at 4°C with capture antibody 1:60 (v/v) in PBS. After washing, the plates were blocked for two hours at room temperature with 1% BSA and 5% sucrose in PBS. Isolated spleen cells in culture medium (RPMI containing 25 mM HEPES and 2 mM L-glutamine 100 U/ml penicillin/ streptomycin) enriched with 10% autologous serum were plated (1.10^5 cells/well) with or without lipopolysaccharide (LPS, E.Coli, O55:B5, Sigma, Zwijdrecht, the Netherlands) at a concentration of 1 µg/ml. The cells were incubated for 16 hours at 37°C in a humidified environment containing 5% CO_2 in air. After washing, the detection antibody (1:60 in reagent diluent) was added to the plate and incubated overnight at 4°C. The plates were washed and incubated with streptavidin-AP (ELISPOT Blue Color Module, R&D systems) diluted 1:60 in reagent diluent for 2 hours at room temperature. After washing with de-ionized water, the spots were visualized after 20 minutes incubation in the dark, using the AP substrate BCIP/NBT (ELISPOT Blue Color Module, R&D systems). After the plates were rinsed with de-ionized water, they were dried at room temperature. Spots were analyzed using an ImmunoScan ELISPOT reader (CTL Europe GmbH, Schwäbisch Gmünd, Germany).

Induction of acute arthritis

Male C75Bl/6 mice (Charles River, Maastricht, The Netherlands), aged 12 weeks at the start of the experiment were acclimatized to the animal housing one-week prior to the start of the experiment. All animals had free access to a standard rodent diet and tap water. Glycine (50 mg/mouse/day) or b-LF (0.1, 1 or 5 mg/mouse/day) were administered daily for five days (day 1 till 5) through gavage. Tap water (vehicle) was applied in the same volume, 200 µl, to control mice. At day two, 6 µl zymosan (3% suspended in PBS) was injected into the joint cavity of the right knee to induce arthritis. The left knee-joint served as an internal control. Joint inflammation was detected 24 hours after zymosan injection. At day 5, 4 days after zymosan injection, mice were bled under general anesthesia and sacrificed. The knees were processed for histology.

Assessment of joint swelling

Joint inflammation (swelling) was measured 1 day after zymosan injection by ^{99m}Tc-pertechnetate (^{99m}Tc) uptake in the knee joints [18, 19]. Briefly, 10 µCi ^{99m}Tc in 0.2 ml saline was injected subcutaneously in the scruff of the neck. After 15 minutes the accumulation of the isotope in the knee due to increased blood flow and edema was determined by external gamma counting. The severity of inflammation was expressed as the ratio of the ^{99m}Tc uptake in the inflamed knee over its non-inflamed counterpart.

Histological processing and analysis of knee joints

Knee joints were dissected, fixed, decalcified, dehydrated and embedded in paraffin [20]. Standard frontal sections of 7 µm were prepared and semi-serial sections were stained with HE for the detection of inflammation. Inflammation was scored by influx of inflammatory cells in the joint cavity and synovium. A score of 0 indicated no cell influx, 1 to 4 was scored according to the degree of cell influx. A corresponding group of sections was stained with Safranin O and counter stained with fast green for cartilage proteoglycan (PG) measurements. Detection of cartilage PG depletion was carried out on the patella. The mean score of the slides of one animal was calculated. Cartilage PG depletion was visualized by a diminished staining of the matrix and quantified by Zeiss image analysis software (KS300 version 3.0, Carl Zeiss, Sliedrecht, The Netherlands).

Statistical analysis

Basal ear thickness was subtracted from the ear thickness at the different time-points after zymosan injection hence representing ear swelling. At 6 hours after injection the supplemented animals and the vehicle injected animals were compared to the zymosan injected (sham supplemented) animals using one-way ANOVA.

The inhibitory effect of b-LF and glycine supplementation at 6 hours after zymosan injection was added up resulting in the expected effect of a combination of the two nutritional supplements. This expected (calculated) effect was compared with the detected effect using two-way ANOVA.

For the cell infiltration area in the ear, the number of TNF-α producing splenocytes, the joint swelling, the joint inflammatory cell infiltration and the joint PG depletion the zymosan injected sham supplemented conditions were compared to the treated animals using one-way ANOVA. PG content of the not injected left knees was detected in the sham supplemented animals and compared to the corresponding zymosan injected right knee by two-way ANOVA.

The overall significance of differences for all calculations was tested using the post hoc Dunnett's test.

The cytokine concentrations in homogenates of the ears were compared to the zymosan injected sham supplemented condition by T-Test.

Statistical analyses were performed in SPSS, P- values <0.05 were considered significant.

Results

Ear (skin)-inflammation

To investigate the effect of glycine and b-LF on the zymosan-induced ear swelling in mice, both components were administered orally for three days. At the second day zymosan (or PBS) was injected into both ears intradermally. Ear thickness was measured before and at different time points after injection. The detected ear swelling peaked around 6 hours after injection and then slowly declined. The swelling of the ears of the zymosan injected, vehicle treated animals at 6 hours (generally the maximal swelling) was set to 100%. In the majority of the tested groups a decrease of the swelling was detectable at the 24 hour time point (Fig. 1A and Fig. 2) as compared to the 6 hour time point. However in some animal groups the recovery seems to be delayed (Fig. 1B). Although there seem to be differences detectable in recovery of the swelling, there is no significant difference between the absolute swellings after 6 hours comparing the zymosan injected controls of the fast with the slow recovering group. Glycine treatment inhibited the zymosan-induced swelling in a dose dependent fashion (Fig. 1A). Maximal inhibition was measured at the highest dose tested (100 mg glycine/day) whereas 20 mg glycine/day did not influence the zymosan-induced swelling. Glycine 50 and 100 mg/day, inhibited the zymosan-induced ear-swelling significantly, from 100.0% to 88.6% ($P<0.05$) and 77.1% ($P<0.05$) respectively (6 hours after injection).

Lactoferrin also inhibited the ear swelling but this inhibition did not show the classical concentration dependency. Low doses of b-LF, 0.1 and 1 mg/day, inhibited the zymosan-induced ear swelling significantly, from 100.0% to 66% ($P<0.001$) and 63.3% ($P<0.001$) respectively (6 hours after injection). The higher doses of 5 and 25 mg/day however, did not influence the swelling, 6 hours after zymosan injection the swelling was 92.3% and 99.6% respectively (Fig. 1B).

After administration of a combination of glycine and b-LF the inhibition of the ear swelling was stronger than the effect of the separate components (Fig. 2). Moreover, 20 or 50 mg/glycine/day in combination with 0.1 mg/b-LF/day displayed a statistical significant synergistic inhibition of the zymosan-induced ear swelling (Table 1).

Inflammatory cell infiltration

From semi serial sections, originating from the middle of the inflammatory region of the ear, the infiltrating cell area was determined and averaged. The effect of a low (0.1 mg/day) and a high (25 mg/day) dose of b-LF on inflammatory cell infiltration was evaluated. The low dose of b-LF was able to reduce the infiltrating cell area significantly from 9007 to 3162 pixels ($P<0.01$) (Fig. 3). The area of the infiltrating cells of the 25 mg/day treated mice was found to be 8453 pixels. These data are in correspondence with the unexpected dose response relationship observed in the ear swelling.

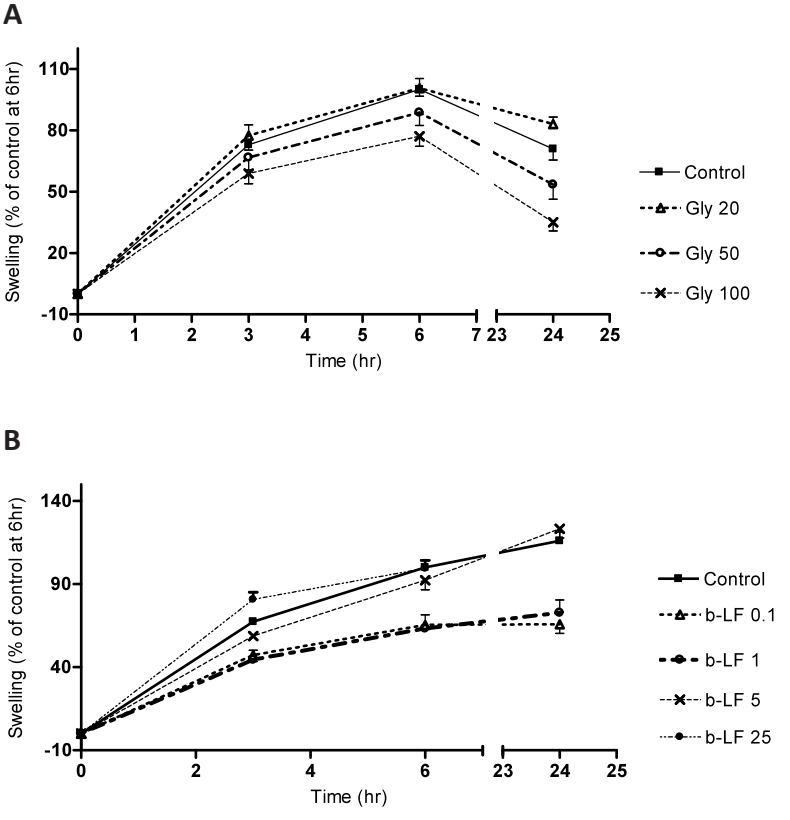

Fig. 1 The effect of glycine (A) and b-LF (B) on zymosan-induced ear inflammation. Vehicle (Control) and different concentrations of glycine (Gly 20, 50, 100 mg/mouse/day) and b-LF (b-LF 0.1, 1, 5 or 25 mg/mouse/day) were orally administered for three days, at day two the ears were injected with zymosan. Ear thickness was measured before and 3, 6 and 24 hours after the injection. The swelling of the ears of the vehicle treated animals at 6 hours (generally the maximal swelling) was set at 100%. Results are shown as means ± SEM, n=6 (control n=18).

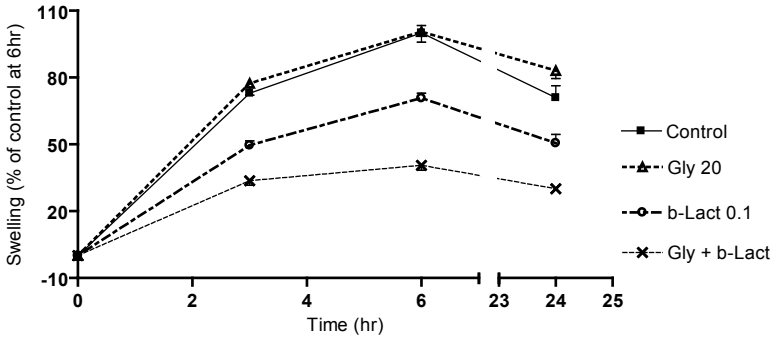

Fig. 2 The effect of glycine, b-LF and a combination of glycine and lactoferrin on zymosan-induced ear inflammation. Vehicle (Control), glycine (Gly 20 mg/mouse/day), b-LF (b-LF 0.1 mg/mouse/day) and a combination of both (Gly/b-LF) were orally administered for three days, at day two the ears were injected with zymosan. Ear thickness was measured before and 3, 6 and 24 hours after the injection. The swelling of the ears of the vehicle treated animals at 6 hours (generally the maximal swelling) was set to 100%. Results are shown as means ± SEM, n=6.

	Sham-injected	**Zymosan- injected**					
Ear	**Control**	**Control**	**b-LF 0.1**	**Gly 20**	**Gly 50**	**Gly 20 b-LF 0.1**	**Gly 50 b-LF 0.1**
Ear swelling (% of 6 hr)	36.6±3.7	100.0±3.9	70.7±2.2	100.6±4.7	88.6±6.2	40.5±2.1	33.4±1.7
Δ swelling			29.3±4.5	+ 0.6±6.1	11.4±7.3	**59.5±4.4**	**66.6±4.3**
Expected (calculated) Δ swelling						**28.7±7.6****	**40.7±8.6***

Table 1 The effect of Glycine, b-LF and a combination on the zymosan-induced ear inflammation. Vehicle (Control), glycine (Gly 20 or 50 mg/mous/day), b-LF (b-LF 0.1 mg/mouse/day) and a combination of both (Gly/b-LF) were orally administered for three days, at day two the ears were injected with zymosan. The ear thickness depicted was the measurement at 6 hours after zymosan injection presented as % of the 6 hour control. The established decrease in swelling of the combination of Gly/b-LF (Δ swelling) was compared to the expected Δ swelling (as calculated, added up, from the separate components), *P<0.05, **P<0.01. Results are shown as means ± SEM, n=6.

Cytokine levels in the ears

Inflammatory cytokine levels in the ear homogenates were detected by Multiplex Bead Immunoassay. Both glycine and b-LF were able to significantly inhibit the zymosan-induced increase in TNF-α and IL-1β, whereas b-LF also inhibited the zymosan-induced IL-6 production. The inhibition of the cytokine production in animals treated with a combination of b-LF and glycine was more powerful in comparison to the single nutritional treatments (Table 2).

