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The influence of irradiation with low-level diode laser on the proteoglycan content in arthrotically changed cartilage in rabbits

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Abstract

The course of arthrosis was investigated on an animal-experimental arthrosis model considering macroscopic aspects, and the proteoglycan and the glycosaminoglycan contents. Based on these parameters, the influence of a low-power diode laser of 692.6 nm wavelength on the progress of arthrosis was investigated. Thirty days following joint instability surgery another operation was made during which the femoral condyles were irradiated using different energy densities. Seven days after the second operation, macroscopic findings were made and the proteoglycan content was established based on the quantitative determination according to Taylor and Jeffre. This method is based on various spectrophotometric absorption behaviours of different concentrations of sulphatized glycosaminoglycans in the presence of dimethylmethylene blue.

Macroscopically, a progressively increasing severity of cartilage changes during the course of arthrosis was detected and the proteoglycan content was found to decrease. The changes in the irradiated joints proved to be less severe, with the higher energy density having a greater positive influence of statistical significance.

Keywords: Cartilage; Laser; Proteoglycans; Spectrophotometry

Introduction

Changes in the preoteoglycan content and, consequently, the glycosaminoglycan content during the course of arthrosis have been unanimously reported in the literature. With the arthrosis progressing, the proteoglycan content decreases which leads to progressing cartilage malacia. A decreasing glycosamine content being heaviest in fibrillated cartilage causes the cartilage to lose its strength. For the biomechanical function, this means that the penetrator can penetrate deeper into the matrix in the case of collisions. At the same time, the cartilage's elasticity is reduced because the cartilage fails to spring back into its original position when released. Under load, the increased deformability, which is strongest particularly with arthrotic defects, may lead to shearing forces which, in turn, result in an increased wear of the cartilage substance. Thus, the abrasive effect of the movement becomes more pronounced on a cartilage, the proteoglycan content of which is reduced. But the reduction of the glycosaminoglycan content also increases the permeability of the cartilage which means that the electrolytic changes may

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cause the physical properties of the cartilage to change further. The changes in the molecular size of the proteoglycans, their glycosaminoglycan side chains, and the proteoglycan aggregation with hyaluronic acid during the progress of an arthrosis were assessed differently in numerous studies. All in all, there is agreement that the gycosaminoglycan-to-protein ratio steadily decreases while the arthrosis progresses and that the increasing lack of hyaluronic acid is responsible for the reduced proteoglycan aggregation.

The following investigations shall evaluate the influence of low-power laser irradiation on changes of cartilage components, especially its proteoglycan content, in an animal-experimental arthrosis model. The quantitative measuring of these changes shall be done by means of photometric absorption measurement in the presence of dimethylmethylene blue. Taylor and Jeffre [1] introduced dimethylmethylene blue as a strongly metachromatic dye for the histochemical detection of sulphated glycosaminoglycans.

Dimethylmethylene blue is a cationic dye which bonds on polyionic molecules like sulphated glycosaminoglycans and changes its colour from blue to purple red during this process. After staining a solution containing glycosaminoglycan with dimethylmethylene blue, the absorption is measured in a spectrophotometer at a wavelength of 525 nm.The wavelength of 525 nm corresponds to the absorption maximum for the product of the reaction between dimethylmethylene blue and chondroitin sulphate.

Material and methods

For the purpose of these investigations an experimental arthrosis model was developed in vivo in 42 adult female rabbits after the animal experiments had been approved (approval no. G0023/92). The animals were divided in four groups (A–D).

Group A consisted of 18 rabbits which were subjected to a joint instability operation. After 3, 7, 17, 20, 30, 40 and 60 days the arthrotic cartilage was pathomorphologically assessed and the glycosaminoglycans were measured. In this group, the glycosamine content changes during the progress of the arthrosis were to be evaluated for cartilage changes. The macroscopic changes were assessed according to the criteria for classification of cartilage changes on human cartilage.

