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AUSTRIAN MARSHALL PLAN FOUNDATION

"Adenosine receptors: new therapeutic target in osteoarthritis treatment"

Bachelor`s Programme

"Medical and Pharmaceutical Biotechnology"

by Hanna Schön

Submitted on: 01/03/2016

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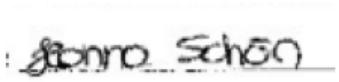

01/03/2016

Date

Hanna Schön

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Abbreviations

ACLT	Anterior Cruciate Ligament
ADA	adenosine deminase
ADP	adenosine di phosphate
AK	Adenosine kinase
AMP	adenosine mono-phosphate
AMPK	AMP-activated protein kinase
ATP	adenosine tri phosphate
AV node	atrioventricular node
B lymphozyten cells	B cells
BMD	bone mineral densitsy
cAMP	cyclic adenosine mono-phosphate
CD73	ecto-5'-nucleotidase
COPD	Chronic obstructive pulmonary disease
C terminal telopeptides of type II collagen	CTX II
Extracellular matrix	ECM
Femoroacetabular impingment	FAI
GDP	guanosinediphasphate
GTP	guanosinetriphasphate
Interleukin 1 receptor	IL 1 receptor
Matrix metallopeptidase 13	Mmp13
MIA	monosodium iodoacetate
MMT	medical mensical tear
MRI	magnetic resonance imaging
Osteoarthritis	OA
Tumor necrosis factor	TNF

Abstract

Osteoarthritis (OA) is a very common disease affecting 15 million of people in Europe. Adenosine, acting at its four receptors, has long been known to regulate inflammation, immune responses and bone and cartilage homeostasis. The importance of adenosine and its receptors in osteoblast, osteoclast, bone marrow homeostasis and chondrocyte physiology and pathology are already demonstrated.

Based on previous *in vitro* and *in vivo* studies, our hypothesis is that adenosine injection in the intraarticular space has a protective effect during OA progression. The aim of the experiment is to evaluate the effect of periodically injection of adenosine in the cartilage development. For this purpose different parameters of cartilage degradation were considered, including the reduction of cartilage degradation, the presence of osteophytes and calcified cartilage and changes in the trabecular and cortical subchondral bone. In order to test our hypothesis a rat animal model was used. OA was induced in 14 weeks old rats by mechanical rupture of the anterior cruciate ligament and consequent knee joint destabilization. Animals were injected in the intratrabecular space every 10 days over a time period of 8 weeks. Injections contain saline, liposome and liposomes with adenosine. After 8 weeks the animals were sacrificed and the knee joints were collected. Samples were scanned in a miro-computer tomography (µCT) machine, in order to study the bone morphology and quantify the cartilage volume. After µCT analysis, the samples were decalcified and the tissue was used for histology. The Safranin-O stained slides were used to calculate the OARSI score for each animal. The results show that adenosine injection prevent OA progression, reduce cartilage calcification and cartilage damage, loss of trabecular bone and cortical bone thickness.

Key words: Osteoarthritis, rat model, Adenosine, injections

Introduction

Osteoarthritis

Osteoarthritis (OA), the most frequent joint disease worldwide, it is a progressive degenerative disease particular defined by loss of articular cartilage. It starts with a breakdown of joint cartilage and pursue with remodelling processes in adjacent bone. Finally resulting in the damage of the bone's articular surface. Consequences of OA are pain and stiffness of the joint with limited mobility. Joints may deform and eventually are destroyed completely (Figure 1). OA can occur in any joint in the body. However most affected are weight bearing joints such as hip or knee joints. (Buckwalter JA, 2004)

Approximately 10% of men and 18% of women over 60 years are affected. (S. Glyn-Jones, 2015) OA can even affect people in their 20s and 30s, as a consequence of joint injury or exorbitantly joint stress. There are various methods to slow down the progression of the disease but an ultimate treatment has not been established.

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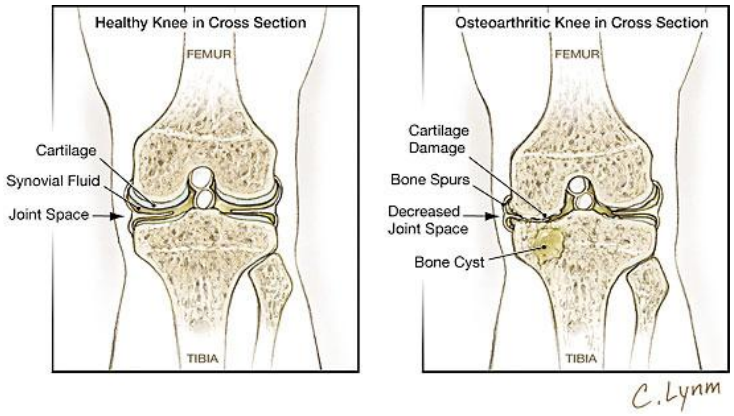

Figure 1 Illustrates the difference between a healthy knee and a knee affected by OA in the cross sectional area (Parmet, 2003)

What causes Osteoarthritis?

Osteoarthritis can be developed with or without the presence of risk factors but the more risk factors a patient has, the greater the likelihood of suffering from this disease.

Especially if the number of risk factors is high, protective measure should be taken in order to lower the risk of Osteoarthritis.

The biggest evidence in understanding the cause of OA is the identification of all risk factors. Possible risk factors involved in the development of OA are: injury, excessive stress or underlying disorder of cartilage, age, obesity, sex. Osteoarthritis can occur in almost any joint in the body. However most affected are those operating underbody weight such as hip or knee joints. In addition the disease is always linked to a breakdown of cartilage in the joints. (Heidari, 2011)

Longitudinal studies have proven that osteoarthritis develops through the operation of different biomechanics on joints. Joint biomechanics are settled by anatomical and functional factors. Risk factors of this group would be joint morphology or hip dysplasia. Beside that tibia and femoral bone morphology as well as limb alignment play an important role in development of Osteoarthritis.

Moreover when it comes to leg length, knee OA is two times more common in the shorter than in the longer leg if the inequality exceeds 1 cm. Despite this strong association, the majority of individuals with abnormal joint biomechanics will not suffer from osteoarthritis. (S. Glyn-Jones, 2015)

A further risk factor for hip OA would be overly sport activity. An excessive exercise during adolescence might lead to the development of femoroacetabular impingement (FAI) morphology. FAI is a situation where the bones of the hip occur in unusual shape. The hip bones rubbing on each other and as a consequence the cartilage is damaged. (S. Glyn-Jones, 2015)

The strongest risk factor in OA is however age due to the assumption of a general erosion. Beside that OA is more common in women then in men, due to the production of oestrogens although the involvement of hormones is not fully understood yet. (Michael C. Nevitt, 2010)

Furthermore the joint capacity can be impaired by a physical violation that causes bone or cartilage damage and has an adverse impact on the meniscus. (Figure 2) Studies have been shown that the risk of developing knee OA is four times higher after the knee has been seriously hurted. Obesity it's another risk factor. Obese patients will increase their risk for knee osteoarthritis by three times. Knee OA is in this case more frequent than hip OA, reasons therefore are still unknown. Strong genetic basis for osteoarthritis could be shown by the realization of family based studies. The prediction of OA development unlikely but it might help getting insights into disease pathogenesis for individual joints. A number of different studies such as epidemiological studies of family history, family clustering, twin studies and exploration of rare genetic disorders have proven evidence of a genetic influence of OA. In addition a relation between OA and the chromosomes 2q, 9q, 11q, and 16p could be proven. (S. Glyn-Jones, 2015)

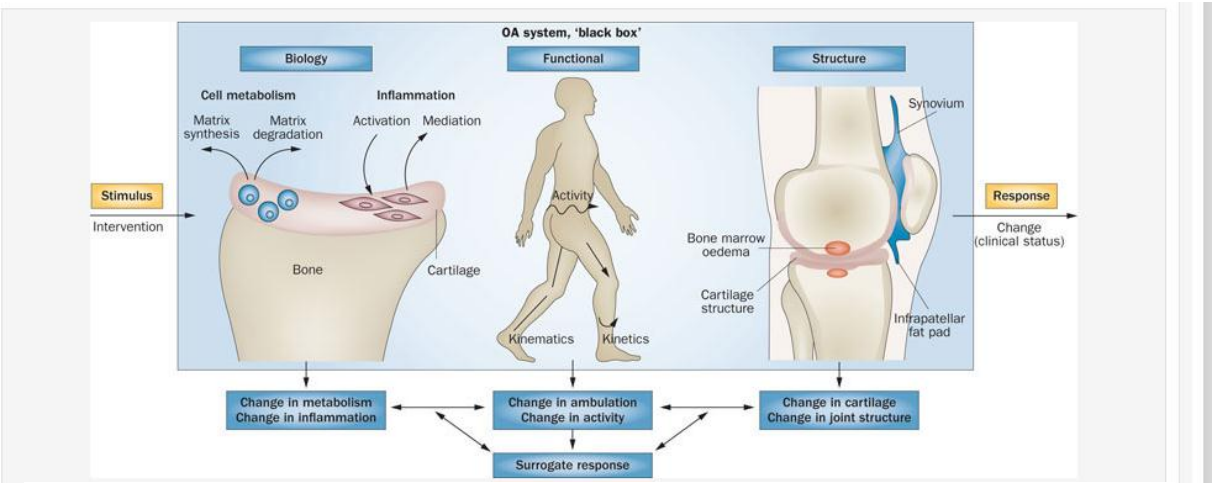

Figure 2 Shows the consequences of Osteoarthritis on the biological, functional and structural level (Andriacci, 2012)

Symptoms

Pain in the joints is a well-known symptom of OA. The intensity of pain is aggravated by damp and cold weather. The —start-up pain is exemplarily for OA. After a long period of rest, the first movements are very painful. Pain gets better as long as the activity is continued. An advanced stage of OA establishes itself by the occurrence of swelling and bruising at the joint as well as joint deformations. Further distinctive symptoms are: joint aching and soreness, particularly with movement, pain after overuse or long periods of inactivity, stiffness after periods of rest, joint swelling and bony enlargement in the middle and end joints of the fingers. (Rizvydeen, 2012)

Treatment

Important aspects concerning treatment of osteoarthritis is the identification of the main risks. This can differ from patient to patient. An early diagnose combined with an efficient treatment can softens the pain. The most significant point when it comes to handling osteoarthritis is lifestyle modification.