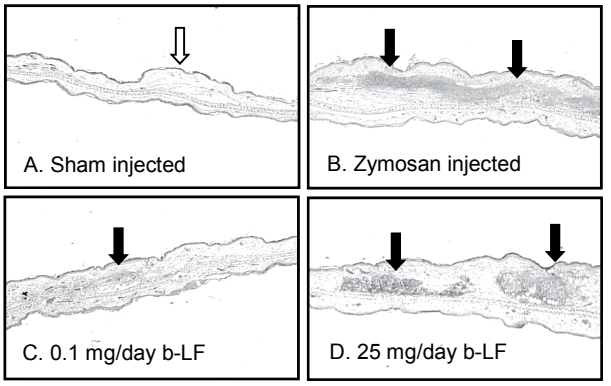

Fig. 3 Pictures of the effect of a low and a high dose of b-LF on the zymosan-induced cell infiltration. Vehicle (B) and b-LF (0.1 (C) and 25 (D) mg/mouse/day) were orally administered for three days, at day two the ears were injected with zymosan. Figure 3A, represents vehicle treated, sham injected animal. Sections of the ears (24 hours after zymosan injection) were HE stained. The areas of inflammatory cell infiltration were depicted with a ➡; the injection-place in the sham injected ear is depicted with a ⇨.

TNF-α producing splenocytes

The mice were sacrificed 24 hour after zymosan injection. The effect of glycine and b-LF on the number of TNF-α producing splenocytes, after *ex-vivo* LPS stimulation, was detected by the ELISPOT assay. Both glycine and b-LF were able to inhibit the zymosan-increase in the number of TNF-α producing cells to basal (initial) levels. In animals treated with a glycine/b-LF combination the number of TNF-α producing splenocytes was even below the initial/ basal number of TNF-α producing splenocytes found in sham injected animals (Table 2).

Joint inflammation

To investigate the effect of glycine and b-LF on zymosan-induced joint swelling in mice, both components were administered orally for five days. At the second day zymosan was injected

into the right knee joint. Knee swelling was measured after 1 day by ^{99m}Tc-pertechnetate uptake. B-LF 0.1 or 1 mg/mouse/day had no effect on the joint swelling. However, b-LF 5 mg/mouse/day and glycine 50 mg/mouse/day were able to decrease the zymosan-induced joint swelling significantly (Table 3).

	Sham injected	Zymosan injected			
Ear	**Control**	**Control**	**b-LF 0.1**	**Gly 50**	**Gly/b-LF**
TNF-α pg/ml	3±1**	23±5	8±2*	12±4	5±1**
IL-1β pg/ml	8±1**	154±40	30±9*	69±28	13±1**
IL-6 pg/ml	16±3**	147±33	63±17*	138±59	33±2**
Spleen	**Control**	**Control**	**b-LF 0.1**	**Gly 50**	**Gly/b-LF**
TNF-α producing cells	115±22**	257±23	127±29**	139±27*	63±15**

Table 2 The effect of glycine, b-LF and a combination on the zymosan-induced cytokine induction in the ear and the number of TNF-α producing spleen cells. Vehicle (Control), glycine (Gly 50 mg/mouse/day), b-LF (b-LF 0.1 mg/mouse/day) and a combination of both (Gly/b-LF) were orally administered for three days, at day two the ears were injected with zymosan. 24 hours after zymosan injection, ears were homogenized and cytokine levels were detected. The spleen cells were isolated and *ex-vivo* stimulated with LPS for 16 hours. The number of TNF-α producing cells out of a total of 200.000 cells was detected. Results are shown as means ± SEM, n=6. *P<0.05, **P<0.01.

Inflammatory cell infiltration into the joint

To investigate the effect of glycine and b-LF on zymosan-induced inflammatory cell infiltration in mice, both components were administered orally for five days. At the second day zymosan was injected into the right knee joint. After 5 days the joints were processed for histology and semi-serial sections were stained with HE. B-LF 0.1, 1.0 or 5 mg/mouse/day had no effect on the infiltration of inflammatory cells into the joint. However, glycine, 50 mg/mouse/day was able to decrease the zymosan-induced cell infiltration significantly (Table 3).

Cartilage proteoglycan depletion

To investigate the effect of glycine and b-LF on zymosan-induced PG depletion in mice both components were administered orally for five days. At the second day zymosan was injected into the right knee joint. After 5 days the joints were processed for histology and semi-serial sections were stained with Safranin O and counter stained with fast green. B-LF 0.1, 0.1 and 5 mg/mouse/day had no effect on the zymosan-induced PG depletion whereas glycine, 50 mg/mouse/day was able to decrease the zymosan-induced PG depletion significantly (Table 3).

	Sham injected	Zymosan injected				
Joint	**Control**	**Control**	**Gly 50**	**b-LF 0.1**	**b-LF 1**	**b-LF 5**
Joint swelling	-	2.0±0.1	1.8±0.0**	2.0±0.1	2.0±0.0	1.8±0.1*
Infiltrate	-	3.6±0.3	2.1±0.3**	3.1±0.4	3.0±0.3	3.0±0.3
PG depletion	36.9±2.4**	87.6±4.1	71.8±4.5**	81.9±4.4	76.0±3.3	83.3±4.4

Table 3 The effect of glycine and b-LF on the zymosan-induced joint swelling, inflammatory cell infiltration and proteoglycan depletion in mouse knee joints. Vehicle (Control), glycine (Gly 50 mg/mouse/day) and b-LF (b-LF 0.1 or 5 mg/mouse/day) were orally administered for five days, at day two the right joint was injected with zymosan. Joint swelling was detected by ^{99m}Tc-pertechnetate uptake measurements at 24 hours after the injection and was depicted as the ratio of the inflamed knee above the not injected knee. At day 5 the knee joints were isolated and processed for histology. Cell infiltration was scored at a scale from 0 (no cells) to 4 (severe cell influx in joint cavity and synovium). Proteoglycan depletion was quantified by computer analysis. Results are shown as means ± SEM, n=8. *P<0.05, **P<0.01

Discussion

The present study demonstrated that a combination of two, orally administered, nutritional components; glycine and bovine lactoferrin, displayed a synergistic anti-inflammatory activity. The effects of glycine on the zymosan-induced ear swelling were concentration dependent whereas only the low doses of b-LF were able to inhibit the swelling. Furthermore, both components diminished: 1) the zymosan-induced increase of inflammatory cytokines in the ear and 2) the zymosan-induced increase of TNF-α producing splenocytes detected after *ex-vivo* LPS stimulation. The individual anti-inflammatory effects of the tested nutritional

components were more pronounced in the zymosan-induced ear-skin inflammation model compared with the zymosan-induced acute arthritis model.

In recent years, evidence accumulated in favor of an anti-inflammatory activity of glycine [3, 7, 9]. The present study demonstrated that these effects are concentration dependent (ear-skin inflammation model) and independent of the model chosen (i.e. ear-skin inflammation model or arthritis model). Glycine attenuated the zymosan-induced increase of TNF-α, IL-1β and IL-6 in the ear-skin model. Moreover, glycine counteracted the zymosan-induced increase in the number of TNF-α producing splenocytes after LPS stimulation. The ability of glycine to inhibit the production of TNF-α and other inflammatory cytokines by different cell types *in vitro* and *ex vivo*, has been described in literature [8, 9]. This decrease in cytokine production might at least partly, result from a decreased number of TNF-α producing spleen cells as indicated in the present study. Furthermore, zymosan is a known Toll-Like Receptor 2 (TLR2)-dectin-1 ligand. The inhibition of zymosan-induced ear-inflammation by the nutritional components indicates an inhibitory effect on TLR-2-induced inflammation. However the changes in the number of TNF-α producing cells, detected after stimulation with LPS (a TLR4 ligand) indicate that glycine not only interferes at the TLR2 level but that after ingestion of glycine and lactoferrin also changes have taken place in reactivity to LPS. Glycine proved to be able to inhibit zymosan-induced-joint swelling, cell infiltration and cartilage proteoglycan depletion. These data were in agreement with a study of Li et al. [7] in which 5% glycine in the diet prevented peptidoglycan polysaccharide-induced reactive arthritis, indicated by a reduced joint swelling and a reduced inflammatory cell infiltration. The cartilage protective activity established by glycine is likely to be a result of the reduced inflammatory cell infiltration. Cytokines produced by inflammatory cells have been demonstrated to be major players in cartilage destruction [21]. Besides this, it has been demonstrated that glycine is a potent inhibitor of calpain activity [22]. Calpains are cysteine proteinases which are present in the synovial fluid of rheumatoid arthritis and osteoarthritis patients and are associated with cartilage degradation [23, 24]. Inhibition of these synovial calpains might represent a second mechanism by which the supplementation of glycine resulted in cartilage protection.

Lactoferrin is a well known natural immune modulator. It might strengthen the immune response [25-27] as well as mediate anti-inflammatory reactions [2]. The present study demonstrated that only the low doses of b-LF (0.1 or 1 mg/day/mouse) were able to inhibit the zymosan-induced ear swelling and inflammatory cell infiltration whereas higher doses (5

or 25 mg/mouse/day) did not affect the inflammatory response in Balb/C mice. The swelling data correspond with the area of infiltrating inflammatory cells in the ears, as detected by histological procedures. After administration of different amounts of b-LF to C57Bl/6 mice in the acute arthritis model only the high dose of 5 mg/mouse/day inhibited the joint swelling. This finding is striking since only lower doses were effective in the ear-skin inflammation model. No effects of b-LF were detected on cell infiltration in the synovium and synovial fluid or on proteoglycan depletion of the cartilage. Literature describes protective effects of lactoferrin in different arthritis models [28-30]. Guillen et al. showed a positive effect of locally injected LF on *Staphylococcus aureus*-induced septic arthritis in mice whereas Hayashida et al. demonstrated a protective effect of orally administrated LF on adjuvant-induced acute and chronic paw inflammation in rats. In the latter study, the relative dose of LF used was comparable to doses used in the current study. Summarizing; the lactoferrin data demonstrated that the anti-inflammatory effects might be reversed concentration dependent and effectiveness might be depending on the "mice" strain, animal species or the test-model used. In addition, literature stated that the way of action of LF might depend on immunological status [31] and mode of administration [32]. In future studies detection of pro- and anti-inflammatory cytokines levels in the serum or immunological tissues in the intestinal region might give more insight into the mechanism behind the observed reversed concentration dependency. The implications of a reversed dose response curve for the use of lactoferrin in foods for special health should be considered carefully.

The anti-inflammatory effects of a combination of glycine and b-LF on the zymosan-induced ear-skin inflammation proved to be, at least in part, synergistic. The tested combinations were able to synergistically reduce the zymosan-induced ear swelling. The decrease in the number of TNF-α producing splenocytes in the combination treated mice ended up below the initial levels (*ex-vivo* LPS stimulated conditions). Other conditions, indicating an amplifying effect of lactoferrin, have been described by Diarra and Leon-Sicairos [33-35]. Both authors described a synergistic effect of LF in combination with an antibiotic. LF as well as the pharmaceutical component were thought to act directly on the intruding pathogen. The present study however demonstrated a synergistic effect of b-LF and glycine, two nutritional components, modulating the inflammatory reaction systemically.

The effect of food compounds on enhancing the immune system or dampening inflammatory processes has been described in numerous articles. The present study indicated that the anti-inflammatory effects of lactoferrin were model dependent. Furthermore, to our knowledge this is the first study demonstrating synergistic anti-inflammatory activity of

two orally administered nutritional components, i.e. glycine and bovine lactoferrin. This concept might offer in the near future a powerful nutritional way in modulating chronic inflammatory diseases.

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Chapter 7

The combination of orally ingested lactoferrin and glycine inhibits arthritis development in a model of collagen-induced arthritis

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Abstract

In literature, it was demonstrated that a mixture of two nutritional components, glycine and bovine lactoferrin, was able to inhibit zymosan-induced inflammation. The present study aims to evaluate the effect of this mixture on arthritis development and serum cytokine levels in the collagen-induced arthritis model in mice.

Arthritis was induced in male DBA/1 mice. Clinical appearance of arthritis was graded. At the end of the experiment serum was collected for cytokine detection.

The mixture of glycine and bovine lactoferrin was able to decrease serum cytokine levels and arthritis development. The present data demonstrate that the tested nutritional mixture was able to reduce inflammation in the collagen-induced arthritis model. Therefore, this nutritional concept might be a new option for the treatment of rheumatoid arthritis.

Introduction

Rheumatoid arthritis (RA) is a systemic inflammatory autoimmune disorder affecting approximately 1% of the general population in the western countries. The disease is characterized by a chronic poly-arthritis, synovial hyperplasia and erosive synovitis, progressive cartilage and bone destruction and an accelerated loss of muscle mass.

Methotrexate and glucocorticoids are classical and efficient treatments of RA [1, 2]. They diminish inflammation and reduce the speed of joint destruction [3-5]. Novel RA therapies include biologicals, targeting pro-inflammatory cytokine activity. Treatment with biologicals results in clinical improvements in RA patients and retardation of radiographic progression [6, 7]. Both the classical agents and the biologicals reduce pain, swelling and progression of joint destruction. They are characterized as disease-modifying antirheumatic drugs (DMARDs).

Modulation of pro-inflammatory cytokine synthesis or activity remains one of the most promising targets for new DMARDs. A pivotal cytokine, playing a key role in the pathogenesis of RA, and a very attractive target for intervention, is tumor necrosis factor alpha (TNF-α) [8]. In 2007 it was demonstrated that the synergistic inhibition of zymosan-induced inflammation by a mixture of orally ingested glycine and bovine lactoferrin (b-LF) was accompanied by a strong decrease in the production of TNF-α [9].