For the macroscopic follow-up beyond 60 days one animal, each, was examined 3, 4, 6 and 8 months post operationem. These animals were taken out of group A for photometric measurement because from the second month the changes proved to be so pronounced that a standardized cartilage removal from the main stress area was no longer possible.

Group B consisted of 8 rabbits which had been subjected to an initial joint instability operation. Thirty days post operationem an arthrotomy and a joint irrigation were carried out. Seven days later the cartilage preparations were pathomorphologically assessed and the glycosaminoglycans were measured using the dimethylmethylene blue method. This group served as reference group towards groups C and D, because for lasing, these groups had to be subjected to an arthrotomy, too.

Group C consisted of 6 rabbits which had been subjected to arthrotomy and cartilage lasing using a diode laser with an energy density of 1 J/cm^2 30 days after the initial joint instability operation. The pathomorphological assessment of the cartilage and the glycosaminoglycan measurements were effected 7 days after the second operation.

The 8 rabbits of group D were subjected to the same procedure as the animals of group C, with an energy density of 4 J/cm^2 having been chosen for lasing.

Joint instability operation

The left leg of each rabbit was subjected to surgery on sterile conditions. After standardized preparation a cutaneous incision about 4–5 cm long was made anteromedially. The articular capsule was opened medial of the patella. Then, the medial collateral ligament was clamped and dissected. Following that the medial meniscus could be luxated and resected in the region of the posterior horn. The anterior cruciate ligament of the knee was then disarticulated under maximum external rotation of the lower leg.

Secondary operation – arthrotomy and joint irrigation (group B)

The knee-joint was opened after usual preparation. After the opening, an extensive irrigation with lactated Ringer's solution was made and the wound was closed.

Secondary operation – arthrotomy and lasing (groups C and D)

After standard preparation for surgery the knee-joint was opened from anteromedial similar to the first operation. The joint was irrigated and fixated by means of two tripoids in 90° flexion position. Prior to that the patella had been fixated lateral to the joint in luxation position. Prior to each lasing, the power was measured with a power meter (model 7101/Scitec Instruments). The laser beam perpendicularly hit the medial femoral condyle. After lasing the wound was closed.

Table 1. Lasing parameters for groups C and D

	Power density (mW/cm^2)	Exposure time (s)	Energy density (J/cm^2)
Group C	4.5	222	1
Group D	4.5	900	4

Laser

Lasing was done using a diode laser with an emission wavelength of 692.6 nm (TOLD 9140/Toshiba). The output power was 20 mW. The lasing parameters for groups C and D are shown in Table 1.

Follow-up examination

The diagnosis of the macroscopic section was established by means of an operation microscope (Opmi 65/Zeiss). The appraisal of the severity of the cartilage defect was made according to the Outerbridge classification.

After establishment of the macroscopic diagnosis the punch biopsies were taken from the main stress area of the medial femoral condyles of the healthy and arthrotic knee-joints. Then, the punch biopsies were fixated in liquid nitrogen and stored at −80 °C. Before the proteoglycan was determined, the wet weight of the frozen samples had been established.

For the extraction of the proteoglycans a method using guanidine HCl and isopyknic CsCl density gradients according to Sajdera and Hascall [2] was applied.

Spectroscopic measurements

Prior to the spectroscopic measurements the specimen were filled up with 1% BSA PBS (bovine serum albumin in phosphate-buffered saline) medium up to 50 ml, each, to ensure colour consistency. After that, 500 µl dimethylmethylene blue solution was added. The stained solution was filled into a cuvette and measured in a spectrophotometer (UV1202/UV-VIS/Shimadzu) with a wavelength of 530 nm. Each specimen was evaluated 3 times and the calculated mean value was used for the statistic preparation.