One of the major risk factors of osteoarthritis is obesity. Especially in the lumbar spine and the knee the pain can get really powerful. A reduction in weight is the first step towards prevention of osteoarthritis. Furthermore in order to reduce the mechanical and inflammatory stressors that are related to Osteoarthritis the body fat needs to be minimized. A combination of exercise, dietary modification, medications, and maybe bariatric surgery will lead to a successful reduction of weight and in the future lower joint pain and increased physical function. The earlier a life modification takes place, the longer will be the lifespan of load bearing joints. (Vincent, 2012)

During the development of the disease patients use different kind of pharmacological treatment with the purpose to reduce the pain and ameliorate the pain. Some of the most used drugs are listed below:

Paracetamol and non steroidal anti inflammatory drugs have an huge impact when in comes to symptom control. Drugs like chondroitin and glucosamine could manifest anti inflammatory and anticatabolic properties in vitro. However in clinical trails the outcome was not promising, which can be due to different drug formulations. Although in both combinations is glucosamine, better results were gained using glucosamine sulphate instead of glucosamine hydrochloride. It is proven that hyaluronic acid in synovial fluid is reduced in OA patients. As a consequence hyaluronic acid was given via intra articular injections but the efficiency and safety is questionable. Also the success was little when lubricin, a glycoprotein which acts synergistically with hyaluronic acids, was used. Another way was tried out by using degenerative enzymes: But only small benefits could be recorded by using doxyclin, an inhibitor of matrix metalloproteinases. Also using other inhibitors of matrix metalloproteinases no satisfactorily results could be achieved. Even worst many result in musculoskeletal toxicity. Also tests with nitiric oxide synthase, a upstream intracellular signalling molecule, could not show any satisfactory results. Bisphosphates was used to inhibit osteoclast activity, which reverse the subchondral bone changes which go along with osteoarthritis. A reduction in urinary CTX II, a biomarker used to monitor OA, concentration could be proven but no difference in joint space narrowing.

The target of most medication is the inflammation, related to OA. Improvement of symptoms could be achieved by intra articular steroid injection but has no benefits for the structure. An improvement over a short time period could be shown by using Anakinra, an antagonist of IL 1 receptor. Also with the monoclonal antibody against interleukin 1 receptor no clinical benefit could be proven. Adalimumab, a monoconal antibody to TNF alpha, show good results when it comes to inflammatory knee osteoarthritis. With recombinant human bone morphogenic protein and fibroblast growth factor cartilage repair in vitro could be proven.

Multipotent mesenchymal stem cells are found in healthy and diseased cartilage. Although it is still unclear if Kartogenin has a beneficial affect in clinical trails, it is known that it promotes chondrocyte differentiation and cartilage repair in animal models of established osteoarthritis. (S. Glyn-Jones, 2015)

If patients are not responding to pharmacological therapy, surgery and knee replacement are their only option. Surgery is most efficient when the underlying reason is a disorientation of bones. For example by reorientation of the acetabulum the progression of OA caused by hip dysplasia is reduced. Hip dysplasia is defined by a wrong shape of the hip, or an incorrect position of the hip socket. As a consequence an increase in force is noticeable, and abnormal erosion on the cartilage. After the surgery not only the symptoms are getting better but also the hip survival rate oversteps 80% at 10 years. Furthermore the long term risk of OA can be reduced. Injured knee alignments can lead in long term to OA. A fast repair can prevent the cartilage from serious damage. Methods to achieve this goal are: transplantation of autologous cartilage or the stimulation of cartilage regeneration. The transplantations of plugs of cartilage and subchondral bone form healthy regions of a joint to areas with injury are refered as Mosaicplasty and osteochondral grafting. Crucial therefor is the quality of the healthy cartilage. Beside that another method exist called Microfracture, in which the production of new cartilage is stimulated. By traumatising the subchondronal bone chondroprogenitor cells are released. This technique is easy, inexpensive and widely used. However all these techniques still have disadvantages and so more advanced techniques needed to be approved. One future idea called autologous chondrocyte implantation is to harvested and cultured chondrocytes before implantation into the cartilage. In resent studies mesenchymal and embryonic stem cells have been used. Furthermore tests with growth factors and cells implanted into three dimensional scaffolds or matrices that support growth have been carried out. But unfortunately the results are not to promising when it comes to OA. However cartilage repair can just be successful if the joint environment is neither biologically nor mechanically hostile. (S. Glyn-Jones, 2015)

Diagnostic methods

In order to collect the medical history (anamnesis) the doctor will first perform a detailed discussion with the patient. By asking different questions to the mobility of the patient the doctor tries to find out the progression of the disease. In a next step a physical examination is done. The doctor assesses the joint position and function. A MRI measurement will give detailed information about the disease pattern. Beside the MRI measurement Biomarkers are frequently used.

There is a variety of biochemical markers such as cytokines, enzymes and extracellular matrix constituents. Their concentrations are dependent on the tissue metabolism and can be proven through blood, urine or synovial fluid. Diagnosis and prediction are their main usage. The number of biomarkers which have been tried out is huge although not all are used in clinical practice Diagnostic biomarkers are deployed to show pathological changes in patients. CTX II (C terminal telopeptide of collagen type II) and cartilage oligomeric matrix protein, with a great performance, are used to prove tissue degradation. It is known that in patients with osteoarthritis the concentration of CTX II in urine and cartilage oligomeric matrix protein in serum is higher compared to healthy people.

Unfortunately biochemical markers are neither sensitive nor specific. The Kellgren Lawrence score is used to diagnose knee osteoarthritis. The reported area under the curve is 0 .70 for urinary CTX II, 0.73 for radiographic joint space width and 0.82 for MRI measurements.

Urinary CTX II and serum cartilageoligomeric matrix proteins can give statements about the progression of hip and knee osteoarthritis. Urinary CTX II works better than the joint space width but the best method would be a MRI measurements. The best way to prognoses structural knee osteoarthritis is to use a combination between MRI measurement and urinary CTX II. However the reliability of biochemical markers in combination with symptoms is low. The interpretation of assay results is a combination between the understanding of the biological activity and the relevance for the clinical osteoarthritis. With the proteomics, study of function and structure of proteins, also the number of biomarkers has gained, because it could prove the specifity of biomarkers to joints. In the future biomarkers should be used to identify

the most appropriate therapies for each patient by a detailed evaluation of osteoarthritis with disease phenotype. In the future combinations between biomarkers, imaging and genotyping should improve diagnostic methods. These day amendments have still been done when it comes to sampling and the fact that the concentration of biomarkers is determined by factors such as diet, physical activity, metabolism. (S. Glyn-Jones, 2015)

Biological Background

- Physiology of Bone Formation, Remodelling

Bone, defined by its rigidity, power of regeneration and hardness, has many essential functions in order to protect the body: it shelter the vital organs, serves as reservoir for calcium homeostasis as well as for growth factors and cytokines, facilitate an environment for marrow and participate in the acid- base balance. In order to adapt to biomechanical changes and micro damages bone undergoes a constant remodelling process. The bones consist of two components: the cortical bone, a solid material surrounding the marrow space and the trabecular bone, which looks like a network made out of trabecular plates. Cortical bone consists of an outer periosteal surface, bearing blood vessels, nerve fibres, osteoblasts and osteoclasts and supports bone formation and an inner endostea surface, a membranous structure covering the inner surface of cortical and cancellous bone.
Moreover it can be distinguished between two types of bones dependent on the order of collagen forming osteoid: woven bone, with order less collagen fibres and lamellar bone, with parallel collagen. As a result of the structure the woven bone is weaker than the lamellar one and is normally produced when osteoblasts rapidly produce osteoid. This normally happens in foetal bones and fractured bones but with time the woven bone is substituted by the stronger lamellar bone. As a consequence all bone, in an adult is lamellar bone.

There is a huge variation in bone cells, one can distinguish between support cells: osteoblast, osteocytes, remodelling cells: osteoclasts, non-mineral matrix of collagen and non-collagenous proteins called osteoid, with inorganic mineral salts deposited within the matrix. In addition bone undergoes different processes: longitudinal, radial growth, modelling and remodelling. The process of formation new bone is called ossification and carried out by osteoblasts. Two important steps are involved: intramembranous ossification, where bone serves as connective tissue and leads to the formation of bones. The second step is called endochondral ossification, where a cartilage model acts as a forerunner. (Usha Kini, 2012)

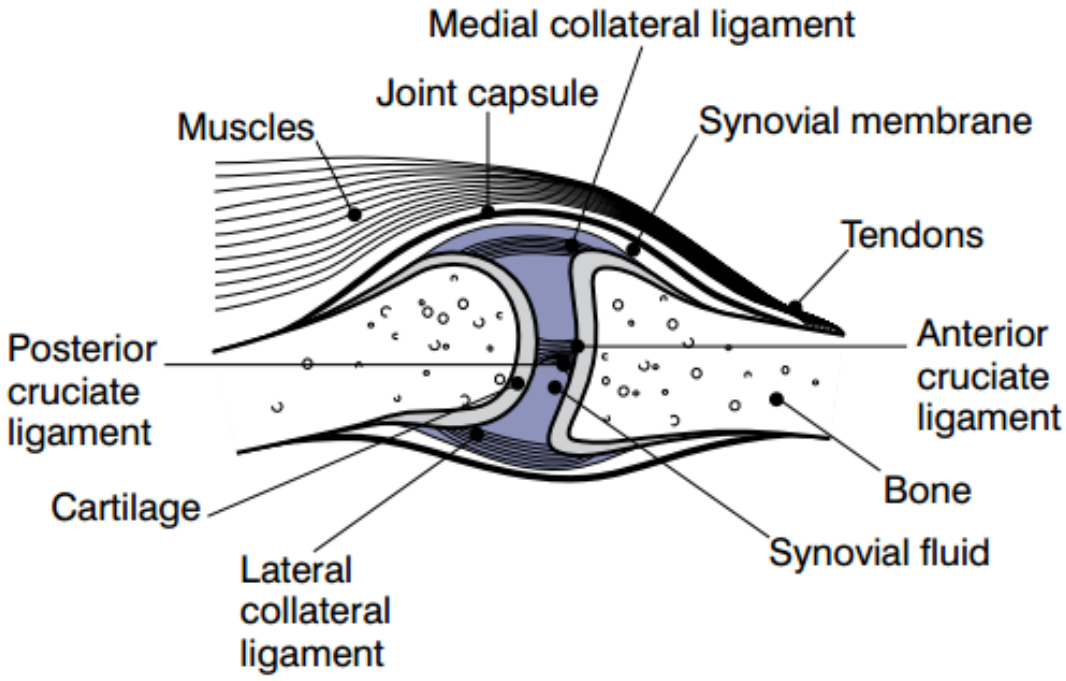

Figure 3 Illustrating structure of a bone (Diseases, 2015)