In the past years it has been indicated that the simple amino acid glycine comprises anti-inflammatory and immune modulatory activities. These effects of glycine on immune responses can partially be explained by activation of the glycine-gated chloride channel (GlyR). GlyRs have been demonstrated on different types of immune cells including macrophages, monocytes, neutrophils and T lymphocytes [10-12]. Activation of the GlyR inhibits production of inflammatory cytokines and other inflammatory mediators [13, 14].

The iron-binding protein lactoferrin (LF) has been demonstrated to play a role in modulating immune responses by its ability to interact with target molecules and cells. Anti-inflammatory effects of lactoferrin are shown by the inhibition of the production of pro-inflammatory cytokines *in vitro* and *in vivo* [15-17] and the up regulation of anti-inflammatory cytokines [18].

The collagen-induced arthritis (CIA) model in mice is an extensively studied RA model. It has been used to provide insight into the underlying disease process of RA and to study the potential of new experimental therapies [19-23]. The present study evaluated the effect of a combination of glycine and b-LF on clinical arthritis development and serum cytokine levels in the CIA model. Future studies on possible joint protective effects of the nutritional

concept will define the possible significance of this nutritional intervention for the treatment of RA patients.

Materials and methods

All experimental procedures using laboratory animals were approved by an independent animal experiments committee (DEC Consult, Bilthoven, The Netherlands).

Induction of CIA

Male DBA/1 mice (Taconic, Lille Skensved, Denmark), aged 9 weeks at the start of the experiment, were acclimatized in the animal housing facility starting two-weeks prior to the start of the experiment. All animals were housed in filter top cages and had free access to water and food. The food was applied as a daily fresh prepared dough, this to simplify food intake in the diseased state. The mice were immunized by a subcutaneous injection of 100 µg native bovine collagen type II (Chondrex, Zurich, Switzerland) emulsified in complete Freund Adjuvant (CFA, Chondrex) , at the base of the tail. An intra-peritoneal booster of 100 µg of collagen type II in phosphate buffered saline (PBS) was given 21 days later. Mice with a clear onset of arthritis at day 21 were excluded from the experiment. After the booster 100% of the animals developed arthritis within 9 days. Starting at day 21 (day of the collagen booster) 200 µl water (solvent) or a mixture of Gly (50mg/mouse) and b-LF (0.1 mg/mouse) in 200 µl water, was administered by a daily gavage.

Assessment of CIA

After the booster the mice were examined three times a week for visual appearance of arthritis. Clinical severity of arthritis of the peripheral joints was graded on the level of "macroscopic" inflammation on a scale of 0 to 4 [24]. 0, no symptoms, 1 mild , 2 moderate, 3 marked and 4 indicates maximal redness and swelling of the paw. The scores of all paws were summarized to obtain the "arthritis score", with a maximum of 16 for each mouse. Mice with an arthritis score of 12 or higher were excluded from the study for ethical reasons. The mean arthritis score for each group was calculated (mean ± SEM). Assessment of the arthritis score was performed by two independent observers.

Serum isolation

Twelve days after the collagen booster the animals were sacrificed. Blood was collected in Eppendorf® tubes and incubated for 1 hour at room temperature followed by 1 hour at

4°C. The serum was collected after centrifugation (5 minutes at 6000rpm in and Eppendorf centrifuge, Eppendorf, 5810-R, VWR, Roden, The Netherlands), and stored at -80°C until cytokine detection.

Cytokine detection

Cytokine detection in serum was performed by using a commercial Multiplex Bead Immunoassay (Biosource, Etten-Leur, The Netherlands), including TNF-α and Interleukin (IL) -1β and IL-6, according to the manufacturers protocol. Cytokine levels were detected using the Bio-Plex system (Bio-Rad, Veenendaal, The Netherlands). Results were calculated using the Bio-Plex Manager Software 3.1 (Bio-Rad).

Statistical analysis

Average values were expressed as mean ± standard error of the mean (SEM). Statistical significance of the glycine and b-LF intervention on the percentage of mice with an arthritis score above 8 was calculated using the Fisher's exact test. For serum cytokine concentrations the results of the CIA (positive control) mice were compared with the glycine and b-LF treated mice and the control mice (without CIA) using one-way ANOVA. The overall significance of differences was tested using the post-hoc Dunnett's test.

Results

Arthritis development

Twelve animals were included in each of the test groups. At day 21, two animals from each test group developed clinical signs of arthritis, and were excluded from the rest of the experiment. After the collagen booster (day 21) the arthritis score was evaluated three times a week. Animals with a score above 12 were sacrificed for ethical reasons as agreed with the ethical committee. The arthritis score of the animals was averaged; arthritis development was depicted in figure 1A. The animals in the glycine and b-LF administered group developed arthritis similar to the untreated group. Since animals with an arthritis score above 12 had to be sacrificed for ethical reasons, it was not possible to follow the effect of the nutritional treatment beyond this clinical stage. By evaluating the number of animals with arthritis scores above a certain threshold, for this an arthritis score of 8 was chosen, all animals can be included in the efficacy calculation. Twelve days after the booster the percentage of animals with an arthritis score above 8 in the control group was significantly higher compared to the percentage of animals with an arthritis score above 8 in the glycine and b-LF treated group (Fig. 1B).

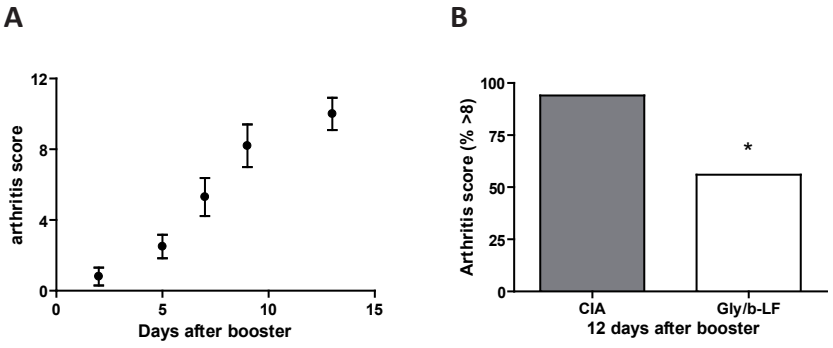

Fig. 1 Arthritis development, depicted as development after the collagen booster (day 21,Fig. A). Twelve days after the booster the percentage of animals with an arthritis score above 8 was calculated (control and the Gly and b-LF intervention) and depicted in Fig. B. The arthritis score is expressed as the mean score ± SEM. Significant differences vs. the CIA condition (positive control) are indicated by * (P<0.05), n=10.

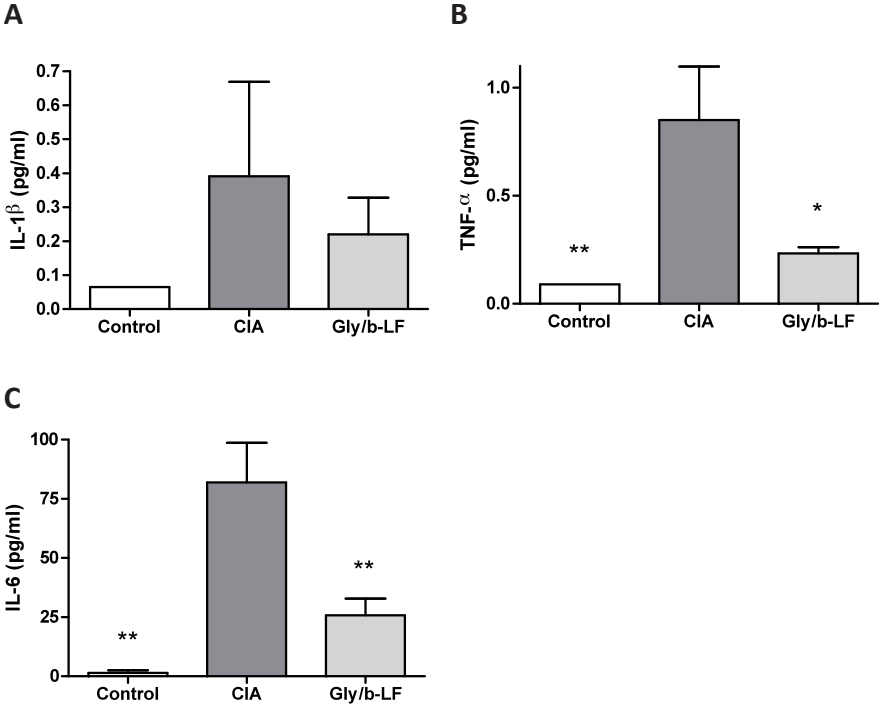

Fig. 2 Mice were sacrificed twelve days after the collagen booster. IL-1β (A), TNF-α (B) and IL-6 (C) levels were detected in the serum of Control mice, CIA mice and in Gly and b-LF treated CIA mice. Values are expressed as mean ± SEM. Significant differences vs. the CIA condition were indicated by * (P<0.05), **(P<0.01).

Serum cytokine levels were detected by Multiplex Bead Immunoassay. Twelve days after the collagen booster, the serum TNF-α and IL-6 concentrations were significantly increased from 0.09 pg/ml to 0.85 pg/ml and 1.49 pg/ml to 81.98 pg/ml, respectively. In both cases this increase was significantly counteracted by intervention with a combination of glycine and b-LF (Fig. 2). IL-1β levels were not significantly affected by arthritis development.

Discussion

All current/regular RA therapies show their own specific side effects including intolerability, gastrointestinal adverse events and increased incidence of common and opportunistic infections [25, 26]. Moreover, many of the new agents are recombinant proteins whose large scale production is extremely expensive. Given the chronic nature of RA, the costs of these treatments are likely to become a social issue. The development of new therapies, preferably orally available, could overcome the concerns on side effects and costs, at least in part.

Although a wide range of cytokines and other inflammatory mediators are expressed in the joints in RA, the arguments that place TNF-α at the heart of the inflammatory process in RA are persuasive [27, 28]. Inhibition of TNF-α via the antagonists infleximab, etanercept and adalimumab has been shown to be an effective and rapid mechanism in order to control disease activity [29]. The results of the present study indicate that orally ingestion of a combination of glycine and b-LF decreases TNF-α production in the CIA mouse model.

Another cytokine modulated by the nutritional intervention is the pro-inflammatory cytokine IL-6. IL-6 has been found in increased levels in RA. It has been indicated to contribute to development of arthritis, stimulation of both B and T cell function, endothelial production of chemokines, synovial fibroblast proliferation and osteoclast formation and activation [30]. IL-6 seems to be induced mainly by TNF-α and IL-1. Blocking IL-6 activity with tocilizumab (a humanized anti-IL-6 receptor antibody) reduced signs and symptoms of RA and provided radiographic benefit [31].

Glycine and b-LF intervention results in an inhibition of arthritis development in the CIA model. This decrease might be brought about by a decrease in the production of TNF-α, resulting in a decreased stimulation of IL-6 production.

The present study demonstrates an anti-inflammatory activity of two orally administered nutritional components, i.e. glycine and bovine lactoferrin in the collagen-induced arthritis model in mice. Future studies on possible joint protective effects of the nutritional concept are ongoing and will for a substantial part define the significance of the nutritional intervention for treatment of RA patients.

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Chapter 8

Locomotion and muscle mass measures in a murine model of collagen-induced arthritis

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Abstract

Rheumatoid arthritis (RA) is characterized by chronic poly-arthritis, synovial hyperplasia, erosive synovitis, progressive cartilage and bone destruction accompanied by a loss of body cell mass. This loss of cell mass, known as rheumatoid cachexia, predominates in the skeletal muscle and can in part be explained by a decreased physical activity. The murine collagen-induced arthritis (CIA) model has been proven to be a useful model in RA research since it shares many immunological and pathological features with human RA. The present study explored the interactions between arthritis development, locomotion and muscle mass in the CIA model.

CIA was induced in male DBA/1 mice. Locomotion was registered at different time points by a camera and evaluated by a computerized tracing system. Arthritis severity was detected by the traditionally used semi-quantitative clinical scores. The muscle mass of the hind-legs was detected at the end of the study by weighing. A methotrexate (MTX) intervention group was included to study the applicability of the locomotion and muscle mass for testing effectiveness of interventions in more detail. There is a strong correlation between clinical arthritis and locomotion. The correlations between muscle mass and locomotion or clinical arthritis were less pronounced. MTX intervention resulted in an improvement of disease severity accompanied by an increase in locomotion and muscle mass.

The present data demonstrate that registration of locomotion followed by a computerized evaluation of the movements is a simple non-invasive quantitative method to define disease severity and evaluate effectiveness of therapeutic agents in the CIA model.

Introduction

Rheumatoid arthritis (RA) is a systemic inflammatory autoimmune disorder affecting approximately 1% of the general population in the western countries. The disease is characterized by a chronic poly-arthritis, synovial hyperplasia and erosive synovitis, progressive cartilage and bone destruction and an accelerated loss of muscle mass, also known as rheumatoid cachexia [1]. The average loss of body cell mass (BCM) among patients with RA is between 13% and 15% [2]. The BMC consists primarily of muscle mass, visceral mass and immune cell mass. A decrease in muscle mass can in part be explained by a decreased physical activity [3]. This decrease in physical activity in RA patients is closely related to pain, characterized by hyperalgesia and spontaneous pain, mostly caused and exacerbated by inflammatory mediators (cytokines, prostaglandins) [4]. Other factors contributing to muscle protein wasting are increased levels of systemic and local markers of inflammation (e.g. TNF-α, IL-1β and IL-6) as well as increased levels of oxidative stress [5].