Results

Progress of the arthrosis

Macroscopic observations

First macroscopic changes could be observed 17 days after the joint instability operation. Decent malacias and colour changes of the medial femoral joint surface according to Outerbridge grade I were found. The joints, which had been examined after 20 days, showed colour changes and pits as well as lacerations (Outerbridge I and II). A section on day 40 post operationem showed malacia with discolouration as well as roughening in the main stress area with indications of an ulcer formation so that this situs was classified grade III. After 2 months besides malacia and yellow-brownish discolouration, ulcer formation and deep fissures were found which overlapped into regions outside the main stress area (arthrosis grades III–IV).

From the third month, the established changes had to be classified grade IV. Osteophytic extracts were found in the condylar region after 3 months and bare sclerosed bone in the main stress area after 4 months. After 6 months the bare bone was visible also outside the main stress area. In the eighth month, severe arthritic changes were observed which were characterized by a complete eburnation of the joint surface.

Spectrophotometric measurements

The measured absorption values of the cartilage specimens of group A were presented as time function in Fig. 1.

From day 7, distinctly lower absorption values are recognized for arthrotically damaged cartilage over the complete time behaviour. None of the measured extinction values of the cartilage specimens taken from damaged knee-joints exceeded the values measured for the healthy side. The decline of the absorption values was nonlinear. The largest differences were noticed between days 20 and 40 post operationem. In this period, the protein content changed most drastically, while the regression of the absorption and of the

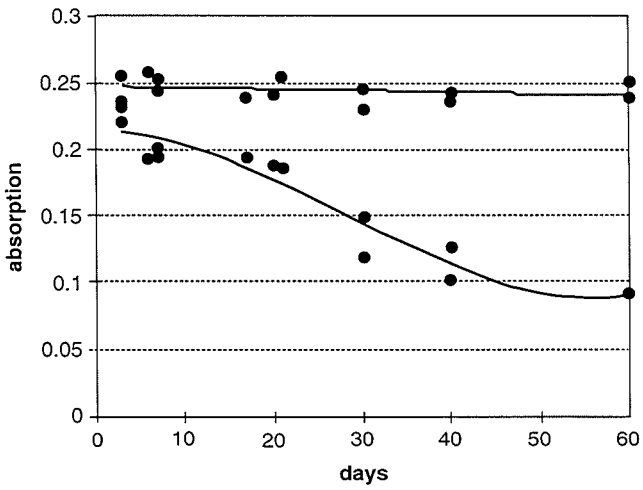

Fig. 1. Extinction values for cartilage specimens (group A), measured 3–60 days after the joint instability operation (upper line: healthy knee; lower line: arthrotic knee-joint).

proteoglycan content became stagnant from day 40 post operationem.

In the light of these results, the animals of groups B–D were subjected to load for a period of 30 days because the changes were then most distinct and therefore easiest to detect.

Cartilage changes after laser irradiation

Macroscopic observations

The changes, according to Outerbridge categorization, in groups B (control group), C (lasing with 1 J/cm^2) and D (lasing with 4 J/cm^2) are shown in Tables 2–4.

Group B. When exposed to load for 30 days after the joint instability operation, the arthrotic change of the knee-joint was mostly of grade III, followed by grade II. Grade I and grade IV damages were observed in none of the investigated cases.

Group C. With 50%, grade II is the value most often assigned. There are 25% more grade II findings compared to the control group. In group C, 37.5% of cases were classified grade III which, compared to the control group (75%) indicates a clearly less severe cartilage damage after lasing with an energy density of 1 J/cm^2.

Group D. With 50%, scale II is the value most often assigned. There are 25% more grade II findings compared to the control group. In group D, 25% of cases were classified grade III which compared to the control group (75%) and group C (37.5%) indicates a clearly less severe cartilage damage after lasing with an energy density of 4 J/cm^2.

Consequently, lasing clearly influences the severity of the cartilage damage, with the irradiation with an energy density of 4 J/cm^2 having a stronger positive effect on the cartilage damage than the irradiation with 1 J/cm^2.