Bone remodelling in OA

Subchondral remodelling is a consequence of to much pressure on the joints, the aim of this process is to repair bone damage, and as a result the normal collagen network is out of order. This process is pushed into the deep layers of cartilage through an increased vascularisation which goes hand in hand with rapid bone turnover. The consequential reduction of aggrecan, leads to stiffness of the cartilage. Through a positive feedback mechanism within the bone, bone density is increased. In a further step the reduction of aggrecan, results in synovial thickening and loss of B cells from the synovial lining. B cells form inhibitors to catabolic enzymes, which as a consequence provides greater availability of collagenases and other enzymes that destroy cartilage. All these reactions can provoke a secondary inflammatory reaction which can speed up the process of cartilage destruction as well. Finally, this process leads to progressive cartilage loss, and in a further step to OA. (Gallant, 2012)

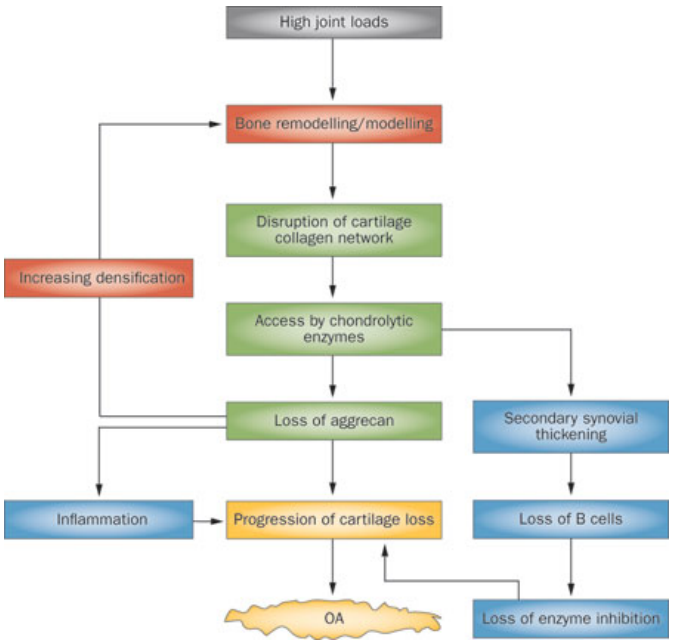

Figure 4 Bone remodelling process (Gallant, 2012)

The key role of cartilage, subchondral bone, and synovium in disease pathogenesis.

Cartilage

The end of bones in joints are covered by Cartilage, a rubbery like material. Articular cartilage with its complex structure and highly specialized can be found in diarthrodial joints. Its main function is to diminish the frictional coefficient by facilitate the joints with a smooth surface. Blood vessels, lymphatics and nerves are absent in articular cartilage. Healing and repair processes are limited in articular cartilage. Injury to articular cartilage is known to cause fundamental musculoskeletal morbidity. Fluid and solid components are crucial for its mechanical attitude.

- Struktur

Articular cartilage is about 2 to 4 mm thick and free of blood vessels, nerves or lymphatics. It is composed of extracellular matrix, which is build up by water, collagen and proteoglycans and other non-collagenous proteins and glycoproteins and contains chondrocytes. This composition allows to keep enough water within the ECM, which is crucial for the mechanical attitude as mentioned above. Articular cartilage is made up of different zones —superficial zone, middle zone, deep zone, and calcified zone .

Within each zone, 3 regions can be marked—pericellular region, territorial region, and interterritorial region. (Alice J. Sophia Fox, 2009) The strength of the cartilage is dependent on type II collagen, the main structural protein of cartilage, together with other collagen types and non-collagenous proteins. Aggrecan and other proteoglycans, which draw water into the cartilage, increase the cartilage`s compressive strength. Chondrocytes control the architecture as well as the biochemical composition. Furthermore they produce various of inflammatory response proteins, such as cytokines – interleukin 1ß, interleukin 6; tumour necrosis factor ∝, metalloproteinases.

In osteoarthritis the innate immune system is activated. Toll like receptors, which are expressed in chondrocytes get activated by damage-associated molecular patterns such as glycosaminoglycan hyaluronan. Beside that also Calcium pyrophosphate and sodium urate cyrstals can bind to chondrocyte toll like receptors.

Amazing is the fact that the expression and activation of the complement system is high in human osteoarthritis joints. Cartilage oligomeric matrix protein serves as an actívator of the alternative complement pathway, whereas proteoglycans (fibromodulin) support the classic pathway. Chondrocytes are also responsible for the expression of glycation end products, which accumulate in ageing tissues. This process could explain the increasing prevalence of osteoarthritis with age. This answer to extracellular matrix components might simply show amplification of established cartilage degradation. Chondrocytes could first be invoked by inflammatory signals coming from other joint structures like as synovium or subchondronal bone. (S. Glyn-Jones, 2015)

- Chondrocytes

OA shows some similar aspects to chondrocyte differentiation during skeletal development by endochondral ossification. For example: articular chondrocyte proliferation, the expression of hypertrophy markers (MMP-13 and collagen X), reorganisation of cartilage matrix by proteases, vascularization and focal calcification of joint cartilage with calcium hydroxyapatite crystals. If the articular cartilage is healthy, chondrocytes resist proliferation and terminal differentiation. But as soon as the cartilage is diseased, chondrocytes grow and develop hypertrophy. Furthermore vascularization and calcification of joint cartilage can be notices. Signalling molecules controlling chondrocyte activities in growth cartilage may be involved in OA pathogenesis. Therefore signalling molecules, which regulate chondrocyte activities during osteoarthritis, are the most promising target of therapy.

Different processes can be observed during OA: proliferation and hypertrophic differentiation of chondrocytes, remodelling and mineralization of the extracellular matrix (ECM), invasion of blood vessels and apoptotic death of chondrocytes.

In growth cartilage the differentiation of chondrocytes is addressed to both positive and negative control, interacting within a signalling network to regulate the progression of the process.

The control of OA occurs by autocrine signals coming from chondrocytes or by paracrine signals coming from cells of surrounding tissues. Survival and proliferation is controlled by the interaction of chondrocytes with their surrounding matrix via cell surface receptors.

Proteinases are fell effectors of ECM degradation and play a huge role in the regulatory networks, by shutting down control elements (endoplasmic reticulum protein 57) and by changing precursors into active agents. Furthermore proteinases can act as mediator by direct cleavage or by release from ECM stores. Most signalling occasion peak at the level of gene expression and so are transcription factors essential in regulation. Different positive and negative feedback mechanisms are known. Articular cartilage is made for life. Articular chondrocytes tracing low metabolic activity under normal conditions, in order to maintain their surrounding ECM including collagens, proteoglycans and non-collagenous matrix proteins. Under healthy conditions, the cells stay in a resting state and do not from proliferate or differentiate. In a diseased state articular chondrocytes start to proliferate and differentiate, combined with marker expression for the over hypertrophic differentiation stage, resulting in apoptotic death and mineralization of the diseased cartilage. OA is seen as a multifactorial disease; but at least part of the process is based on hypertrophic differentiation.

Differentiation of chondrocytes results in an extended metabolic activity of articular chondrocytes, a turnover in the expression of ECM molecules, and a changed pattern of proteases. The differentiation leads to a disturbed cartilage homeostasis preferring degenerative changes. Signalling factors involved during endochondral ossification were also proven to play a role in OA cartilage, but just under diseased conditions.

As soon as signalling triggers the regulation of ECM or activation of proteases such as MMP-13, differentiation changes can be related to a potential activator of OA. However it is important to be aware that the information was gained by using spontaneous, transgenic or surgically induced mouse models of OA but research was not done in large animals, which probably would better reflect human OA pathophysiology (Dreie, 2010)

Subchondral bone

The subchondral bone is located below the cartilage. Its main function is to observe shock, but it is also important when it comes to cartilage metabolism. The subchondral layer is rich of vascularisation and vulnerabilistation. Some terminal vessels are in close contact with the deepest hyaline cartilage layer. Approximately 50% of glucose, oxygen and water requirements of cartilage flow through these vessels. The subchondral layer is full of variation in bony structure, local metabolism, hemodynamic and vascularization. In osteoarthritis the thickness of the subchondral region is increased. It has been shown that the layer gets thicker as soon as the articular cartilage starts to wear away. Moreover a production of cytokines and growth factors could be proven in the subchondral bone layer, which triggers inflammation and changes in cartilage tissue. (imhof, Sulzbacher, Grampp, Czerny, Youssefzadeh, & Kainberger, 2000) Cortical bone serves as connecting piece between the calcified cartilage under the tidemark and the underlying trabecular bone. In OA both the cortical and the trabecular bone are affected.
It could be proven that cartilage degradation can be due to changes in subchondral bone and osteophyte formation, which is known as an unusual bone growth. Another possibility would be remodelling of subchondronal bone.