The collagen-induced arthritis model (CIA) in mice is an extensively studied RA model. It has been used to provide insight into the underlying disease process of RA and is frequently used to study the potential of new experimental therapies [6-8]. The development and severity of arthritis in the CIA model is mostly detected by a semi-quantitative clinical scoring system based on the severity of arthritis in the peripheral joints [9]. Despite of being the most widely used rodent model for RA, its use for studying arthritic pain has been reported just recently [10]. Moreover, only in a small number of studies locomotion was one of the readouts in the CIA models [11, 12].

The present study evaluated locomotion (changes which are at least partially pain-induced), muscle mass (changes might be inflammation and locomotion-induced) and clinical arthritis scores in the CIA model. The study aims to determine the applicability of locomotion and muscle mass changes as readout parameters in the CIA mouse model and its relevance for intervention studies.

Methods

All experimental procedures using laboratory animals were approved by an independent animal experiments committee (DEC Consult, Bilthoven, The Netherlands).

Induction of CIA

Male DBA/1 mice (Taconic, Lille Skensved, Denmark), aged 9 weeks at the start of the experiment were acclimatized in the animal housing facility starting two-weeks prior to the

start of the experiment. All animals were housed in filter top cages and had free access to a water and food. The food was applied as a daily fresh prepared dough, this to simplify the food intake in the diseased state. The mice were immunized by a subcutaneous injection of 100 µg native bovine collagen type II (Chondrex, Zurich, Switzerland) emulsified in complete Freund Adjuvant (CFA, Chondrex) , at the base of the tail. An intra-peritoneal booster of 100 µg of collagen type II in phosphate buffered saline (PBS) was given 21 days later. Mice with a clear onset of arthritis at day 21 were excluded from the experiment. After the booster 100% of the animals developed arthritis within 9 days. To evaluate the effect of pharmaceutical treatment on arthritis development and locomotion one group of animals was treated with Methotrexate, a frequently used disease-modifying anti-rheumatic drug (DMARD). Methotrexate (MTX, Emthexate PF, Pharmachemie B.V., Haarlem, The Netherlands) was injected three times a week (1 mg/kg, intra-peritoneal) starting at the day of the collagen booster (day 21). Control mice were injected with PBS.

Assessment of CIA

After the booster the mice were examined three times a week for visual appearance of arthritis. Clinical severity of arthritis of the peripheral joints was graded on the level of "macroscopic" inflammation on a scale of 0 to 4 [9]. 0, no symptoms, 1 significant , 2 moderate, 3 marked and 4 indicates maximal redness and swelling of the paw. The scores of all paws were summarized to obtain the "arthritis score", with a maximum of 16 for each mouse. Mice with arthritis score of 12 or higher were for ethical reasons excluded from the study. The mean arthritis score for each group was calculated (mean ± SEM). Assessment of the arthritis score was performed by two independent observers.

Assessment of locomotion

Twice, before the arthritis induction and at 9 days after the collagen booster, mice were placed individually in an acrylic movement box of 60 x 40 cm. Spontaneous, exploratory locomotion of the animals was detected by a camera which was positioned above the "movement" boxes. The movements were registered for 5 minutes, starting 2 minutes after the mice have been placed into the boxes. The movements were evaluated by a computerized tracing system and image analyzer (EnthoVision 3.1, Noldus, Wageningen, The Netherlands). The moved distance (in cm) for each group was calculated and averaged (mean ± SEM). Changes in moving distance were calculated for each mouse as % of initial movement.

Detection of skeletal muscle mass

Twelve days after the collagen booster the animals were sacrificed and the different skeletal muscles from the hind leg, tibialis anterior (TA), gastrocnemius, soleus and extensor digitorum longus (EDL) were dissected and weighted.

Statistical analysis

Averaged values are expressed as mean ± standard error of the mean (SEM). Correlations were calculated using the Pearson's linear regression model. The changes induced by MTX were calculated by the independent-samples T-Test.

Results

Arthritis development

Arthritis development was detected in two independent experiments. Twelve animals were included in each of the test groups. At day 21, two animals from each test group developed clinical arthritis, as detected by the arthritis score, and were excluded from the rest of the experiment. After the collagen booster (day 21) the arthritis score was detected three times a week, animals with a score above 12 were sacrificed. The score of the different animals was averaged; the arthritis development was depicted in figure 1.

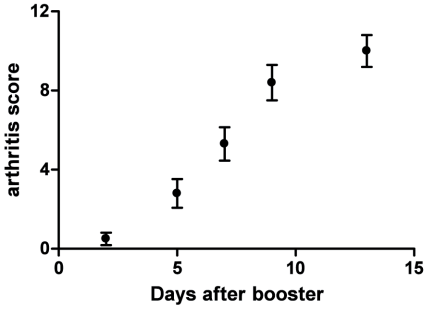

Fig. 1 Arthritis development, depicted as development after the collagen booster (day 21). Values are expressed as the mean score ± SEM, n = 17.

Assessment of locomotion

To investigate the reproducibility of the locomotion test, locomotion was tested twice in a control group of animals, with an interval of 5 days. The results were depicted in figure 2A.

There is a significant correlation (Pearson r = 0.55, p = 0.0002) between the distance moved by the different animals at the first detection day and the distance moved at the second detection day. The presence of “lazy” and “active” animals indicates a need for individual calculation of movement changes. The average movement of each mouse as detected in two measurements before the start of arthritis induction was set at 100%. At day 30, 9 days after the collagen booster, locomotion was tested again and changes in movement, as a percentage of the initial movement, were calculated. The individual movement changes were depicted against the individual arthritis score at the detection day. There is a clear correlation (Fig. 2B) between the decrease in locomotion and the arthritis score (Pearson r = -0.78, p = 0.0001).

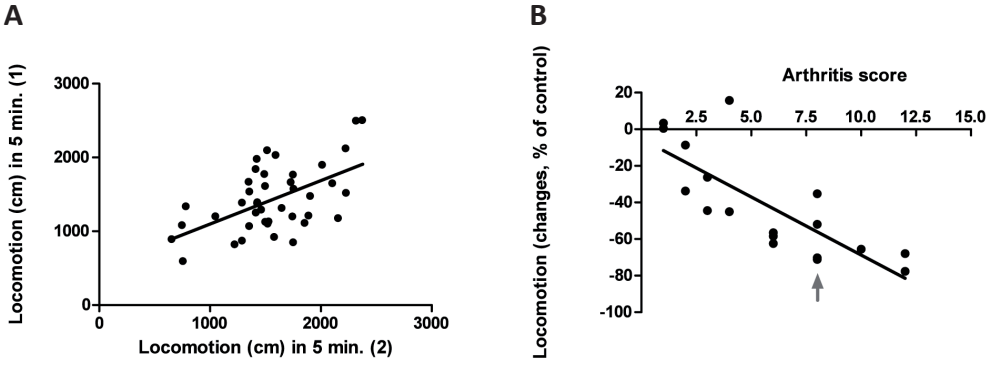

Fig. 2 Evaluation of locomotion in healthy and arthritis animals. Locomotion was tested at 2 different time point (1 and 2), with 5 days interval, in a control group of animals (A, n=42). At day 30, 9 days after the collagen booster locomotion was tested and changes were expressed as % change of the initial locomotion against the arthritis score of the animal (B, n=17).

The locomotion pattern of the mouse in figure 2B which is marked with an arrow was depicted in figure 3. Figure 3A shows the locomotion pattern of the mouse before arthritis initiation while figure 3B indicates the movement pattern after arthritis development (arthritis score at the time of the movement detection is 8).

In order to test the relevance of the locomotion parameter for testing therapeutics, the effect of MTX treatment on the locomotion and the arthritis score were detected. At nine days after the collagen booster the MTX treatment resulted in a decreased arthritis score of 48 % (Fig. 4A) and an increase of locomotion of 60% (Fig. 4B).

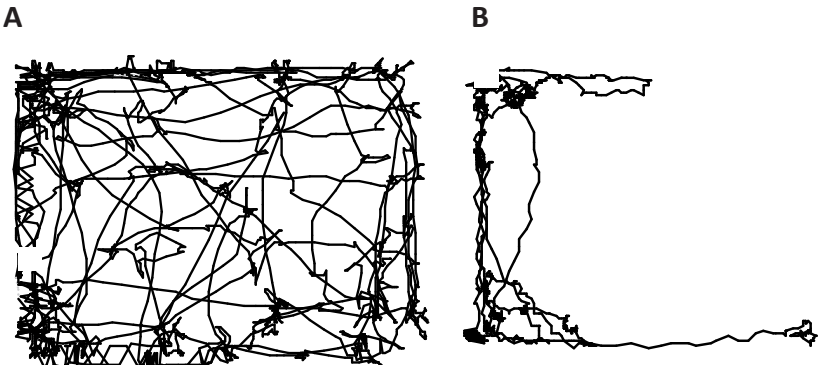

Fig. 3 Reproduction of the locomotion pattern before arthritis development (A) and at 9 days after the collagen booster (B) The mouse depicted corresponds to mouse which is marked with an arrow in figure 2B (arthritis score = 8).

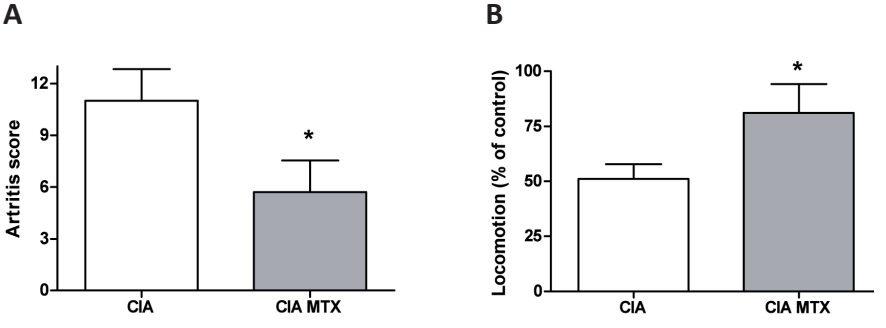

Fig. 4 The effects of MTX treatment on the arthritis score and locomotion, as detected nine days after the collagen booster, were depicted in figure A and B respectively. Values are expressed as the mean ± SEM. Significant differences vs. the CIA condition were indicated by * ($P<0.05$).

Muscle mass

After the mice were sacrificed at 12 days after the collagen booster the different skeletal muscles from the hind legs were dissected and weight. The changes in total weight of the skeletal muscles of hind legs correlated significantly (Pearson $r = 0.53$, $p = 0.02$) with the % of the initial locomotion as detected 9 days after the collagen booster (final movement study) (Fig. 5A) and with the arthritis score (Fig. 5B, Pearson $r = -0.49$, $p = 0.03$). Comparison of the individual muscles with locomotion or arthritis score did not result in a significant correlation (data not shown). MTX treatment, starting at the day of the collagen booster, was able to significantly reduce the decrease in muscle mass of the TA (Fig. 6A), the EDL (Fig. 6B) and gastrocnemius (Fig. 6C). The effect of MTX treatment on the soleus was however not significant (Fig. 6D).

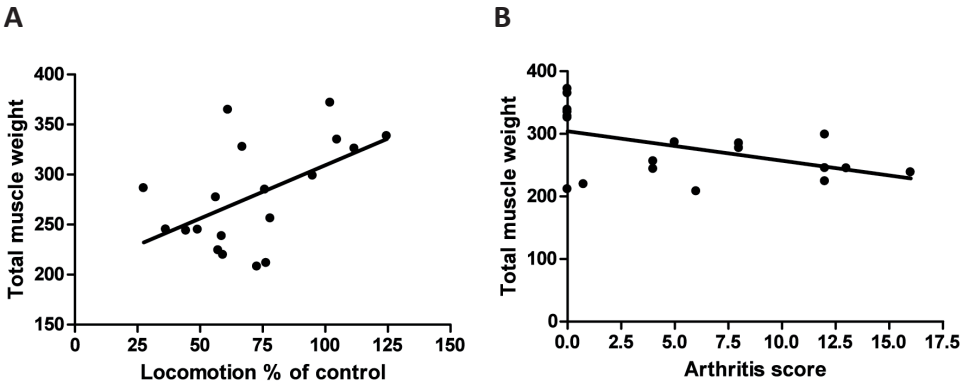

Fig. 5 Correlation between muscle weight and locomotion (A) and arthritis score (B). The total weight (mg) of the different skeletal muscles from the hind legs (TA, gastrocnemius , soleus and EDL), weight 12 days after the collagen booster, was depicted as a function of the % of locomotion after arthritis development (A, n=20) and arthritis score (B, n=20).