Table 2. Arthrosis scores in group B (control group)

Arthrosis grade	Number	Percentile
I	0	0
II	2	25
III	6	75
IV	0	0
Total	8	100

Table 3. Arthrosis scores in group C (lasing with 1 J/cm^2)

Arthrosis grade	Number	Percentile
I	1	12.5
II	4	50
III	3	37.5
IV	0	0
Total	8	100

Table 4. Arthrosis scores in group D (lasing with 4 J/cm^2)

Arthrosis grade	Number	Percentile
I	2	25
II	4	50
III	2	25
IV	0	0
Total	8	100

Spectrophotometric measurements

The results of spectrophotometric measurements in groups B (control group), C (lasing with 1 J/cm^2) and D (lasing with 4 J/cm^2) are shown in Tables 5–7.

Group B. Considering the minimal and maximal extinction values of the healthy and arthrotic knee-joints one will notice that the extinction values and thus the proteoglycan content of the specimens from arthrotically changed cartilage are distinctly below the values of healthy cartilage. Based on the differences in the absorption maximum, minimum and mean values, the measured absorption values are expected to decrease 30 days after a joint instability operation by between 34% and 39%.

Group C. The minimal and maximal extinction values of the arthrotic knee-joints, which had been lased with 1 J/cm^2, are below the extinction values measured for healthy cartilage. The difference in the absorption minima (32%) is smaller than the difference in the absorption maxima of the control group (39%). The differences in the absorption maxima are equally high in groups B and C (34%). The mean extinction value of group C is lower by 33% on the arthrotic side than on the contralateral undamaged side. This difference is lower by 2% than the relevant difference in the control group.

When lasing with an energy density of 1 J/cm^2 it was noticed that the absorption was by 0.007 higher, on average, than for the damaged knee-joints of the control group. This corresponds to a 4.7% higher mean extinction value. It was found in a t-test for unpaired random samples that the influence of a laser irradiation with an energy density of 1 J/cm^2 is reflected by higher absorption values at a 25.6% ($p = 0.256$) error probability. Although the laser irradiation was observed to positively influence arthrotic joint damages, this was statistically irrelevant in view of a 5% error probability.

Table 5. Results of spectrophotometric measurements in group B (control group)

	Number	Minimal extinction value	Maximal extinction value	Mean	Standard deviation
Healthy knee-joints (right)	8	0.223	0.258	0.243	0.012
Arthrotic knee-joints (left)	8	0.14	0.17	0.157	0.011

Table 6. Results of spectrophotometric measurements in group C

	Number	Minimal extinction value	Maximal extinction value	Mean	Standard deviation
Healthy knee-joints (right)	8	0.213	0.283	0.246	0.026
Arthrotic knee-joints (left)	8	0.145	0.183	0.164	0.014

Table 7. Results of spectrophotometric measurements in group D

	Number	Minimal extinction value	Maximal extinction value	Mean	Standard deviation
Healthy knee-joints (right)	8	0.224	0.258	0.241	0.013
Arthrotic knee-joints (left)	8	0.171	0.192	0.184	0.007

Group D. The extinction values measured for the cartilage of the arthrotic knee-joints lased with 4 J/cm^2 was below the value range measured for the contralateral healthy cartilage in group D, too. The difference between the minima of the two knee-joint sides was but 25% and between the maxima 25%.

The difference between the mean values of the two sides is 0.06 apart. This is 25% of the mean value for cartilage from non-operated knee-joints in group D. The difference between the mean values for damaged knee joints in groups B and D is 0.024 in favour of the knee-joints which had been treated with an energy density of 4 J/cm^2. This corresponds to 15.2% of the mean value of the control group. Consequently, lasing with an energy density of 4 J/cm^2 influences the absorption values and the proteoglycan content stronger than laser irradiation with 1 J/cm^2.