Synovium

Joints and tendon sheaths are connected by the synovium, a thin layer of tissue. Its main function is to keep the environment balanced within the joint and tendon sheath. The synovium can act as a membrane, determining the in and out flow. Beside that it is the reason why joints stay smooth. The term synovitis is used as soon as the layer become irritated and thickened under OA conditions. (Jonathan Cluett) Synovitis is a common characteristic of osteoarthritis. In advanced osteoarthritis, proliferation of synovitis and tissue hypertrophy happen which lead to an increased vascularity. Hyaluronic acid and lubricant are important factors in synoviocytes synthesis. They are responsible for an optimum joint functions and reduce OA proliferation. Inflammatory mediators and degenerative enzymes are set free by chondrocytes and osteoblasts.
Synovitis forecasts the development of symptoms and cartilage loss. (S. Glyn-Jones, 2015)

Adenosine and its receptors

Purinergic signalling

Purinergic receptors are membrane receptors that are responsible for different physiological and pathological functions in response to release of ATP or adenosine. The existence of three classes of purinergic receptors could be proven: P1, P2X and P2Y. P1 receptors respond to adenosine and the nucleotides ATP and ADP; this receptors are part of the G protein coupled receptors family. The PI purinoceptors response better to adenosine and AMP than ADP and ATP. Antagonist to those receptors are methylxanthines including caffeine. As soon as the PI purinoceptros are occupied the activity of adenylase cylase is switched and the level of cAMP is higher. P2 purinoceptors are more sensitive to ATP compared to AMP and adenosine.In 1980 it was demonstrated that there are two different extracellular adenosine receptors. The activation of the first receptor inhibits adenylate cyclase whereas the activation of the second receptor stimulates adenylate cyclase. Soon the name A1 and A2 appeared in the literature. In addition the two receptors also show different agonist profiles. Furthermore the existence of two types of A2 receptors were proven. The difference lies in the needed concentration of adenosine analogues for activation of the two receptors. Molecular cloning brought more information about the three adenosine receptor subtypes. Further research shows the existence of another adenosine receptor which was named A3. It was demonstrated that A3 receptor is concentrated in the peripheral tissue in the brain and immune system, where it is part of the release of allergic mediators from mast cells. (G.Burnstock, 2010)

Adenosine

Adenosine, a purine nucleoside and main endogenous agonist at the P1 receptor class, is composed of the nucleobase adenine and a ribose connected by a glyosidic bond. As soon as an aberration between the rates of ATP synthesis and ATP utilization happens, adenosine is formed in the cell. An example therefore is a limitation in oxygen and glucose supply. Adenosine is involved in both physiologic and pathologic biochemical processes. The molecule can bind to four transmembrane receptors: A1, A2A, A2B and A3. Adenosine receptors belong to the G-protein coupled receptor family. These receptors are prevalent in the whole body and have different functions like the control of cell proliferation and signal transmission of inflammation. Adenosine receptors are known as auspicious drug target. The main reason therefore is their strong presence in almost all cells. Moreover different agonist and antagonist, with high affinity and specify for adenosine receptors were synthesize. Signal transfer happens due to the activation of heterotrimeric signalling reaction. All four subtypes of receptors are encoded by different genes. The receptor subtypes can be distinguished by the variation of their affinity for adenosine analogues or in their favoured mechanism of signal transduction. Metabolic distress is excuded by purine nucleoside adenosine, which is moulded in the extracellular space by breakdown of ATP that has been released and cleaved by ectoATPases/apyrases and ectonucleotidases. In hypoxic tissue the level of adenosine is increased. But due to the instability of adenosine only an increase of local adenosine receptor signalling can be recorded during hypoxic stress. (G.Burnstock, 2010)

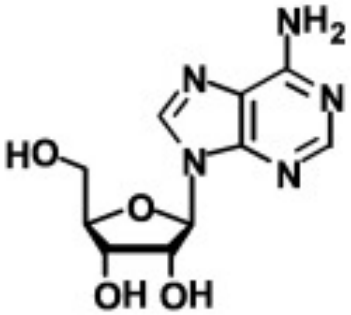

Figure 5 Structure of adenosine molecule (Alice Gaudin, 2015)

Adenosine metabolism

Nucleotides including ATP, ADP, GTP and GDP are highly concentrated in a cell and have a short half life time. Those molecules are degraded in the related nucleoside by phosphatases. For example AMP and adenosine are transformed back to ATP by different kinases. Under normal conditions the adenosine concentration in a cell is low.

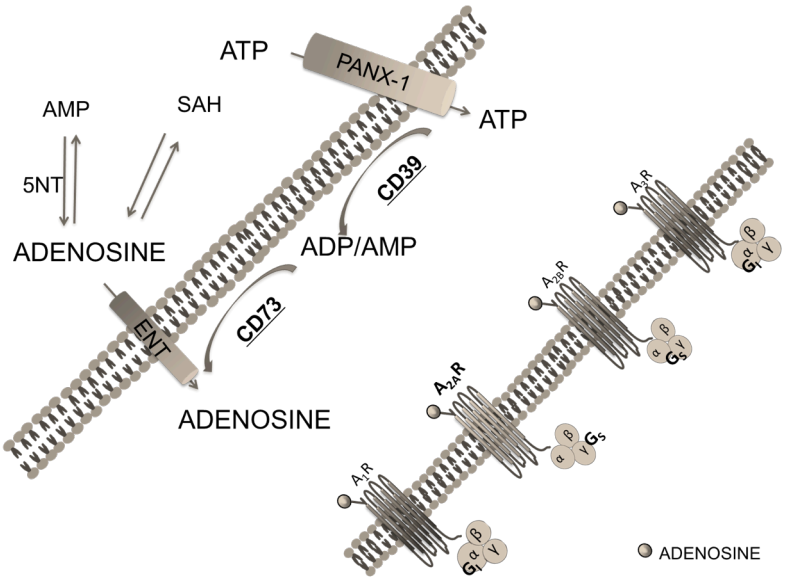

Figure 6 Adensoine metabolism

Adenosine kinase (AK) is the regulatory enzyme in the cycle of ATP breakdown and formation and is responsible for the transformation of AMP back to ATP. Within a cell ATP and adenosine are permanent recycled this process is dependent on the energy requirement of a cell. The execution happens via series of dephosphorylation and phosphorylation steps conducted by various enzymes. If metabolic stress occurs the recycling process is disturbed. As a consequence an increased phosphatase activity leads to a high level of adenosine. Furthermore extracellular adenosine is clustered due to an increased diffusion and active transport of nucleotides out of the cell carried out by an enzyme called ecto 5`nucleotidase (CD73). Intracellular adenosine level is regulated by AK and extracellular adenosine level is regulated by adenosine deaminase (ADA) which degrades adenosine to inosine. AMP activated protein kinase (AMPK) is a further really important regulator enzyme in the ATP adenosine pathway. The Km value, stands for the substrate concentration at which the reaction rate is at half maximum, is higher for ADA compared to AK. This fact indicates that AK is activated at a lower concentration of adenosine. The ATP metabolic pathways in and outside are cell are completely distinct. Intracellular ATP-adenosine cycle is controlled by cytosoloc 5` nucleotidase and AK, extracellular ATP is converted not only to adenosine but also to inosine regulated by CD73 and ADA.

Beside that enzyme levels are not always kept constant, therefore in hypoxia the adenosine level is increased due to a increased CD73 expression. In addition also the amino acid L homocyteine in connection with the enzyme S-adenosyl homocysteine can have an influence on adenosine. (Evans, 2012)

Adenosine and its receptors

The four adenosine receptors are A1, A2A, A2B and A3. Those receptors detect local changes in adenosine concentration. The all belong to the family of G- linked receptors. A2 receptors work on G_s, but A1 and A3 interact with G_i and G_o.

A1: The A1 receptor is responsible of the effects of adenosine in the heart. A stimulation of the A1 receptor leads to an inhibition of nerve cells. Adenosine causes a pharmacological cardioversion which means that an abnormally fast heart rate is converted to a normal rhythm. That means that Adenosine can lowering the heart rate and especially slow down the AV nodal conduction.

A2A: The A2A receptors encourage the anti-inflammatory effect. The function of these receptors include: improvement of neural communication, coronary vasodilatation promotion and effect the platelet.

A2B: A2B receptors are similar to A2A receptors, but still their function is not totally clear yet. Although they are found in the entire body, they are especially found on human mast cells. This can be important for asthma treatment.

A3: A3 receptors are known to be key target in stimulation as well as inhibition of growth cells. Dependent on the concentration of adenosine present A3 receptors can have good or bad effects. (Lara Hopley, 2006)

Potential therapeutic use

Adenosine agonist as well as antagonist can have potential therapeutic use.

Heart: It has the power to relax vascular smooth muscles, which is done by an reduction in calcium uptake. In addition Adenosine can also reduce the vascular tone. The disadvantages should not be neglected: by reducing conduction time through the AV node the heart can be affected. The affect of the drug is moderate and leads to a reduction in systolic, diastolic, and arterial blood pressure. The problem is that Adenosine is quickly degraded. Here especially the A1 receptor and propably A3 receptors are important.

Neurology: Furthermore adenosine receptors might be helpful when it comes to drug development in Parkinson's disease , schizophrenia and Alzheimer`s disease. Especially A2A antagonists may be helpful in Parkinson`s disease, but this could not be proven yet. The reason therefor is the expression of the adenosine receptors in the dopamine rich regions of the brain. (Bertil B. Fredholm, 2003)

Pain: Another field of action could be pain management, especially Adenosine receptor agonists might be useful.

Asthma: It is known that adenosine receptors are expressed on inflammatory cells involved in asthma and COPD. The inhibition of the A2B receptors can help in those diseases. (Lara Hopley, 2006)

Furthermore it is known that adenosine plays a huge role in regulatory processes of bone remodeling. The presence of all four adenosine receptors in bone has been well reported. Studies could prove an involvement of adenosine in osteoclast formation and function through A1 receptor. Although the involvement in bone formation, remodeling of adenosine could be proven deeper understanding needs to be gained in bone metabolism in order to develop new therapies for osteoporosis. (He W1, 2011)

Agonist and Antagonist

Table 1 shows Adenosine receptor and its agonists and antagonists

receptor	Agonists	Antagonists
A1	R-PIA, CPA TCPA, CVT-3146, CVT-510, GR 79236	DPCPX, N-0861, CVT-124 (=BG9719), KW-3902, FR166124, FK453, WRC-0571 , CPX , FSCPX
A2A	CGS 21680, APEC, 2HE-NECA	SCH 58261, ZM 241385, CSC, KF17837
A2B	None	Enprofylline, IPDX, MRS 1754
A3	IB-MECA, CI-IB-MECA3'-Aminoadenosine-5'-uronamides	MRS 1067, MRS 1097 L-249313, L-268605, CGS15943, KF26777,

(Lara Hopley, 2006)

Animal Models of Osteoarthritis

Animal models are crucial in order to make predictions about the efficacy of drugs and to study the disease mechanisms of Osteoarthritis especially changes in tissues of the joint such as cartilage, bone, synovium, synovial fluid, tendon, ligaments and joint capsule. It is not easy to study Osteoarthritis development in human patients. In a later step of the drug development the drug, with the most promising in vivo effect in animal models is used for further clinical trails. It is advised to use skeletally mature animals, 12 weeks or older. For example it is known that young, growing rats develop less cartilage pathology. (N.Gerwin, 2010) A slow progression, high variability, unknown initiating factors of biomechanical, immunological or genetic origin make it hard to study Osteoarthritis in humans. It is unlikely to see early signs of OA in patients. Serious sights appear at a more advanced stage of the disease, after cartilage is degenerated and the joint space is narrowed. Therefore animal models play an important role when it comes to new anti OA drug development. In order to evaluate safety, dosage and toxicity of a new possible drug. To make a biomarker analysis, another important step in drug development, serum, urine, and synovial fluid needs to be collected.