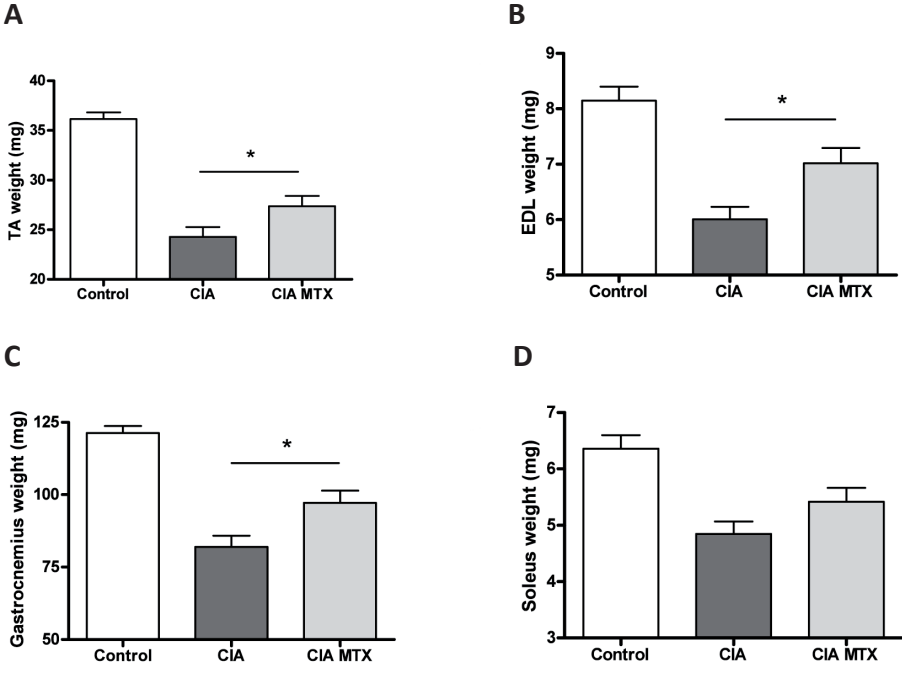

Fig. 6 The effect of MTX treatment on the weight of the different skeletal muscles, TA (A), EDL (B), gastrocnemius (C), soleus (D) weight 12 days after the collagen booster. Values are expressed as the mean ± SEM. Significant differences between the CIA condition and the MTX treated CIA condition were indicated by * ($P<0.05$).

Discussion

The mouse model of CIA has been proven to be a useful animal model for RA research because it shares many immunological and pathological features with human RA [13, 14]. In the present study it was demonstrated that there is a strong correlation between the macroscopic arthritis score and locomotion. This locomotion test, by which mice were placed in a new surrounding, strongly correlates with the open field-tests performed to study incidence and duration of certain behaviors [15]. Corresponding results have been indicated by Inglis et al. studying hyperalgesia [10] and Millecamps et al. studying behavior in a monoarthritic rat model [11]. However, in these studies the correlation between locomotion and the disease severity was not tested. To evaluate possible corruption of the locomotion values by habituation, the effect of repeated measurements was tested. No differences in walking distance were detected in control mice between different days of assaying (5 tests with an interval of 3-7 days between each test, data not shown). The repeated detection of locomotion in control mice did reveal however, a mouse dependent initial locomotion. These results stress the need for determination of the initial locomotion distance for each individual mouse. These initial values were set by averaging the locomotion values detected on two separate days before the start of the experiment. The present data indicate that the quantitative detection of locomotion strongly corresponds to clinical changes as detected by the semi-quantitative detection of disease severity. Also the total mass of the skeletal muscles of the hind legs, as detected at the end of the experiment, revealed to correlate to locomotion. These results suggest a direct relation between movement and muscle mass. However, movement is not the only factor effecting the muscle mass in the CIA model. In contrast to the fact that food intake by RA patients does not differ from the intake by healthy people [16, 17] a strong significant decreased food intake by the CIA animals has been detected (data not shown). Moreover, TNF-α which is believed to be a central mediator of muscle wasting in RA exerts a powerful influence on muscle protein turnover resulting in a net muscle protein wasting [18, 19]. Increased serum levels of TNF-α were detected in arthritic mice at the end of the experiment (data not shown). Besides, direct effects of pro-inflammatory cytokines on muscle metabolism, they play a role in hyperalgesia resulting in decreased movement. These interactions might indicate that muscle mass might be a perfect biomarker for disease severity. However, the significant decrease in food intake hampers the correlations between muscle mass loss and arthritis score although a weak correlation between total muscle mass and arthritis score could be detected.

A MTX intervention group was included to study in more detail the applicability of locomotion detection for testing treatment effectiveness of pharmaceuticals or other disease

interventions. MTX is the most frequently used DMARD. Although the precise mechanisms in the treatment of RA are not completely clear, MTX exerts a variety of pharmacological actions resulting in suppression of the disease activity and reduced joint damage [20, 21]. In the present CIA study the effects of MTX treatment on arthritis score, locomotion and muscle mass were studied. In agreement with previous publications [22] MTX inhibited the arthritis development in the CIA model. Moreover, treatment with MTX results in an increased locomotion. The MTX data are in agreement with the finding that the arthritis score displays an inverse correlation with locomotion. A protective effect of MTX treatment was also detected on the muscle masse of the TA, the EDL and gastrocnemius. Although the correlation between muscle masse and arthritis score was weak, a clear modifying effect by MTX could be detected in the separate muscles.

Conclusion

The present data indicate that movement detection by camera followed by a computerized evaluation of the locomotion is a simple non-invasive quantitative method to follow disease development or disease modulation by interventions in the CIA model.

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Chapter 9

Summary and Remarks

Nutraceutical concepts for osteoarthritis and rheumatoid arthritis management

Pharmaceutical treatment of osteoarthritis (OA) patients is hampered by the incapability of the currently available medications to alter disease outcome successfully. Moreover, the commonly prescribed non-steroidal anti-inflammatory drugs (NSAIDs) and coxibs are associated with a number of potentially serious adverse events including gastrointestinal bleeding and an increased incidence of ischemic cardiovascular events [1]. As a consequence, OA patients look for alternative therapies in an attempt to modulate both pain and the structural changes that occur within and associated with a degenerating joint. Numerous nutraceuticals are being used by OA patients, among which glucosamine, chondroitin and collagen hydrolysate are proposed to supplement the substrates required for cartilage repair. Other nutraceuticals used in OA management include avocado-soybean unsaponifiables and SKI306X which have been indicated to modulate inflammation as well as cartilage metabolism. Clinical data suggest a potential role for these nutraceuticals in OA pain management. However, the ability of these nutraceuticals to change disease outcome needs to be confirmed by further research in order to establish clinical benefits.

Medical treatment of rheumatoid arthritis (RA) improved significantly during the past decades. Inhibition of the inflammatory activity in RA patients results in a reduction in pain and a delay in joint and bone destruction. However, the majority of the patients will, at least for a certain period during the course of their disease, seek for separate/additional therapies for amelioration of symptoms of pain and stiffness. Nutraceuticals used by RA patients include amongst others, fish oil, evening primrose oil, SKI306X and ginger, all of which have been indicated to modulate inflammation.

Nature offers an almost inexhaustible amount of disease modulating components. Many of these components have been investigated in detail and are used, mostly in synthetic from,

as drugs. Another group of natural products is offered as nutraceuticals. Nutraceuticals are used by arthritis patients in an attempt to modulate pain, mobility and joint destruction. Most of the nutraceutical therapies are not or only limited studied for effectiveness, safety and underlying mechanisms. The research presented in this thesis evaluates the mode of action of two of these natural products, SKI306X and apocynin. In addition, new possible cartilage modulating (*Grifola frondosa* extract and *Centella asiatica* triterpenes fraction) and anti-inflammatory (glycine and bovine lactoferrin) natural components were identified and there mode of action was studied.

Identification of new cartilage modulating nutraceuticals

At present there is still no cartilage protective agent for the treatment of OA patients available. Moreover, only a limited number of components have been indicated to modulate cartilage metabolism *in vitro* and in animal models. There is a clear need for new cartilage protective agents and a better understanding of potential cartilage protective components such as chondroitin sulfate, collagen hydrolysate and SKI306X. The work presented in this thesis describes two different avenues; the screening of a plant and mushroom library and screening of "Pubmed" documented literature, by which new cartilage modulating agents were identified and selected for further elucidation for their way of action and potential role in OA management (chapter 2 and 3).

***Grifola frondosa* extract**

In chapter 2 an assay detecting direct effects of nutraceuticals on glycosaminoglycan (GAG) synthesis by primary chondrocytes is validated. Detection of GAG production by interleukin (IL)-1β-stimulated bovine chondrocytes in monolayer cultures was described to be a quick and valid model system to test and screen novel compounds for their potential cartilage matrix synthesis stimulating effects. An active hit, a mushroom extract from *Grifola frondosa,* was selected for further evaluation. The selected *Grifola frondosa* extract was able to stimulate GAG synthesis and inhibit proteoglycan (PG) degradation by bovine chondrocytes. These cartilage protective effects were accompanied by an increase in transforming growth factor (TGF)-β production which might explain at, least partially, the way of action of the selected *Grifola frondosa* extract.

***Centella asiatica* triterpenes fraction**

Literature indicates that there is interconvertibility between the phenotype of fibroblasts and

chondrocytes. Yabu et al. showed that fibroblasts in the presence of bone morphogenetic protein (BMP) transform to a chondrocyte-like cell type [2]. Moreover, chondrocytes in cultures are able to convert into fibroblast-like cells [3]. The second way of identifying new cartilage modulating components was based on this interconvertibility. Different nutraceuticals including, *Panax ginseng* and *Centella asiatica*, which were shown to stimulate fibroblasts *in vitro* or support wound healing *in vivo*, were selected from literature. These specific ingredients were screened using the assay as described in chapter 2. A *Centella asiatica* triterpenes (CAT) fraction, that showed strong stimulating effects on GAG synthesis, was selected for further evaluation. Chapter 3 describes the effects of this CAT fraction on cartilage metabolism and on zymosan-induced inflammatory arthritis [4]. In line with the effects of *Centella asiatica* extracts on fibroblasts, the *in vitro* matrix and nitric oxide (NO) production by chondrocytes was modulated by this CAT fraction as well. Moreover, it was demonstrated that the tested *Centella asiatica* fraction was able to inhibit the zymosan-induced cartilage degradation *in vivo* although the zymosan-induced inflammatory cell infiltration and joint swelling were not affected. The *in vitro* data indicate that this cartilage protective activity could at least partially be brought about by an inhibition of the NO production by chondrocytes. Recent literature shows that asiaticoside and asiatic acid, both triterpenes which could be isolated from *Centella asiatica*, inhibit lipopolysaccharide (LPS)-induced NO, prostaglandin (PG)E_2 and pro-inflammatory cytokine production by macrophages *in vitro* [5]. Inhibition of pro-inflammatory cytokine production by synovial macrophages or macrophages infiltrating the synovium after zymosan injection could account for the *in vivo* cartilage protective effects by the CAT fraction as well. Moreover, madecassoside, another *Centella asiatica* triterpene, and asiaticoside have been indicated to inhibit arthritis development in collagen-induced arthritis (CIA) models in mice [6, 7]. These data are supportive for an anti-inflammatory capacity of a number of the *Centella asiatica* triterpenoids.

Future experiments (*Grifola frondosa* and *Centella asiatica*)

The presented studies show that both the *Grifola frondosa* extract and the *Centella asiatica* triterpenes fraction used are cartilage protective *in vitro*. More research is needed to demonstrate a possible disease modifying osteoarthritic activity.

Future experiments could include animal studies to elucidate the effectiveness of the *Grifola frondosa* extract *in vivo*, activity guided fractionation might enable identification of the active component(s) in the *Grifola frondosa* extract.

Besides the cartilage protective effects of the triterpenes fraction of *Centella asiatica*,

literature describes an anti-inflammatory capacity of a number of triterpenenes present in *Centella asiatica*. It would be of interest to identify the cartilage protective component(s) present in the tested triterpenes fraction. Knowledge of the active components will be useful for developing detailed mixtures of triterpenes with cartilage protective and/or anti-inflammatory activity, exclusively designed for OA or RA patients.

Evaluation of the way of action of SKI306X and Apocynin

Two nutraceuticals described earlier in literature as having anti-inflammatory and/or arthritis modulating activities are examined in more detail in chapter 4 and 5. Chapter 4 describes *in vitro* effects of SKI306X on cartilage metabolism and on the production of pro-inflammatory cytokines by PBMC, while chapter 5 describes the *in vivo* effects of apocynin on cartilage metabolism and inflammation using two different mouse models.

SKI306X

SKI306X is on the market in Australia and Korea as a remedy for OA patients. It consists of a mixture of three herbal extracts, *Clematis mandshurica*, *Trichosanthes kirilowii* and *Prunella vulgaris*. Clinical studies in OA and RA patients show analgesic effects and/or functional improvements of the effected joints by SKI306X treatment [8-10]. The active ingredients of SKI306X are suggested to be the oleanolic acid from *Clematis mandshurica*, rosmarinic acid and ursolic acid from *Prunella vulgaris*, and 4-hydroxybenzoic acid, 4-hydroxy-3-methoxybenzoic acid and trans-cinnamic acid from *Trichosanthes kirilowii*.