Contrary to the irradiation at an energy density of 1 J/cm^2, the positive laser effect on the measured extinction values observed at an energy density of 4 J/cm^2 proved to be statistically significant ($p > 0.001$) as against the reference group.

Discussion

The study made was aimed at evaluating the influence of irradiation using a low-energy laser with a wavelength of 692.6 nm on the progress of arthrosis in arthritically changed cartilage in rabbits. The progress of arthrosis was investigated both macroscopically and by spectrophotometric detection of changes in the proteoglycan and glycosaminoglycane content in an animal-experimental arthrosis model.

The spectrophotometric detection by means of dimethylmethylene blue proved to be a very specific method for quantitatively comparing the glycosaminoglycan contents in the cartilage specimens. Compared to the detection by immunohistochemical and microscopic methods [3], which are highly sensible, too, the spectrophotometric determination was better to objectify by far.

The sensitivity of the spectrophotometric analysis as against macroscopy is demonstrated in group A. Changes caused by experimental arthrosis induction were detected much earlier through a decline in the measured absorption values than by macroscopic observation. The influence of the degenerative changes on the proteoglycan content of the cartilage was visible after 7 days already based on the spectrophotometric measuring results, while initial macroscopic changes were observed as late as after 17 days, only.

The largest changes per time unit of the measured absorption values were found after 30 days. At that time, the macroscopic changes corresponded to a mean to severe degree of damage. After day 17, a correlation between the macroscopic change and changes in the amount of the measured absorption values was noticed.

For future investigation the spectrophotometric quantitative detection of glycosaminoglycans can be considered a simple and precise method. The influence of various factors on the progress of arthrosis can be reliably evaluated in vivo by means of this method.

Biostimulation studies made so far have shown that essential biostimulating effects would have to be expected within a wavelength range from 600 to 980 nm. The existence of distinctive action spectra in the wavelength range of 580–860 nm for various biological responses of cells irradiated with monochromatic light was postulated by Karu [4]. The laser used in this study should additionally emit light with a wavelength from that range in which the energy absorption by water is lowest. The laser energy used should mainly be absorbed by and have its largest effect on the organic tissue [5]. The chosen energy densities (1 and 4 J/cm^2, resp.) are within the low-energy density range where photochemical effects are to be expected.

Previous studies on the biostimulation of laser light were evaluated by means of histological methods, but not subjected to the quantitative spectrophotometric analysis described above. By means of an operation microscope macroscopic assessments were made showing that the findings for knee-joints, which had been subjected to low-level laser therapy, were often less severe than for knee-joints not subjected to lasing. Cartilage tissue irradiated with an energy density of 4 J/cm^2 also showed often lower degrees of arthrosis than cartilage tissue irradiated with 1 J/cm^2 or cartilage tissue of the control group. Consequently, the best results were achieved following a low-level laser irradiation with 4 J/cm^2. This means that a siginificantly larger effect on the absorption values is achievable by extending the exposure time while keeping the power unchanged. It could be shown that the decline of proteoglycans during the progress of an arthrosis can be delayed significantly better when irradiated with an energy density of 4 J/cm^2 than with an energy density of 1 J/cm^2. This indicates that a low-level laser irradiation during the progress of an arthrosis leads to an increase in the metabolic rate in the hyaline cartilage. The proteoglycan synthesis seems to accelerate due to the laser irradiation.

The mechanism of light regulation of cell metabolism and the reason for the synthesis enhancement are still understood only fragmentarily. As Karu [6] has pointed out, cytochrome *c* oxidase is a possible photoacceptor when cells are irradiated with monochromatic red to near-IR light radiation, and its interaction with radiation leads to 4 effects: changes in the redox properties of respiratory chain components following photoexcitation of their electronic states, generation of singlet oxygen, localized transient heating of absorbing chromophores, and increased superoxide anion production with subsequent increase in concentration of the product of its dismutation, H_2O_2.