An optimal model is required in order to achieve best results.

1. The reproducibility in a suitable time window is highly important. The time frame in human OA is a critical point because progression and development is in most cases slow. The perfect time window from induction to clinically significance are one to twelve months. When it comes to the interpretation of efficacy of therapy the difference in progression between human and animal model should be considered.
2. In order to be able to study early, mid and late stage of development of Osteoarthritis the time windows should be comparable. Frequently animal models are too aggressive or the disease advancement happens to fast. Stagnation is known to be a huge problem. Comparison in disease development should be possible between the individual animals.

3. Mammalian species are preferred when it comes to animal models. In addition various criteria need to be fulfilled: it should be controllable, cheap, easy to house, large enough for analysis steps,..
4. The most difficult aspect when it comes to animal models it the combination between human and animal pathology. For example late stage OA is well known in humans whereas early stage Osteoarthritis is almost unknown. Genomic analysis of ACLT rats has proven that the expression of many genes is converted in a similar way in rats as well as in humans. A main disadvantage is that often only the single joint tissue is taken into consideration and the joint as an organ is neglected. Further problems occur in combination with temporal differences and genetic differences. An example therefore would be the lake of collagenase, matrix metalloproteinase (MMP)-1 in adult rats and mice. When it comes to the interpretation of the results, awareness of those differences is needed.
5. The best option would be: what works in animals works in patients. Unfortunately many therapies that achieve good results in animal models are less useful in humans and vice versa.

Many different animal models exist but none of them is able to fulfil all the criteria mentioned above. However in order to achieve good, reliable results, the choice of a suitable animal model is highly important.

Osteoarthritis induction methods

There are three existing methods to induce OA in small animals: intraarticular injection of a variety of agents, spontaneously occurrence of OA and surgically induction. Especially in the rat model spontaneous osteoarthritis is extremely uncommon, most often surgically induced medical mensical tear (MMT) is applied followed by anterior cruiate ligament transection (ACLT) (N.Gerwin, 2010)

1. Usage of intraarticular injection of a variety of agents in order to induce pathologic changes by using enzymes, cytokines, transforming growth factors, chemicals. A high number of the used agents cause an acute local inflammation at the site of injection, which makes the comparison to natural disease process in human OA difficult. MIA (monosodium iodoacetate) is the most common used agent for intraarticular injections. It is known as an inhibitor of glycolysis and induces significant chondrocyte apoptosis and joint inflammation.
2. In specific genetically modified mice OA can occur spontaneously. This method is especially used in order to gain information about the role of specific molecules in the disease process. Inbred strains of mice, guinea pigs and macaques are known for the occurrence of spontaneous OA. The main problem when it comes to this method is that the underlying mechanism of developing OA does not necessarily need to be the same as in human. In addition higher variability and extended time frame happen.
3. Surgically induced OA is the most common method where a destabilization of joints is produced. Which is the most common cause for secondary OA in human. Surgically induced OA includes ACLT and meniscal injury which are well known from human. The main advantage of this method is the temporal control of the disease induction and the progression of the disease is predictable.

   ACLT

   ACLT surgery leads in adult rats to progressive cartilage degeneration. Cartilage degeneration happens slow and the progression is not fast. There is a lack of Osteophytes, if present they are really small. Fibrocartilage replaces articular cartilage, this phenomena is most likely detected in the lateral femoral condyle. ACLT result in less severe disease compared to other models. So also the threshold for detecting protective effects of therapeutic interventions is expected to be lower. In general various strains of rats are used but for the ACLT especially the Lewis rats are preferred. (N.Gerwin, 2010)

Species choice

The choice of the species is highly important in order to achieve reliable results. Not all induction methods go along with all type of animal models. For example ACL transection can have different effects dependent on the species. This is due to anatomic and biomechanical differences. Other factors that can influence the progression of the disease are age, gender, maturity of the animal, level of exercise.

Outcome measures

Although OA does not only include cartilage degeneration mostly analysis focus on cartilage degeneration. One oft he most popular scoring system is described by Mankin. The most widely used non invasive measurement of the disease for human is JSN but it can only be applied in a late stage of the disease. Another method which get more and more used in human as well as animal models is the MRI. In addition the aim to find a universal but specific biomarker is high. (Smith, 2008)

Methods

- Experimental design

OA was induced by mechanical rupture of the anterior cruciate ligament and consequent knee joint destabilization.
Animals were inject in the intratrabecular space every 10 days over a time period of 8 weeks.
Injection contain saline, liposome and liposomes with adenosine (n=6 for each group). For each group, 3 of the animals received the first injection right after the ACL rupture (prevention group) and other 3 after 1 week (treatment group).
After 8 weeks the animals were sacrificed and the knee joints were collected.

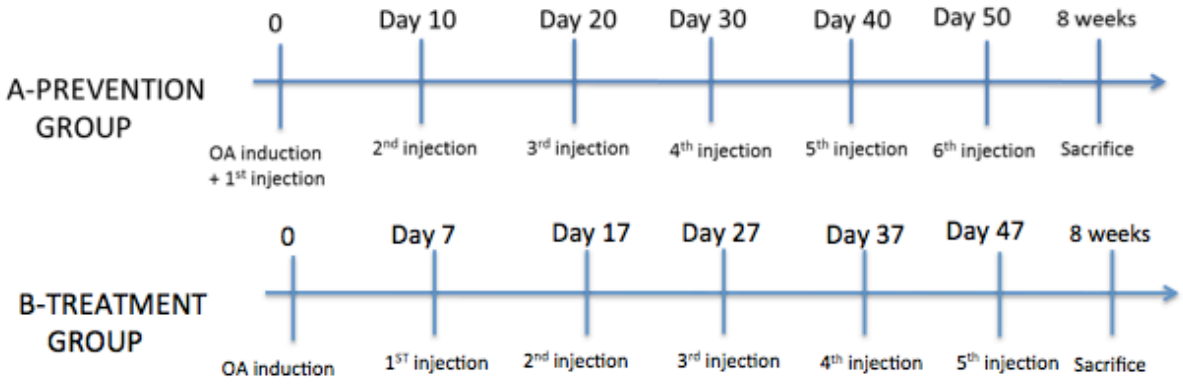

Figure 7 3 of the animals received the first injection right after the ACL rupture (prevention group) and other 3 after 1 week (treatment group).

- Animal

Mice employed in this study were kept under regular lighting conditions (12h light/dark cycle) and given food and water ad libitum.
Protocols for experimental procedures involving the use of animals were approved by the New York University School of Medicine Institutional Animal Care and Use Committee.
We used 14 weeks old male Sprague Dawley rats.

- ACL rupture

The rat is placed into the injection box. The rat is now put to sleep with the aid of isoflurane, a compound used for inhalational anesthesia. As soon as the rat is unconscious it is put in a special machine (Figure 6) It is highly important that the level of isoflurane is kept constant otherwise the rat will wake up again. The knee of the rat is clamped within a special machine. The machine was bought from a company called Bose. In order to be able to induce the ACL rupture the Oran Kennedy lab developted a specific protocol for this procedure.

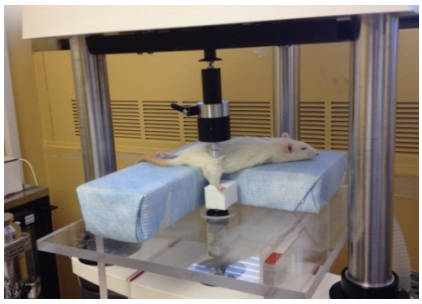

Figure 8 machine used for ACL rupture

The leg of the rat is fixed within a stony block and a metal stump. The metal stump is moved further down to the stony block and as a consequence stress is applied to the leg. As soon as the very end point is reached the anteriar cruciate ligament is ruptured. This leads to an instability in the knee and as a further consequence to OA. After the ACL rupture took place the ruptured knee is shaved and the injections are given. With the injections also pain killers are spouted. In a further step the weight of the rat is taken. After completion of the procedure the rat is put back into its cage.

- Treatment

The rats are treated for 8 weeks with an injection each 10 days. The injections include saline, Liposomes alone and Liposomes in combination with Adenosine.
In order to create the liposomes in a first step Soybean oil (S7381- 250 ML), Ethyl alcohol, Pure (E7148- 500ML) were mixed together.
To those containing the agonist, Adenosine (A4036-5G) was added.
L alpha Phosphatidylcholine (P4279-100MG) and Cholesterol (C8667-500MG) were assorted and well vortexed. And in a further step added to the previous mixed solution containing Soybean oil, Ethyl alcohol and dependent on the injection adenosine.
A further vortexing step is carried out bevor Saline and Glycerol (G6279-1L) are added.

The finished solution needs to be vortexed a gain for a few minutes.
The injections can be stored at 4°C over night.

- Tissue harvesting

After 8 weeks the animals were sacrificed and the knee joints were collected.
Soft tissue and muscles were removed from the bone. The femur is seperated from tibia.
The bone was fixed in Paraformaldehyde (32% Solution, EM Grade (15714)). From the original 32% Solution a 4% solution was prepared. The samples were left in paraformaldehyde for two days on the shaker at -4C.
In order to decalcify the bones, they were put into a solution containing EDTA for the following 40 days. A pH of around 7 is highly important.
After completion of the 40 days the samples were left with the micro CT core.