The inhibitory effect of SKI306X and its individual herbal extracts on IL-1β-induced proteoglycan degradation and NO and PGE_2 production by bovine cartilage explants, described in chapter 4, indicate a cartilage protective effect [11]. Moreover, the SKI306X also inhibits lipopolysaccharide (LPS)-stimulated tumor necrosis factor (TNF)-α, IL-1β and PGE_2 production by human peripheral blood mononuclear cells (PBMC), demonstrating an anti-inflammatory and possibly an analgesic capacity.

Apocynin

Apocynin can be isolated from the Himalayan herb *Picrorhiza kurrooa*, which is well known in traditional Indian medicine. Apocynin has been indicated to inhibit arthritis incidence and to decrease joint swelling in CIA models [12, 13]. The studies presented in chapter 5 show that orally ingested apocynin partially counteracts the zymosan-induced inhibition of PG synthesis in the mouse knee joint. Moreover, apocynin is able to induce a cyclooxygenase

(COX) inhibitory effect similar to the NSAID ibuprofen in mice [14].

Future experiments (SKI306X and Apocynin)

For SKI306X, promising *in vitro* and animal studies, suggesting the mode of action, have been performed. Moreover, clinical trials indicate an analgesic effect. The treatment duration in the described clinical trials with SKI306X in OA and RA is 4-6 weeks; clinical trials in which the patients are treated for longer periods are required to determine whether SKI306X is able to slow down the structural disease progression as measured by joint space narrowing. Research on mechanisms of action and animal studies using different arthritis models shows that apocynin might be an interesting candidate for further clinical studies in OA and/or RA patients. Although it has been indicated that apocynin has a very low toxicity (LD50: 9g/kg) [15], there are currently, no clinical trials on bioavailability or functional effectiveness in OA or RA patients registered.

Glycine and lactoferrin as new tools in inflammation and arthritis management

In the past years, evidence accumulated indicating that the simple amino acid glycine has anti-inflammatory and immune-modulatory activities. Li et al., for instance, demonstrated that dietary glycine was able to prevent peptidoglycan polysaccharide-induced arthritis in rats [16]. Chapter 6 describes the effects of orally ingested glycine in a model of zymosan-induced arthritis in mice. Glycine was able to counteract the zymosan-induced joint swelling, inflammatory cell infiltration and PG depletion [17].

Lactoferrin (LF) is a widespread iron-binding protein and member of the transferrin family. It is produced by exocrine glands (it is present in for instance tears and milk) and might be released by degranulating neutrophils at the side of infection and inflammation. Literature indicates anti-inflammatory effects of LF by inhibition of pro-inflammatory cytokine production [18-20] and up-regulation of anti-inflammatory cytokines [21].

Chapter 6 shows that both glycine and bovine (b)-LF are able to decrease ear swelling in a zymosan-induced ear-skin inflammation model. The glycine effects were concentration dependent. B-LF (0.1 or 1 mg/mouse/day) inhibited the inflammatory response although higher doses (5 and 25 mg/mouse/day) were not effective. A combination of b-LF and glycine counteracted the zymosan-induced ear swelling synergistically [17]. After testing the zymosan-induced ear-skin inflammation, spleen cells were stimulated *ex-vivo* with LPS; both glycine and b-LF were able to decrease the number of LPS stimulated TNF-α producing spleen cells. The combination of glycine and b-LF decreased the number of TNF-α-producing

cells to lower levels compared to the effect induced by the individual components.
Chapter 7 describes the effect of the combination of orally ingested glycine and b-LF in a murine model of CIA. Intervention with glycine and b-LF results in a decrease in TNF-α and IL-6 cytokine serum levels and more importantly inhibition of arthritis development.

Future experiments (glycine and b-LF)

The combination of glycine and b-LF shows a strong anti-inflammatory effect in models of acute and chronic inflammation.
Future studies on possible joint-protective effects of the glycine-b-LF mixture will define the significance and relevance of this nutraceutical intervention for the treatment of RA patients. Via *in vitro* testing of glycine on activated synovial membranes and stimulated cartilage explants the modulatory capacity on the different joint tissues can be evaluated.
Replacement of glycine by glycine rich food components will result in a more natural/ gradual release of glycine into the blood stream. The effectiveness of glycine-rich nutritional interventions should be compared to the effects induced by free glycine.

Rheumatoid arthritis and muscle loss

One of the characteristics of RA is an accelerated loss of muscle mass, also known as rheumatoid cachexia [22]. The average loss of body cell mass (BCM) among patients with RA is between 13 and 15% [23]. The BMC consists primarily of muscle mass, visceral mass and immune cell mass. A decrease in muscle mass can in part be explained by a decreased physical activity [24]. This decrease in physical activity in RA patients is closely related to pain, characterized by hyperalgesia and spontaneous pain, mostly caused and exacerbated by inflammatory mediators (cytokines, prostaglandins) [25]. Other factors contributing to muscle protein wasting are increased levels of systemic and local markers of inflammation (TNF-α, IL-1β and IL-6) as well as increased levels of oxidative stress [26].
The experiments described in chapter 8 were initiated with the aim to identify the interaction between arthritis severity, locomotion, muscle mass and, serum cytokine levels in the CIA model in mice. This, to define the suitability of the CIA model for the evaluation of muscle mass supporting interventions in RA. It was found that there is a strong correlation between clinical arthritis and locomotion. The correlations between muscle mass and locomotion or muscle mass and clinical arthritis were less pronounced [27]. In contrast to the observation that food intake by RA patients does not differ from the intake by healthy people [28, 29] a strong significant decrease in food intake by the CIA animals was detected. This indicates

that food intake is a significant variable in the CIA model influencing muscle mass.

Future experiments (muscle loss)

As mentioned above, a strong correlation was observed between arthritis severity and locomotion in de CIA mouse model. Muscle mass changes might be mediated by these changes in locomotion. However, the decreased intake of food will affect all correlations which include muscle mass. To evaluate the effect of a specified nutritional product on muscle mass changes in the CIA model the change in food intake should be taken in account and will most likely hamper easy conclusions.

Overview of the tested components and there way of action

In OA the balance between synthesis and degradation is disturbed as a result of an altered exposure of the chondrocytes to various cytokines and growth factors. Modulation of this disturbed balance could include inhibition of the release, activity or actions of the catabolic cytokines IL-1β and TNF-α or stimulation of anabolic factors like Insulin-like growth factor (IGF)-1, BMP and TGF-β [30]. The *Grifola frondosa* extract, as well as the *Centella asiatica* triterpenes fraction and SKI306X have been shown to modulate cartilage metabolism *in vitro* (Fig. 1). These modulations might play a role in restoring the balance.

Inflammation is considered to be the main target in manipulating clinical outcome as well as cartilage metabolism in RA patients. Modulators of inflammation described in this thesis that may play a role in RA intervention are SKI306X, apocynin and the combination of glycine and b-LF (Fig. 1)

The results of the tested ingredients described in this thesis are gathered in table 1. In figure 1 the different mechanisms involved in modulating cartilage metabolism or inflammation are depicted.

Grifola frondosa extract (Chapter 2)	
Cartilage/Chondrocytes (IL-β stimulated)	
PG synthesis ↑↑ PG degradation ↓↓ TGF-β2 production ↑↑	
***Centella asiatica* triterpenes fraction (Chapter 3)**	
Cartilage/Chondrocytes (IL-β stimulated)	**Joint (zymosan-induced acute arthritis)**
NO production ↓↓ PG degradation ↓↓ PG synthesis ↑↑ Proliferation ↑↑	Cell infiltration ≈ Swelling ≈ PG depletion ↓↓
SKI306X (Chapter 4)	
Cartilage/Chondrocytes (IL-β stimulated)	**LPS stimulated human PBMC**
PG degradation ↓↓ NO ↓↓ PGE_2 ↓↓	TNF-α and IL-1β ↓↓ PGE_2 ↓↓
Apocynin (Chapter 5)	
Joint (zymosan-induced acute arthritis)	**Ear (Zymosan-induced acute ear inflammation)**
PG synthesis ↑↑	Swelling ↓↓ ***Ex vivo* LPS stimulated whole blood** PGE_2 ↓↓
Glycine and Bovine Lactoferrin (Chapter 6 and 7)	
Joint (Collagen induced arthritis)	**Ear (Zymosan-induced acute ear inflammation)**
Swelling ↓↓ **Serum** TNF-α and IL-6 ↓↓	Swelling ↓↓ Cell infiltration ↓↓ TNF-α , IL-1β and IL-6 in the ear ↓↓

Table 1. Overview of the nutraceuticals studied in this thesis including the observed modulatory effect; ↓↓ indicates a decrease, ↑↑ indicates an increase and ≈ indicates no change, of the defined parameter.

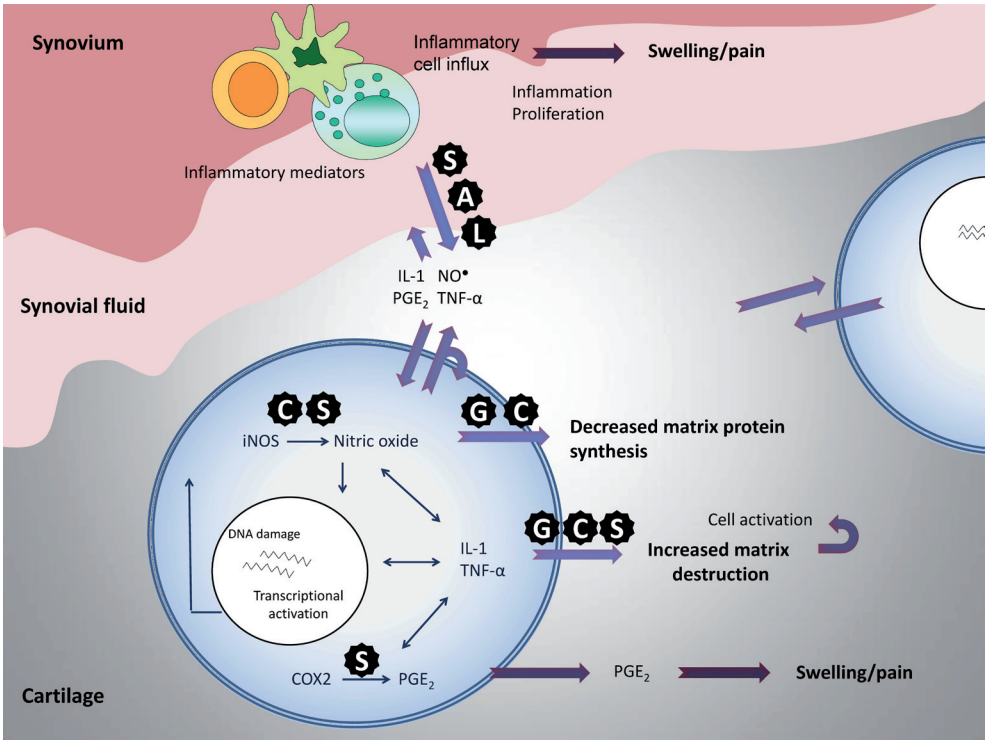

Fig 1. A simplified schematic representation of the different pathways resulting in cartilage matrix degradation. Inflammatory cytokines like IL-1β and TNF-α can be produced by the chondrocyte, synovial cells and inflammatory cells homing in the synovium or the synovial fluid. These inflammatory cytokines are able to up-regulate the production of NO and PGE_2 by the cells in the synovium as well as by chondrocytes. These catabolic factors lead to the production of proteinases and cartilage degradation accompanied by a reduced matrix synthesis. The components studied in this thesis are depicted at the site of action (as indicated in this thesis), G *Grifola frondosa* extract, C *Centella asiatica* triterpenes fraction, S SKI306X homogenate, A Apocynin, L Glycine and Lactoferrin.

Perspectives

The amount of natural products claiming a health benefit is increasing. These so-called nutraceuticals cover a broad range of products including nutrients, herbal preparations and dietary supplements.

The functional activity of nutraceuticals can vary depending on the source of the raw material, the biological variability, the storage conditions, the way of preparation and stability. It is of high importance for the efficacy and safety of these products that the quality

is maintained and therefore can be controlled. To achieve these challenging objectives the composition of the nutraceuticals has to be defined and standardized. Identification of the active constituent(s) or, if not possible, characteristic and typical marker compounds, plays a key role in enabling this standardization process. Moreover, standardized products facilitate validated research on mechanism of action, efficacy and safety.
This thesis describes the effects of three different botanical preparations: *Grifola frondosa*, *Centella asiatica*, and SKI306X. For SKI306X a number of the active constituents have been described and the composition of the preparation is based on these active constituents. The manufacturing processes of the *Grifola frondosa* extract and the *Centella asiatica* triterpenes fraction are also standardized. However, the active ingredients causing the depicted cartilage protective effects are not known and standardization has been executed on marker components. The apocynin and glycine experiments described in chapter 5, 6 and 7 were performed with purified components. The use of purified components simplifies comparative research. The activity of the bovine lactoferrin used in this thesis, although purified, can vary depending on the iron saturation of the preparation used.

Standardization of the production process and an accurate definition of the composition of nutraceuticals will facilitate proper safety and efficacy evaluations and opens a way to build on the evidence based efficacy of these natural based therapies. Increased evidence of efficacy and safety might allow the nutraceuticals to become an integral part of healthcare.