The cytochrome absorption makes the photon act as a carrier of biological energy as the cytochrome system in the mitochondria can absorb the photon and stimulate electron transport, which generates bioenergy in the form of ATP from ADP [7]. The cytochromes being part of the electron transport chain absorb extremely strong in the wavelength range used in our study, which speaks in favour of this theory.

Various studies investigated the effects of the diode laser (Ga–Al–As) on cartilage tissue. Here, it was possible to show stimulatory effects on the proliferation and the proteoglycan synthesis in human cartilage cells [8]. The irradiation of the arthrotic cartilage in animal experiments showed similar results. After irradiation with a low-level laser a good cartilage regeneration was achieved. [9,10].

Zusammenfassung

Einfluss der Bestrahlung mit niederenergetischem Diodenlaser auf den Proteoglykanabbau im arthrotischen Kaninchenknorpel

In einem tierexperimentellen Arthrosemodell wurde der Verlauf der Arthrose nach makroskopischen Gesichtspunkten und nach Aspekten des Proteoglykan- bzw. Glykosaminoglykangehaltes des Knorpels untersucht. Der Einfluss der Knorpelbestrahlung mit niederenergetischem Diodenlaser mit einer Wellenlänge von 692.6 nm auf den Verlauf der Arthrose wurde nach gleichen Parametern evaluiert. 30 Tage nach einer Gelenkinstabilitätsoperation wurden während einer zweiten Operation die Femurkondylen mit unterschiedlichen Energiedichten bestrahlt. Sieben Tage nach der zweiten Operation wurden sowohl makroskopische Befunde erhoben als auch der Proteoglykangehalt auf Basis der quantitativen Bestimmung nach Taylor & Jeffre bestimmt. Dieses Verfahren beruht auf den unterschiedlichen spektrophotometrischen Absorptionsverhalten der verschiedenen Konzentrationen von sulfatierten Glykosaminoglykanen in Anwesenheit von Dimethylmethylenblau.

Makroskopisch fand sich eine progrediente Zunahme der Schwere der Knorpelveränderungen im Verlauf der Arthrose, ebenso konnte eine Abnahme des Proteoglykangehaltes nachgewiesen werden. Des Weiteren fanden sich bei den bestrahlten Gelenken mildere Veränderungen, wobei hier die höhere Energiedichte einen größeren positiven Einfluss mit statistischer Signifikanz hatte.

Schlüsselwörter: Knorpel; Laser; Proteoglykane; Spektrophotometrische Messungen

Sumario

Influencia de la radiación con láser de diodo de baja energía en el descenso del proteoglicano en el cartílago de conejos afectado por artrosis

La evolución de la artrosis se ha examinado en un modelo experimental-animal, considerando los aspectos macroscópicos y el contenido de proteoglicano y

glicosaminoglicano del cartílago. La influencia de la radiación del cartílago con láser de diodo de baja energía con una longitud de onda de 692.6 nm en el desarrollo de la artrosis se evaluó con los mismos parámetros. Treinta días después de una operación para estabilizar la articulación, los cóndilos femorales fueron radiados con diferentes densidades de energía en una segunda operación. Siete días después de la segunda operación, se presentó el diagnóstico y se estableció el contenido del proteoglicano sobre la base de la determinación cuantitativa según Taylor y Jaffe. Este procedimiento se basa en el diferente comportamiento de absorción espectrofotométrico de las diferentes concentraciones de glicosaminoglicanos sulfatados en presencia de azul de dimetilmetileno.

Macroscópicamente, durante el transcurso de la artrosis, se observó un incremento progresivo del cuerpo de las alteraciones en el cartílago, también pudo comprobarse un descenso en el contenido del proteoglicano. Además se encontraron en las articulaciones radiadas alteraciones más bajas y la más alta densidad de la energía ejerció una mayor influencia positiva con significado estadístico.

Palabras claves: Cartílago; Láser; Proteoglicanos; Mediciones espectrofotométricos

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