- Micro- CT

After the animals were sacrificied and the bones were seperated from the soft tissue the analysis step follows. In previous experiments the correct concentration and incubation time of the contrast agent Hexabrix were stated in order to recreate segmentation of femoral articular cartilage from enhanced µCT scans. The optimum proportion turns out to be 60% of Hexabrix and 40% of PBS solution. The incubation time was determined to be 6 hours at 37C. After completion of the incubation time the samples were send to uCT core. Micro CT is a 3D microscopy, in which the internal structure of objects is displayed as an image nondestructively with very fine resolution. The scanned pictures were sent back and further processed.

- Ctan

Ctan is the software to analyze micro-CT datasets in 2D and 3D for morphometric and densitometry.
Note of terminology:
Region of interest (ROI) – refers to the selected region
Volume of interest (VOI)- refers to the incorporation of all the ROIs across all the selected image levels
First the wanted set of micro-CT results are loaded up. After the upload is completed several buttons will appear in the main toolbar. Next the region of interest is selected. Those volumes of interest changed dependent on what needed to be analyzed.

Femur: Find the point were the growth plates break from each other. The high density tissue inside the growth plates go away with time. The first slide of interest is the one where everything is disappeared. The last slide of interest is the one where the two sides are still connected to each other by trabecular bone.
Tibia: From the femur go directly to the beginning of the tibia. By moving away the trabecular bone is increased and as soon as the lower left part shows a trabecular part the first slide of interest is reached. At the growth plate a cavity gets visible as soon as it becomes apparent the last slide of interest is reached.
Cortical bone: The cortical bone is analyzed at the mid-shaft. The mid shaft is the top of the range which is taken into consideration. The region of interest is about 62 slices.
Trabecular bone: To determine the upper side search for the point where growth plate breaks and substract 25 slides . From this upper side substract 258 slides and this will be the bottom.

The most important part of the femur and the tibia is the very top section, because here the damage took place. In the next step the regular shape of the region of interest is selected and the unwanted part is now cut out. With a special tool called invert edit ROI the outside of the ROI becomes inside and vice versa.

The slides are the saved. When it comes to rat sample every single slide needs to be done, because the differences between them is huge.

Analysis

As soon as all samples are done you can go on to the analysis part. Here the calibration and the correct parameters are important. In the tool “Histogram”, in the calibrate section the “Attenuation coefficient” can be found. By clicking “Calibrate” the needed values can be filled in.

For the BMD analysis the attenuation coefficients were 0.25 phantom, AC=0.01926 and 0.75 phantom, AC=0.04286. After pressing OK the following equation will show up: BMD = (Ac-0.00746/0.0472)g/cm3, Binarization range 86-255

For the analysis the before prepared samples are uploaded. The bottom “costume processing”, “image inside ROI view” and the “batman icon” need to be pressed.

Cortical bone: A new screen is shown and the bottom “internal” is pressed and following items from the occurring list in 2D analysis are selected: threshold, despeckle, ROI shrink-wrap, 2D analysis. The correct order is highly important.

Threshold: high 255 and low 103

Despeckle: remove inner object in 2D space

ROI shrink wrap: 2D and stech over holes <6 pixles

2D analysis: all parameters

Once the parameters are set all samples need to be added. As soon as VOI is pressed and they are added. The icon is shown in red. Click on ROI and the ROI screen will appear.

By clicking on the file, the icon will be green. When all samples are added, start is pressed and the samples will be analyzed.

For the trabecular bone: In the tool “internal” a new list of items need to be selected to analyze in a 3D analysis: Threshold, Despeckle, 3D analysis. The correct order is highly important.

Threshold: high 86 and low 22

Despeckle: in 3D remove white speck within volex size <1

3D analysis: all results

Once the parameters are set all samples need to be added. As soon as VOI is pressed and they are added. The icon is shown in red. Click on ROI and the ROI screen will appear.
By clicking on the file, the icon will be green. When all samples are added, start is pressed and the samples will be analyzed.
The results will be found in the excel file.
BMD (Bone mineral density) analysis is done manually using the CTAN program: open image in VOI file, then select region of interest icon and load ROI and the binary section and a new set of folder appear . In the graph write the threshold values and then clicks on from dataset and analysis will be done. Check on the list: the first mean is for trabecular and the second mean for cortical BMD value.
After this step the prepared slides can be converted into binary images. The Histogram dialog is opened and higher and lower global threshold levels are determined. By performing those steps the bone mineral density can be measured. Data can be easily captured and ready data table (e.g. in excel) are possible. By using the BatMan analysis parameters such as bone volume, bone surface, trabecular thickness, trabecular separation, number, cortical section can be find out.

- Safranin O'staining

This staining method is specially used for the detection of cartilage, mucin and mast cell granules on formalin-fixed, paraffin-embedded tissue section and in frozen sections. This type of staining is especially recommended because Safranin O stains are specific for cartilage glycosaminoglycans and PGs. The cartilage will appear in oragen to red, the nuclei normally in black and the background will be stained in blue to green. After the staining is completed the slides are closed with multi media. The process of staining was done according to the NovaUltra^{tm} Safranin O Strain Kit (Cat# IW-3011). In order to perform later on the histologic scoring it is important that the three sections appear on the slides. The mean value for each parameter of the three sections per knee is taken.
In the first step the sections are deparaffinized by 2 changes of xylene (UN1307). Each change lasts 10 minutes.

Then the sections are hydrate by 2 changes of 100% alcohol. Each change lasts for 10 minutes. Followed by 90% alcohol and 70% alcohol for 1 minute each, and the rinse in distilled water. All EtOH solutions were prepared out of the Ethyl alcohol, Pure (E7148-500ML). As a next step the slides are stained Weigert's Iron Hematoxylin Solution (Cat# IW 3011B) for 5 minutes. A dilution of 1:2 in water is used. Then the slides are put under running tap water for a few minutes and rinsed briefly with distilled water. The slides are next stained in Fast Green Solution (Cat# IW-3011C) for 10 minutes, followed by acetic acid (Cat# IW 3011D) for a few seconds. Again the slides are briefly rinsed with distilled water. In a last staining step Safranin O Solution (Cat# IW- 3011A) is added for 3 minutes. A solution of 1:2 in water is prepared.The slides are rinsed briefly in distilled water and then dehydrated through 90% alcohol for 1 minute, 2 changes of 100% alcohol for 5 minutes each. All EtOH solutions were prepared out of the Ethyl alcohol, Pure
(E7148-500ML). Then the slides are cleared by 2 changes of xylene (UN1307). The first xylene change is carried out for 5 minutes the second one foor 10 minutes. The slides are covered using mounting medium (SP15 – 100).

- Analysis

Pictures are taken from those stained samples and are exactly observed on the computer. Cartilage degeneration, osteophytes score, calcified catilage, growth plate thickness are taken into consideration. Here only the tibia is of relevant.

1. Cartilage degeneration: The cartilage is divided into three zones and each of them is scored for 0-5 dependent on the degradation.
2. Osteophytes: the biggest osteophyte from each segment is counted (from the deepest point at the chonro-osseous junction to the surface of the overlying cartilage)
3. Clacified cartilage: the section showing the most damaged is taken into counting. It should give information about the effects of agents on OA associated changes in the subchrondral bone
4. Growth plate thickness: the growth plate thickness is measured medially and laterally

Results

The aim of experiment is to show a reduction in the development of OA in OA rat model after periodically injection of adenosine in liposomes. The effects are demonstrated by factors including the reduction of cartilage dregradation, the presence of osteophytes and calcified cartilage and changes in the trabecular and cortical subchondral bone. Expected is a stagnation in the development of Osteoarthritis and an improvement of cartilage and bone structure.

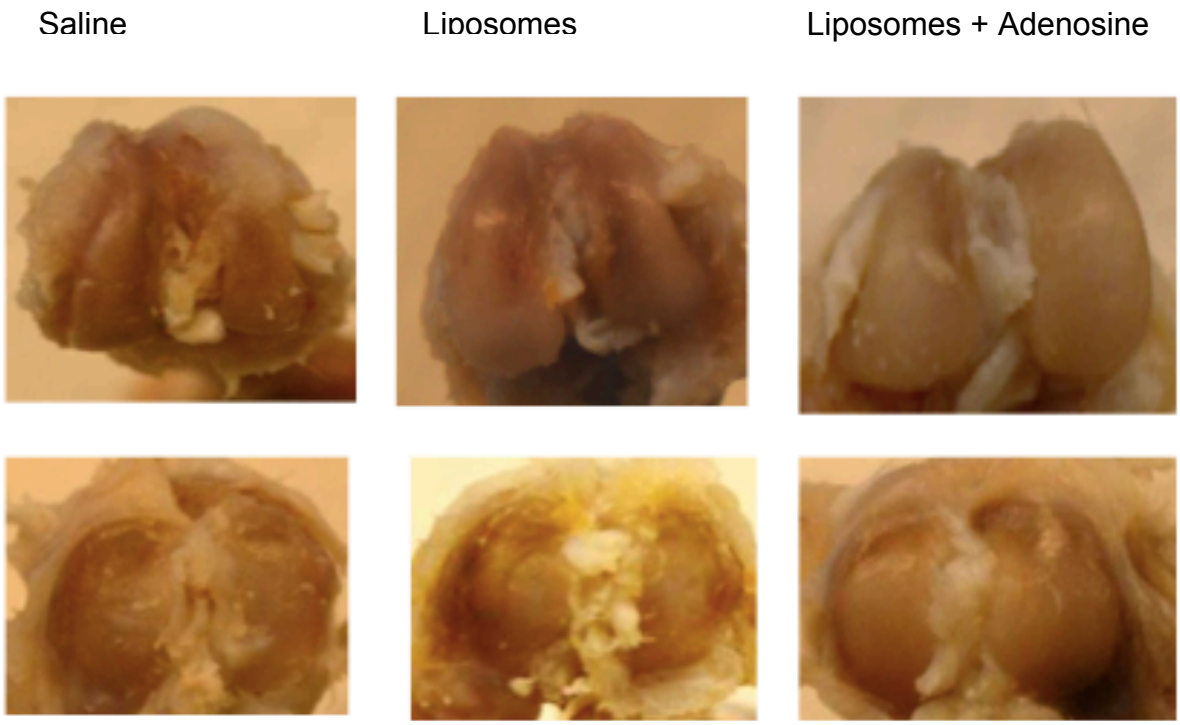

Figure 9 Results after 8 weeks treatment and 10 days injections. Pictured are tibia and femur. Injections include saline, lipsomes alone and liposomes in combination with adenosine.