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Chapter 10

Samenvatting
Affiliations of co-authors
Curriculum Vitae
List of Publications
Dankwoord

Samenvatting

Bestudering van de effecten van nutraceuticals op het kraakbeen metabolisme en ontstekingsreacties

Reuma is een verzamelnaam voor meer dan 100 verschillende vaak chronische aandoeningen. Het grootste deel van de mensen die lijden aan reuma hebben dagelijks te maken met pijn en stijfheid in de gewrichten. De behandeling van reumatische aandoeningen bestaat voornamelijk uit het bestrijden van de pijn en het remmen van de veelal aanwezige ontstekingsreacties. Volledige genezing is meestal niet mogelijk.

Twee veelvoorkomende vormen van reuma zijn artrose en reumatoïde artritis (RA). Zowel het verlies van het op de uiteinden van de botten aanwezige kraakbeen, als ontstekingsreacties spelen een belangrijke rol in deze twee ziektebeelden. Een groot percentage van de patiënten die lijden aan artrose of RA, gebruiken naast de door de behandelende arts voorgeschreven medicatie regelmatig alternatieve producten. Veel van dergelijke alternatieven worden in de vorm van voedingssupplementen aangeboden en pretenderen de gezondheid te ondersteunen en/of een modulerend effect te hebben op fysiologische of ziekteprocessen. Dergelijke producten worden doorgaans nutraceuticals genoemd. De patiënten verwachten dat deze alternatieven, die vaak uit de natuur afkomstig zijn en een lange historie van gebruik hebben, het ziektebeeld op een veilige manier positief kunnen beïnvloeden. Voor een groot deel van de nutraceuticals geldt dat de klinische effectiviteit, de kwaliteit van het preparaat, de bijwerkingen of mogelijke interacties met andere alternatieven of de door de behandelende arts voorgeschreven medicatie niet of onvoldoende wetenschappelijk onderzocht zijn. Dit proefschrift beschrijft het effect van een aantal nutraceuticals op het proces van kraakbeenafbraak en -ontsteking.

Artrose

Artrose, ook wel osteoartritis (OA) genoemd, is een veelvoorkomende gewrichtsaandoening waarvan de incidentie toeneemt met de leeftijd. OA wordt gekenmerkt door kraakbeenschade, fibrose van het synoviale weefsel (binnenste laag van het gewrichtskapsel), ontsteking in het synoviale weefsel en de aanwezigheid van osteofyten (nieuw bot dat ontstaan is op de rand tussen bot en kraakbeen).

Het kraakbeen is de belangrijkste speler in OA. Het bestaat voor een groot deel uit een extracellulaire matrix met daarin water (60%-80%), collageen (10%-20%), proteoglycanen (5%-15%) en andere eiwitten. De kraakbeencellen die in deze matrix liggen ingebed, zorgen voor de aanmaak van nieuwe matrix en de afbraak van oude matrixcomponenten. In gewrichten die zijn aangetast door OA is de balans tussen de aanmaak en de afbraak van matrix eiwitten verstoord. Signaalstoffen (cytokines) die bijdragen aan deze verstoring zijn interleukine (IL)-1 en tumor necrosis factor (TNF)-α. Beide cytokines, die voornamelijk worden geproduceerd door de kraakbeencellen zelf, onderdrukken de aanmaak van proteoglycanen en collageen en stimuleren de afbraak van de verschillende matrix bestanddelen. TNF-α drijft vooral acute ontstekingsreacties aan, terwijl IL-1 een belangrijke rol speelt bij het instant houden van chronische ontstekingsreacties en de afbraak van kraakbeen. Door de centrale rol die IL-1 speelt bij de kraakbeenafbraak in OA patiënten, wordt de ontwikkeling van een therapie die de werking of de productie van IL-1 verminderd gezien als een goede optie om het ziekteproces te beïnvloeden.

Deze ontstekingsprocessen en de veranderingen in het OA gewricht die hiermee gepaard gaan, leiden tot pijn, stijfheid en functieverlies. OA patiënten worden behandeld met ontstekingsremmers die vaak de pijn in het gewricht verminderen. Deze ontstekingsremmers zijn echter niet in staat de ontstane kraakbeenschade teniet te doen of het voortschrijden van het ziekteproces te stoppen. Er bestaat een grote behoefte aan componenten die de afbraak van kraakbeen kunnen stoppen of de aanmaak van nieuw kraakbeen stimuleren. Dit proefschrift beschrijft twee verschillende routes waarlangs nieuwe kraakbeen beschermde stoffen kunnen worden geïdentificeerd, welke worden beschreven in de hoofdstukken 2 en 3.

Reumatoïde artritis

Reumatoïde artritis (RA) is een systemische auto-immuunziekte die voor komt bij 1% van de bevolking. De ziekte wordt gekenmerkt door chronische ontsteking van de gewrichten die leidt tot pijn, stijfheid, beschadiging van het kraakbeen en bot, en functieverlies. Het synoviale weefsel dat de binnenkant van het gewricht bekleedt, is in de door RA aangetaste

gewrichten sterk verdikt en geïnfiltreerd door ondermeer monocyten, T cellen, B cellen en verschillende soorten dendritische cellen. Deze ontstekingcellen zijn de belangrijkste spelers in het RA ziekte proces en produceren grote hoeveelheden cytokines, zoals IL-1 en TNF-α. Deze cytokines houden de ontsteking in stand wat resulteert in kraakbeen en bot afbraak.
De behandelmethoden voor RA zijn in de afgelopen jaren sterk verbeterd. Remming van de ontsteking resulteert in een afname van de pijn en in een vertraging van kraakbeen en bot schade. Ondanks de sterk verbeterde behandelmethoden zoekt een groot deel van de patiënten gedurende bepaalde periodes in het ziekteverloop naar aanvullende of alternatieve therapieën en vindt deze vaak in nutraceuticals met ontstekingsremmende eigenschappen.
Uit de wetenschappelijke literatuur blijkt dat de twee nutraceuticals SKI306X en apocynine naast een mogelijke kraakbeenbeschermende werking, ook een ontstekingsremmende werking hebben. Het werkingsmechanisme van deze twee nutraceuticals is nader beschreven in de hoofdstukken 4 en 5. De hoofdstukken 6 en 7 beschrijven de ontstekingsremmende capaciteit van het aminozuur glycine en het uit rundermelk geïsoleerde eiwit lactoferrine.

Identificatie van nieuwe kraakbeenbeschermende nutraceuticals

Grifola frondosa

Hoofdstuk 2 beschrijft een techniek waarmee grote hoeveelheden grondstoffen getest kunnen worden op hun eventuele kraakbeen beschermende effectiviteit. Kraakbeencellen die geïsoleerd worden uit stierenkraakbeen, afkomstig van restafval uit het slachthuis, zijn in kweek gebracht. De gekweekte kraakbeencellen produceren verschillende matrix eiwitten, onder andere glycosaminoglycanen (GAGs, onderdeel van de veel grotere proteoglycanen). De GAGs komen vrij in het kweekmedium. De hoeveelheid vrijgekomen GAGs is bepaald met een biochemische test (dimethylmethylene blue assay). De geïsoleerde kraakbeencellen zijn gekweekt in aanwezigheid van IL-1. Het cytokine IL-1 remt, in overeenkomst met het effect dat waargenomen wordt in het kraakbeen van OA patiënten, de productie van de GAGs. Het effect van 600 verschillende planten en paddenstoelenextracten op de door IL-1 aangetaste GAG productie is getest. Van de geteste extracten blijken er 22 de door IL-1 geïnduceerde remming van de GAG-productie te kunnen tegenwerken. Eén van deze 22 mogelijk kraakbeenbeschermende fracties, een extract van de eetbare paddenstoel Maitake (*Grifola frondosa*), is nader onderzocht. Het geteste Maitake-extract is in staat om *in vitro* (in kraakbeencelkweken) de GAG productie te stimuleren, zowel in afwezigheid als ook in aanwezigheid van IL-1. Naast de GAG-productie stimuleert het Maitake-extract ook

de productie van de groeifactor Transforming Growth Factor (TGF)-β2. Uit de literatuur is bekend dat TGF-β in staat is de IL-1 effecten tegen te werken. Het door de kraakbeencellen geproduceerde TGF-β zou een deel van de werking van het Maitake extract op de productie van GAGs kunnen verklaren. Naast een remming van de GAG-synthese is IL-1 ook in staat de kraakbeencellen aan te zetten tot de productie van enzymen die het kraakbeen afbreken. Het effect van het Maitake-extract op de kraakbeenafbraak is getest met behulp van stukjes kraakbeen (explants) afkomstig van jonge stieren. De stukjes kraakbeen zijn gekweekt in aanwezigheid van IL-1. Het IL-1 zet aan tot afbraak van de proteoglycanen uit de kraakbeen matrix. De hoeveelheid vrijgekomen proteoglycanen kan bepaald worden in het kweekmedium. Het Maitake-extract is in staat om naast het stimuleren van de IL-1 geremde synthese, ook de IL-1 gestimuleerde afbraak van de proteoglycanen tegen te werken. Toekomstige onderzoeken moet uitwijzen of het Maitake-extract ook in dieren of in een later stadium in patiënten met OA, een kraakbeenbeschermende effectiviteit vertoond.

Centella asiatica

Uit verschillende publicaties blijkt dat de uiterlijke kenmerken van kraakbeencellen onder specifieke omstandigheden kunnen veranderen in kenmerken behorend bij bindweefselcellen en omgekeerd. Verschillende natuurlijke stoffen waarvan is aangetoond dat ze bindweefselcel activiteit of wondheling stimuleren zijn getest in het IL-1 gestimuleerde kraakbeencel model zoals beschreven in hoofdstuk 2. Een extract van *Centella asiatica*, bleek in staat de GAG synthese sterk te stimuleren en is geselecteerd voor nader onderzoek.

Hoofdstuk 3 beschrijft de effecten van het geselecteerde *Centella asiatica* extract op kraakbeencel kweken en een diermodel. In IL-1 gestimuleerde stieren kraakbeen explants is het effect van het *Centella asiatica* extract op de proteoglycaan afbraak getest. Het extract blijkt in staat te zijn de IL-1 geïnduceerde afbraak van proteoglycanen uit stieren kraakbeen explants te remmen. Gelijktijdig wordt de IL-1 geïnduceerde productie van nitraatoxide (NO) geremd. NO wordt door de kraakbeencellen geproduceerd na stimulatie met IL-1. Het is een belangrijk intermediair in de door IL-1 opgewekte kraakbeenschade. Remming van de NO productie wordt gezien als een mogelijke therapie voor mensen die leiden aan OA. Naast een remming van de proteoglycan afbraak is het *Centella asiatica* extract in staat de IL-1 geïnduceerde remming van de proteoglycaan synthese door de kraakbeencellen tegen te werken.
In muizen is gewrichtsontsteking geïnduceerd door zymosan, een glycaan afkomstig uit de celwand van gist, te injecteren in het gewricht. De muizen hebben dagelijks oraal het *Centella asiatica* extract of het oplosmiddel van het extract toegediend gekregen. De toediening

van het extract is 6 dagen voor de zymosan injectie begonnen. De zymosan geïnduceerde proteoglycan afbraak is lager in de *Centella asiatica* extract behandelde muizen, in vergelijking met de muizen die alleen het oplosmiddel toegediend hebben gekregen. De zymosan geïnduceerde zwelling van het gewricht en de infiltratie van ontstekingscellen in het gewricht worden niet beïnvloed door de *Centella asiatica* toediening. De resultaten uit de studie tonen aan dat het *Centella asiatica* extract in staat is de zymosan geïnduceerde kraakbeenschade te remmen zonder dat de ontstekingsreactie in het gewricht afneemt.

Toekomstig onderzoek moet uitwijzen welk van de vele in het extract aanwezige componenten het kraakbeen beschermende effect veroorzaakt. Klinische studies moeten de effectiviteit aantonen van het extract, of de actieve componenten uit het extract, voor OA patiënten.

Bestudering van het werkingsmechanisme van SKI306X en apocynine

Van een groot aantal nutraceuticals bestaan er indicaties dat ze ontstekingsreacties en/of de ernst van OA of RA kunnen moduleren. In de hoofdstukken 4 en 5 worden de effecten van twee van dergelijke nutraceuticals nader beschreven. Hoofdstuk 4 beschrijft de effecten van SKI306X op kraakbeen explants en op de productie van cytokines door humane bloedcellen. Hoofdstuk 5 beschrijft de effecten van apocynine op het kraakbeenmetabolisme en op acute ontsteking in twee verschillende muis modellen.