After 8 weeks the legs of the animals were collected, soft tissue was removed and femur and tibia separated. Then pictures were taken right away before any further treatment.

Figure 9 clearly demonstrate the differences between the various compounds. No recovery at all could be registered in those knees saline was injected at regular intervals. Cartilage as well as bone look worst. Slightly better results could be achieved by administer Liposomes alone. But still the appearance of cartilage and bone is impaired. The best results were achieved by a combination between Liposomes and Adenosine.

3D reconstruction of the cartilage of the femur

With the aid of the software admira the above mentioned huge differences in loss of cartilage between the diverse injections administered periodically could be demonstrated even better. Figure 10 shows that the worst results are scored by saline injections and the best results are scored by liposome plus adenosine injections.

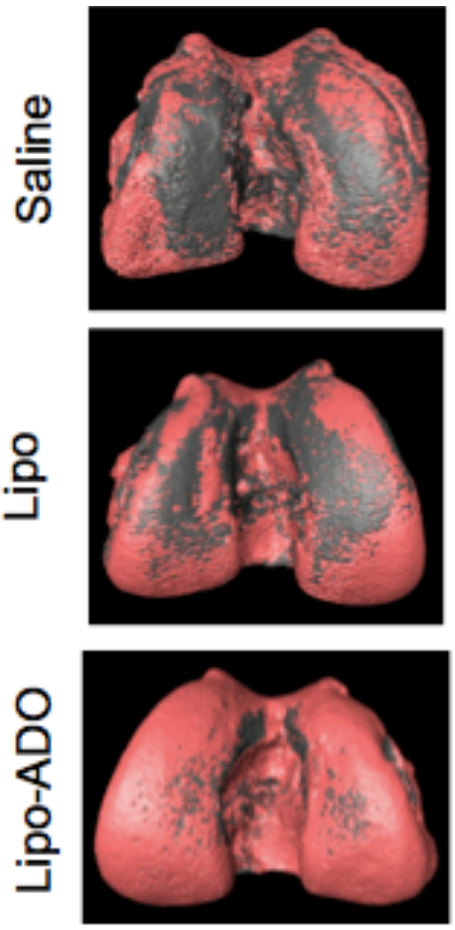

Figure 10 3D reconstruction oft he cartilage of the femur with the aid oft he admira software

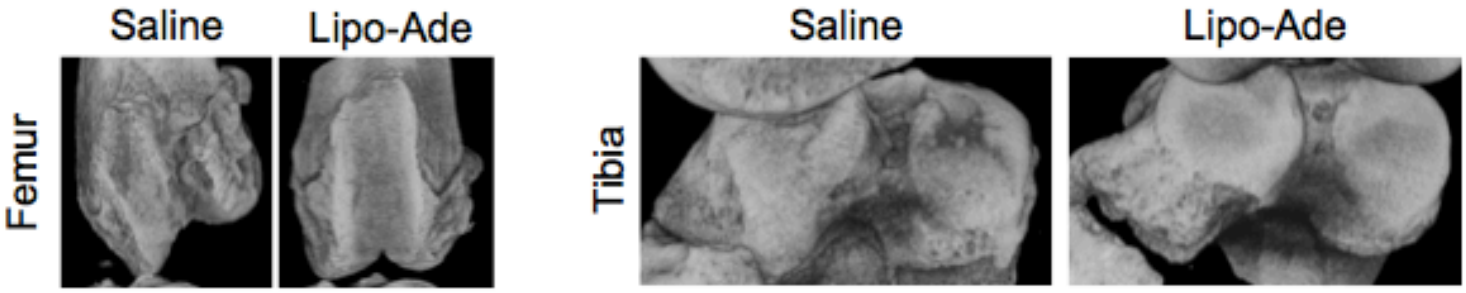

Figure 11 Picutures show huge effect of Liposomes- Adenosine in comparison to Saline alone in both Tibia and Femur.

The appearance of those knees treated with Liposomes- Adenosine injections is much better compared to those with saline injections only.

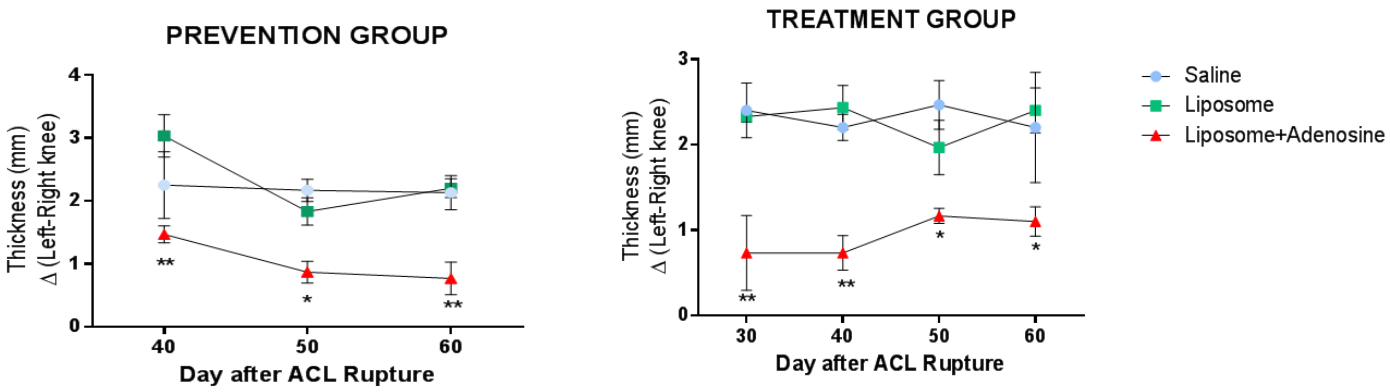

Figure 12 Thickness of left and right knee after ACL rupture

2-way ANOVA
*, p<0.05 ; **, p<0.01; ***, p<0.001

When it comes to the measurement of the thickness of the knee the results can be clearly separated. Best results could be obtained with Liposome – Adenosine injections.

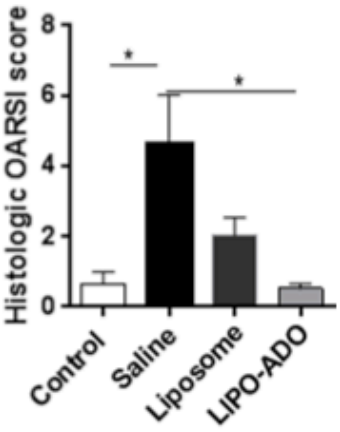

Figure 13 OARSI score was calculated for each animal. Results show a clear tendance that Liposome-Adeosine injections prevent OA progression

The OARSI score was calculated for each animal and supports the results gained by the uCT analysis. As shown in Figure 13 the worst progression happens with Saline injections, almost no OA prevention took place. Best results are gained with Lipsome- Adenosine injections. Almost the same results as with the control group are achieved.

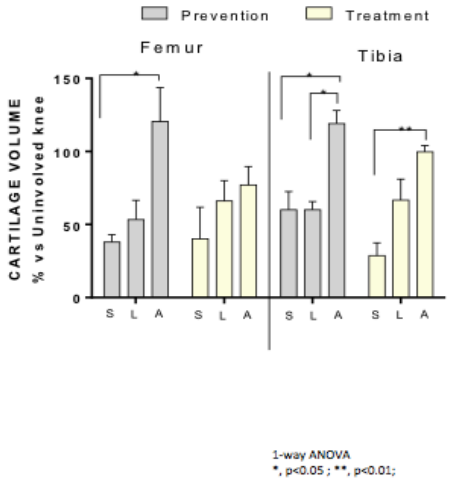

Figure 14 Cartilage volume in % of femur and tibia

Figure 14 shows that there is a huge difference in cartilage volume between the different treatments. Again worst results are gained by saline injections slightly better ones by Liposome-alone injections and best effect has Adenosine-Lipsome injections.

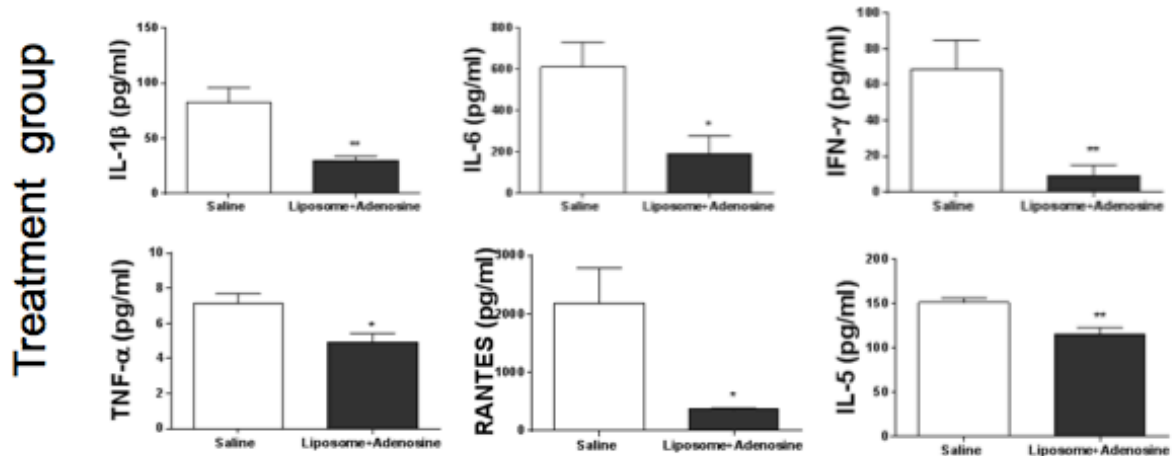

Figure 15 Adenosine injection reduces inflammatory cytokines in the peripheral blood of OA rats

Figure 15 shows a clear reduction in inflammatory cytokines in the peripheral blood of OA rats after Liposome Adenosine injections. Picture shows the difference between Saline injections and Liposome-Adenosine injections.

Figure 16 shows the presence of MMP 13. It is noticable that the highes amount is in those knees treated with Saline and get less in those knees Liposomes have been injected. The lowest presence is noticable in the knees Liposomes in combination with adenosine have been injected.

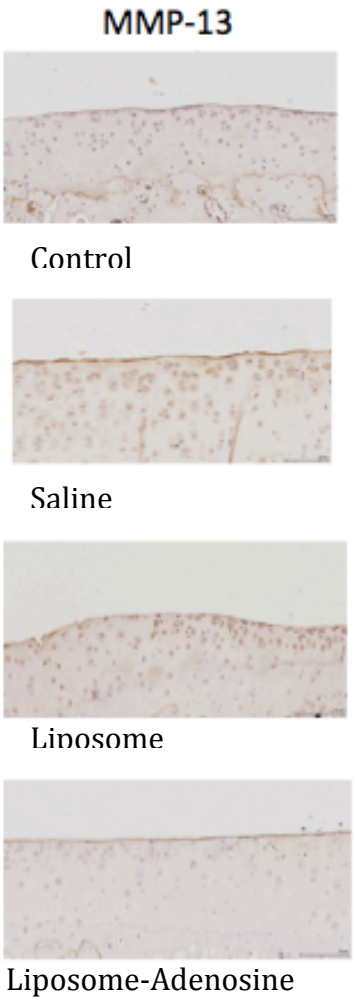

Figure 16 Pictures show presence of mmp13

Figure 17 shows the cartilage surface. Calcified cartilage and a huge damage can be noticed with Saline. Less calcified cartilage but still damaged cartilage is the result of Liposome injections. Almost a perfect cartilage surface, with no damage is reported with Liposome- Adenosine injections.

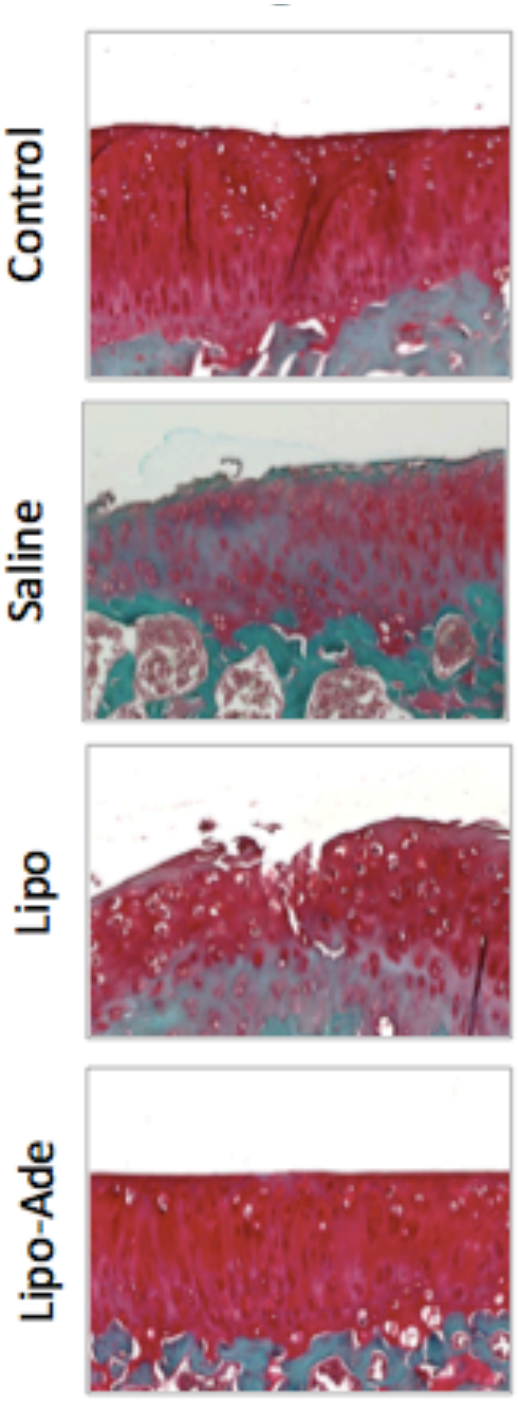

Figure 17 Safranin O staining

Discussion

Expectation

Three different types of injections were administered at regular intervals:
Saline – no expected effect
Liposome – low to moderate effect
Liposome + Adenosine – high effect
After 8 weeks and a periodical injection of every 10 day the animals were sacrified.

Procedure

The bone structure of the knee joint includes femur, tibia and the patella. Femur and tibia are connected by the anteriar cruciate ligament (ACL), one of the four main ligaments within the knee. The ACL runs diagonally in the middle of the knee and gives the knee its stability.
Beside the ACL rupture other methods exist to induce OA: by injections, surgery or spontaneously.
The reasons why for this experiment the ACl rupture was chosen are following:

- ACL rupture is used to induce OA because it is one of the most common injuries of the knee in an active human beeing.
- The time point of when the rupture is happening is predictable
- It is the best method when it comes to the rat model
- ACLT result in less severe disease compaired to other models.
  ACLT surgery leads in adult rats to progressive cartilage degeneration. Cartilage degeneration happens slow and the progression is not fast. There is a lack of Osteophytes, if present they are really small. Fibrocartilage replaces articullar cartilage.

There are a few points needed to be considered when it comes to the accomplishment of the ACl rupture. It is highly important that the animals are around 14 weeks. If they are too young a dislocation of the femur will happen and as a consequence the rat has to be sacrifised. The optimum rupture will be around 90% for femur as well as for tibia. In addition it is important that the rupture is accurant. If not only the ACL is ruptured but also other ligaments are impaired the rat will be exposed to too much distress.

Outcome

Between the several injections huge differences can be noticed. Unfortunately it can not be claimed that the results are significant due to the low number of animals involved. For each group, 3 of the animals received the first injection right after the ACL rupture (prevention group) and other 3 after 1 week (treatment group). Although the results are not significant a strong tendency can be observed.
In Figure 9 pictures from tibia and femur are taken right after they have been cleaned from soft tissue. Here those taken from Saline look worst. Especially the difference between Saline and Liposomes + Adenosine is clear. The difference between Saline and Lipsomes is not that huge especially in the tibia. Also when it comes to the swelling of the knee, as shown in Figure 12 Saline and Liposomes are almost the same but they differ from those knees treated with Liposomes+ Adenosine. There is only a slightly more deviation at day 50. But in total the level of inflammation and the thickness of the knee is high. The swelling of the knee is almost reduced by the half. There is a small rise in thickness after day 40. Reason therefore is maybe an activated immune reaction or decline effect of the injection.
Figure 10 shows the loss of cartilage surface in OA rats with the aid of the admira software. And is clearly observable that the biggest lost had happened when the knee was treated with Saline. To be sure about the loss of cartilage, the cartilage volume was calculated as shown in Figure 13. And Not only that there is a huge loss noticalbe in those treated with Saline there is alos a gain of cartilage noticable in those treated with Adenosine + Lipssomes. In the prevention group the effect is bigger but still there are differences observable in the treatment group. Why thre is even a gain in cartilage is not clear yet but it shows that lipsomes+ adenosine give the best protection. Figure 14 shows the OARSI score where Cartilage degeneration Osteophytes, Clacified cartilage, Growth plate thickness are taken into consideration. And also here clear differences are visible. Figure 17 shows the Safranin Ó staining and here the Lipsome- adensoine surface looks quite similar to the Control. But not only the cartilage is different also changes in bones can be obervable as shown in figure 11.

Saline

There was no effect expected for saline. This compound was used to show the impact of an ACL rupture without any further treatment.
Saline does not protect the knee from the process of cartilage degeneration or bone deformation. No recovery at all could be registered in those knees saline was injected at regular intervals. Cartilage as well as bone look worst. Although it is known that in the ACL ruptur animal model osteophytes are not that common still a few could be noticed. Osteophyte fromation can be normally related to changes in bone formation. Causes therefore can be degenreation, mechanical instability or disease. As soon as a mechanical damage of the joint happened calcification and new bone formation can occur. And those processes are observable in those knees treated with saline only. Beside changes in bone formation also a huge loss of cartilage can be noticed. Saline solution is salt dissolved in water. So nothing that could stop cartilage degradation, bone deformation or later on Osteoarthitis.
This group shows the full impact of the acl rupture.

Liposomes

Liposomes are spherical vesicle, which are made of phosphatidylcholine. They normally serve as a vehicle for transportation of nutrients and pharmaceutical drugs. But they can not bind the A2A receptor.
Slightly better results could be achieved by administer Liposomes alone. But still the appearance of cartilage and bone impaired. The extent of the total effect is not clear yet. But still there is an effect observable. The apprearance of knee is better than with saline. Researchers discuss about a possible delay of the disease. But until now no evidence could be found.

Liposomes + Adenosine

The biggest effect was expected from Liposomes in combination with adenosine. Adenosine was used bevor but due to a really short half life time it was hard to deliver and to develop the effect. The lipsomes make a slow duty possible. Adenosine receptors can be found in the whole body and have various functions such as control of cell proliferation and signal transmission of inflammation. A2A receptors can be found in bone and are always active. One possible hypothesis why there is such an improvment in cartilage and bone appearance is that the injected adenosine block all the receptors. It has been shown that adenosine has an affect on A2A receptor but because of the short half life time it was never relevant. The combination between Liposomes and Adenosine increase this half life time and so also the effect is increased. In addition, it has an impact on the inflammation side because on the cartilage surfac less mmp 13 can be shown as pictured in Figure 16. Mmp13 is critical for OA progression and pharmacologic inhibition of MMP13 is an effective strategy to decelerate articular cartilage loss in a murine model of injury-induced knee OA.

Problems

Problems can occur at the ACL rupture. The animals are exposed to enormous stress. If more than just the acl is ruptured the pain would be to big for the rat and the animal needs to be sacrificed immediately.
Furthermore the production of the lipsomes is quite difficult and one can never be sure that the adenosine is also in the lispome.

Conclusion

The aim of experiment was to show a reduction in the development of OA in OA rat model after periodically injection of adenosine in liposomes. The results show that a reduction of OA progression by periodically injections of adenosine in Liposomes is possible. Especially compared to the results gained with saline injections the effect is impressive. Beside a reduction of development also a cartilage production could be noticed. There is almost no damage observable on the cartilage surface and the level of infalmmation is low. Those knees treated with Liposomes+ Adenosine look almost the same as the control group.

Unfortunately the results can not be claimed tob e significant because of the low number of animals participated in the study. For each group, 3 of the animals received the first injection right after the ACL rupture (prevention group) and other 3 after 1 week (treatment group). But still a strong tendancy is observable.

Further studies will be done to prove not only a tendancy but also to make the results more significant.

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