SKI306X

In Australië, New Zeeland en Korea wordt SKI306X verkocht ter behandeling van OA. SKI306X is samengesteld uit drie verschillende plantenextracten afkomstig van 1) de wortels van de *Clematis mandshurica*, 2) de wortels van de *Trichosanthes kirilowii* en 3) de bloemen en de stam van de *Prunella vulgaris*. De afzonderlijke ingrediënten van SKI306X worden traditioneel gebruikt voor de behandeling van ontstekingsverschijnselen. Klinische studies in OA patiënten hebben aangetoond dat SKI306X de pijn en de afname in bewegingsvrijheid van de gewrichten kan verminderen. Hoofdstuk 4 beschrijft de effecten van SKI306X en de individuele extracten van SKI306X op IL-1 gestimuleerde stieren kraakbeen explants en op lipopolysaccharide (LPS*) gestimuleerde humane perifere bloed mononucleaire cellen

* *LPS is een karakteristieke component geïsoleerd uit celmembraan van gram negatieve bacteriën*

(PBMCs). Zowel SKI306X als de individuele extracten waaruit SKI306X is samengesteld, zijn in staat om in explants de door IL-1 geïnduceerde productie van NO en proteoglycanen te remmen. Deze effecten kunnen wijzen op een kraakbeen beschermend effect. Daarnaast zijn SKI306X en twee van de afzonderlijke extracten in staat de productie van de cytokines IL-1 en TNF-α door LPS gestimuleerde PBMC te remmen. Deze remming is een indicatie voor een ontstekingsremmend effect van SKI306X en zou een verklaring kunnen zijn voor de uitkomst van de verschillende klinische studies, waarin een afname van pijn en toename van bewegingsvrijheid werd waargenomen. Om te bepalen of SKI306X in patiënten ook een kraakbeenbeschermend effect heeft zijn klinische studies nodig met een langere tijdsduur.

Apocynine

Apocynine kan worden geïsoleerd uit het in de Himalaya voorkomende kruid *Picrorhiza kurrooa*, een kruid dat wordt gebruikt in de traditionele Indiase geneeswijze. In de literatuur is beschreven dat apocynine het optreden van artritis en de gewrichtszwelling in verschillende diermodellen van collageen geïnduceerde artritis (CIA) kan remmen. Bovendien tonen studies in kraakbeencellen aan dat apocynine de ontstekingsgemedieerde afname van proteoglycaan synthese kan remmen en dat apocynine de cyclooxygenase (COX)-2 expressie in monocyten kan voorkomen. COX-2 speelt een belangrijke rol bij ontstekingsreacties. Het COX-2 enzym induceert de productie van het prostaglandine E_2 (PGE_2). PGE_2 speelt tijdens ontstekingsreacties onder andere een rol in het ontstaan van koorts en pijn.

In hoofdstuk 5 is de werking van apocynine in het muizenmodel van zymosan geïnduceerde gewrichtsontsteking beschreven. De muizen kregen apocynine via het drinkwater toegediend. Door de door zymosan geïnduceerde gewrichtsontsteking neemt de proteoglycaan synthese in de knieschijf sterk af. Apocynine kan deze afname van proteoglycaan synthese remmen. De ontstekingsremmende effecten van apocynine zijn onderzocht in een model van zymosan geïnduceerde ontsteking in de oorschelp. In deze studie is ibuprofen meegenomen als positieve controle. Door injectie van zymosan in de oorschelp wordt een ontsteking opgewekt die gepaard gaat met een zwelling van de oorschelp. In de groep dieren die apocynine en ibuprofen toegediend hebben gekregen, is de zwelling verminderd ten opzichte van de onbehandelde dieren. Het bloed uit de onbehandelde, de apocynine en de ibuprofen behandelde dieren is na isolatie gestimuleerd met LPS. De PGE_2 productie in de LPS gestimuleerde bloedmonsters van de apocynine en de ibuprofen behandelde dieren was lager dan in de onbehandelde groep.

Uit bovengenoemde dierstudies blijkt dat orale toediening van apocynine de zymosan geïnduceerde ontstekingsreactie en de zymosan geïnduceerde reductie van de kraakbeen-

synthese kan tegenwerken. De relevantie van deze vindingen voor OA en RA patiënten moet in klinische studies worden aangetoond.

Remming van ontstekingsreacties door glycine en lactoferrine

Uit recent wetenschappelijk onderzoek is gebleken dat het aminozuur glycine een ontstekingsremmende capaciteit bezit. In hoofdstuk 6 worden de effecten van glycine in het zymosan geïnduceerde gewrichtsontstekingmodel bestudeerd. Orale inname van glycine remt zowel de door zymosan geïnduceerde zwelling als de infiltratie van ontstekingscellen in het gewricht. Via de remming van de ontsteking wordt ook de zymosan geïnduceerde proteoglycaan afname in het kraakbeen verminderd.

Lactoferrine is een veel voorkomend ijzerbindend eiwit dat ondermeer wordt gevonden in lichaamsvloeistoffen die worden uitgescheiden zoals tranen en melk. In de literatuur staat beschreven dat lactoferrine ontstekingsreacties kan tegenwerken door remming van de cytokine productie.

In hoofdstuk 6 wordt de ontstekingsremmende capaciteit van glycine en lactoferrine op de zymosan geïnduceerde ontsteking in de oorschelp beschreven. Zowel glycine als lactoferrine remmen de door zymosan geïnduceerde oorzwelling. De combinatie van glycine en lactoferrine remt de zwelling sterker dan de som van de afzonderlijke effecten (synergie). Na het opofferen van de dieren zijn de miltcellen geïsoleerd en gestimuleerd met LPS. Na stimulatie is het aantal TNF-α producerende cellen in de miltcelkweek bepaald. Het aantal TNF-α producerende miltcellen in de met glycine of lactoferrine behandelde dieren is lager dan het aantal TNF-α producerende cellen in de onbehandelde groep. Behandeling met de combinatie van glycine en lactoferrine verminderde het aantal TNF-α producerende cellen sterker dan ze afzonderlijk doen.

Hoofdstuk 7 beschrijft de effecten van de glycine en lactoferrine combinatie in een diermodel voor reumatoïde artritis. Het collageen geïnduceerde artritis model (CIA) is een geaccepteerd RA model dat veel overeenkomsten vertoond met deze ziekte in mensen. CIA kan met collageen injecties worden opgewekt in muizenstammen, die gevoelig zijn voor de ontwikkeling van auto-immuun ziekten. De ziekteverschijnselen beginnen vaak met een ontsteking in de teen van de muizen waarna de hele poot ontstoken raakt. De ernst van de ontsteking aan de poten is een maat voor de artritisontwikkeling. Orale behandeling van muizen met een combinatie van glycine en lactoferrine kan de artritis ontwikkeling in het CIA model remmen. Daarnaast wordt ook de TNF-α en IL-6 verhoging in het serum geremd door de glycine-lactoferrine behandeling.

Samenvattend kan geconcludeerd worden dat zowel glycine als lactoferrine een ontstekingsremmende werking hebben. De combinatie van de twee stoffen resulteert in een synergistiche remming van de ontstekingsreactie. De resultaten uit het CIA model tonen aan dat de combinatie van glycine en lactoferrine in staat is de ontwikkeling van gewrichtsontsteking te remmen. Toekomstige studies moeten de toepasbaarheid van deze combinatie voor het gebruik door RA patiënten aantonen.

Reumatoïde artritis en spiermassa verlies

Het meest duidelijke kenmerk van RA is ontsteking in de gewrichten. Een ander veel voorkomend verschijnsel in RA patiënten is het verlies van spiermassa. Zowel de gewrichtsontstekingen als het spiermassaverlies dragen bij aan het functieverlies in RA patiënten. De afname van spiermassa is sterk gerelateerd aan een afname in lichamelijke activiteit. Andere factoren die bijdragen aan het spiermassaverlies zijn de systemische en locale verhoging van cytokines (TNF-α, IL-1 en IL-6) en de oxidatieve stress.
Hoofdstuk 8 beschrijft een experiment dat is opgezet om de interactie tussen de ernst van de artritis, de bewegingsintensiteit, de spiermassa en de cytokine niveaus in het serum in het CIA model te bepalen. Dit alles om vast te stellen of het CIA model eventueel geschikt is voor het testen van spiermassa beschermende nutraceuticals. De resultaten tonen aan dat een sterke samenhang bestaat tussen de ernst van de artritis en de bewegingsintensiteit. De correlatie tussen spiermassa en de bewegingsintensiteit of de ernst van de artritis, is minder duidelijk. Onderzoek in RA patiënten heeft aangetoond dat het voedingspatroon normaal gesproken niet verandert. Dit is echter in tegenstelling tot de sterk verminderde voedselopname in de CIA muizen. Deze verandering in voedselopname door de zieke muizen is een extra variabele die van grote invloed is op de spiermassa. Deze extra variabele bemoeilijkt in hoge mate het trekken van conclusies uit experimenten waarin de effecten van voedingsinterventies op de spiermassa getest worden.

Tot slot

Voor een groot deel van de nutraceuticals geldt dat de klinische effectiviteit, de kwaliteit van het preparaat, de bijwerkingen of de mogelijke interacties met de door de behandelend arts voorgeschreven medicatie, niet of onvoldoende wetenschappelijk is onderzocht. De functionele activiteit van een nutraceutical is afhankelijk van een groot aantal factoren: de herkomst van het ruwe materiaal, de biologische variabiliteit, de opslag condities, de manier

van opwerken en de stabiliteit van het product. Zowel voor het bepalen van de activiteit als voor het garanderen van de veiligheid, is het van belang dat de kwaliteit van de producten kan worden gewaarborgd en gecontroleerd. Om controles mogelijk te maken moet de samenstelling van de nutraceuticals worden beschreven en gestandaardiseerd. Identificatie van de actieve component of, indien dit onmogelijk is, het gebruik van markercomponenten kunnen het standaardisatieproces ondersteunen.
Dit proefschrift beschrijft de effecten van drie plantaardige preparaten: *Grifola frondosa*, *Centella asiatica* en SKI306X. Voor al deze preparaten is het productie proces gestandaardiseerd. Van SKI306X zijn een aantal werkzame componenten beschreven, de samenstelling van het preparaat is gebaseerd op vaste hoeveelheden van deze componenten. De actieve kraakbeen beschermende componenten in het Maitake en het *Centella asiatica* extract zijn niet bekend. Standaardisatie is in deze twee gevallen uitgevoerd op in het preparaat aanwezige marker componenten. Het gebruik van gezuiverde stoffen vereenvoudigt het wetenschappelijk onderzoek, omdat wordt uitgegaan van een goed omschreven actieve componenten. De apocynine en glycine experimenten zijn uitgevoerd met gezuiverde componenten. Ook het gebruikte lactoferrine is een gezuiverde component. Toch kan de lactoferrine activiteit, afhankelijk van de hoeveelheid ijzer die aan het eiwit gebonden is, variëren. Om vergelijkend onderzoek mogelijk te maken, moet de verzadiging van het lactoferrine preparaat duidelijk omschreven worden.

Een goede omschrijving van de productsamenstelling van nutraceuticals opent de weg naar gedegen wetenschappelijk onderzoek. Na het wetenschappelijk aantonen van de effectiviteit en de veiligheid van de specifieke nutraceuticals kunnen deze worden overwogen als een integraal onderdeel van de gezondheidszorg.

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Curriculum Vitae

Anita Hartog-te Kortschot werd geboren op 5 februari 1964 te Angerlo. In 1980 behaalde zij het MAVO diploma aan de Prinses Beatrix MAVO in Doesburg. In datzelfde jaar startte zij het voorbereidend jaar van de hogere beroepsopleiding HBO-A aan de Opleiding voor Laboratorium personeel Arnhem Nijmegen (OLAN), de huidige Hogeschool Arnhem Nijmegen. In 1983 behaalde zij het HBO-A diploma in de richting Cytologie & Histologie.
Van 1983 tot 1988 was zij achtereenvolgens werkzaam op de afdeling Anatomie en Embryologie van de Universiteit van Amsterdam, de afdeling Anatomie van de Vrije Universiteit Amsterdam en op de afdeling Nierziekten van het UMC St. Radboud in Nijmegen. Vanaf 1985 volgde zij de HBO-B avondopleiding aan de voormalige laboratoriumopleiding Amsterdam, de huidige Hogeschool van Amsterdam. In 1988 behaalde zij het HBO-B diploma in de richting Medische Biologie met als hoofdvak Immunologie.

Van 1988 tot 2001 was zij werkzaam als senior analiste op de afdeling Celfysiologie van het UMC St. Radboud onder leiding van Prof. Dr. R.J.M. Bindels. In deze periode nam zij deel aan verschillende onderzoeksprojecten waarin het werkingsmechanisme van ionkanalen in de nier werd bestudeerd.

Vanaf 2001 is Anita Hartog-te Kortschot werkzaam bij Danone Research - Centre for Specialized Nutrition in Wageningen, voorheen Numico - Research. Hier startte zij als junior onderzoeker op een project waarin de effecten van TGF-β remming op wondheling en littekenvorming werden bestudeerd.

Van 2002 tot 2006 nam zij deel aan een door Senter gefinancierd project getiteld: “Onderzoek

naar de structuur functie relatie van nieuwe botanische extracten en de validatie voor de behandeling van gewrichtsziekten". Dit onderzoek heeft de basis gevormd voor een aantal in haar proefschrift verschenen artikelen.

Na beëindiging van dit project heeft zij deelgenomen aan verschillende projecten binnen het immunologie platform. Vanaf 2008 is zij als onderzoeker betrokken bij twee onderzoekslijnen:

1. het preklinische en klinische onderzoek naar de effecten van verschillende voedingscomponenten op het immuunsysteem in ouderen en
2. het Top Instituut Pharma project "CXC chemokine receptors: potential targets from chronic inflammatory diseases".

De eerste resultaten uit het Top Instituut Pharma project maken deel uit van dit proefschrift.

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Anita

"*In de wetenschap gelijken wij op kinderen, die aan de oever der kennis hier en daar een steentje oprapen, terwijl de wijde oceaan van het onbekende zich voor onze ogen uitstrekt*" (Newton).