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PÔVODNÁ PRÁCA

ÚČINOK ZEEL comp. NA EXPERIMENTÁLNU OSTEOARTRÓZU V KOLENE KRÁLIKA

M. Stančíková, M. Bély[1], K. Švík, H.W. Metelmann[2], M. W. Schmolz[3], R. Ištok, R. Fano

EFFECTS OF ZEEL Comp. ON EXPERIMENTAL OSTEOARTHRITIS IN RABBIT KNEE

Research Institute of Rheumatic Diseases, Piešťany, Slovak Republic
Vedúci: prof. MUDr. J. Rovenský, DrSc
[1]National Institute of Rheumatology and Physiotherapy, Budapest, Hungary
Vedúci: Dr. G. Bálint
[2]Biologische Heilmittel Heel GmbH, Medical Research Department, Baden-Baden, Germany
Vedúci: H.W. Metelmann
[3]Experimental and Diagnostic Immunology, GmbH, Reutlingen, Germany
Vedúci: Dr. rer. nat. M.W. Schmolz

Súhrn

Pozadie problému: **Zeel comp. je roztok homeopatických extraktov, ktorý sa používa v antihomotoxickej liečbe osteoartrózy. Klinická štúdia u pacientov s gonartrózou ukázala priaznivý účinok tejto látky.**
Cieľ práce: **Sledovanie účinku Zeel comp. na artikulárnu chrupku počas vývoja osteoartrózy u králikov po prerušení predného skríženého väzu (PPSV).**
Metódy: **V štúdii sa použilo celkovo 12 králikov samcov kmeňa New Zealand White (NZW). Králiky sa po chirurgickom výkone PPSV pravého kolena a po jednoduchej artrotómii kontralaterálneho ľavého kolena náhodne rozdelili do dvoch skupín po 6 zvierat: osteoartrotická neliečená skupina (OA) a skupina liečená Zeel comp. OA skupina dostávala do pravého operovaného kolena s PPSV intraartikulárnu injekciu sterilného fyziologického roztoku a liečená skupina podobným spôsobom Zeel comp. Súčasne obe skupiny dostávali do ľavého kolena (slepá, tzv. sham kontrola) sterilný fyziologický roztok. Zeel comp. a fyziologický roztok sa aplikovali intraartikulárne dvakrát za týždeň, ihneď po operácii, celkom 9 týždňov až po eutanáziu zvierat. Sledovali sa tieto parametre: makroskopická morfológia, histológia, močový pyridinolín (Pyr).**
Hlavné výsledky: **Morfologické zmeny v osteoartritickej skupine bez liečby boli viditeľné na mediálnej aj laterálnej oblasti kondylov operovaných kolien (PPSV), ale výraznejšie na mediálnom kondyle. Povrch artikulárnej chrupky bol zdrsnený, s hypertrofickými zmenami a závažnými eróziami. Závažnosť poškodenia chrupky bola všeobecne nižšia v skupine liečenej Zeel comp. v porovnaní s artritickými kontrolami. Morfologické zmeny na kontralaterálnej sham kontrole boli malé alebo žiadne. Bodové hodnotenie poškodenia chrupky makroskopickou morfológiou preukazovalo signifikantne nižší rozsah poškodenia v skupine Zeel comp.**

Summary

Objective: **Zeel comp. is a mixture of homeopathic extracts used in antihomotoxic medicine of osteoarthrosis. The clinical study in patients with gonarthrosis showed a pronounced beneficial effect of this compound.**
The aim of study: **The evaluation of the Zeel comp. effect on the articular cartilage of knee joint during development of osteoarthrosis in rabbits following anterior cruciate ligament transsection (ACLT).**
Methods: **A total of 12 New Zealand White (NZW) male rabbits were used in this study. The rabbits were randomly divided into two groups of 6 animals after surgical ACLT of right knees and simple arthrotomy of their contra-lateral left knees: osteoarthritic untreated group (OA) and Zeel comp. treated group. OA group received intra-articular injection of sterile saline and the treated group injected in the same manner with Zeel comp. into the right ACLT knee. At the same time both groups received intra-articular injection of sterile saline into the left knees (Sham controls). Zeel comp. or saline were injected intra-articularly twice a week, immediately after surgery until sacrifice at 9 weeks. The parameters tested were gross morphology, histology as well as urinary pyridinoline (Pyr).**
Most important results: **Morphological changes in osteoarthritic group without treatment were seen on both, medial and lateral region, but markedly on medial condyle of the ACLT right knee. The articular cartilage was characterised by a rough and hypertrophic appearance with severe erosions. The severity of cartilage damage was generally lower in Zeel comp. group in comparison with OA group. The gross morphological examination of contralateral sham controls revealed very little or no changes. Gross morphological grading of cartilage damage showed significantly lower extent of damage in Zeel comp. group.**

Histologické dôkazy degenerácie chrupky sa pozorovali v kolenách s PPSV liečených aj neliečených králikov. V artikulárnej chrupke OA kontroly sa zistili tieto degeneratívne zmeny: zdrsnený povrch, strata povrchovej vrstvy, erózie, fibrilácie a/alebo fisúry, nepravidelné rozmiestnenie a tvar chondrocytov. V osteoartritickej skupine liečenej Zeel comp. boli znaky chrupkovej degenerácie femorálnych kondylov limitované. Priemer celkového histopatologického skóre skupiny Zeel comp. bol signifikantne nižší v mediálnych kondyloch (20,7±0,64, p<0,05) v porovnaní so skupinou OA, ktorá dostávala fyziologický roztok (23,40±0,54). Výsledok bol podobný aj pri pozorovaní laterálnych kondylov.

Biochemické stanovenia ukázali signifikantne vyššie koncentrácie Pyr v moči OA skupiny oproti zdravým králikom počas celej štúdie (9 týždňov), indikujúce vyššiu kolagénovú degradáciu chrupky a subchondrálnej kosti u osteoartritických zvierat. Králiky liečené Zeel comp. mali tieto koncentrácie pozorovateľne nižšie v porovnaní s OA kontrolou.

***Záver*: Výsledky tejto štúdie ukázali, že hoci intraartikulárna aplikácia Zeel comp. celkom nezabránila rozvoju poškodenia kĺbu u králikov s PPSV na modeli osteoartrózy, výsledky našich morfologických a histologických sledovaní, ako aj biochemických zistení demonštrovali signifikantné a bazálne zníženie závažnosti poškodenia chrupky kondylov a chondrocytov.**

Kľúčové slová: králik, osteoartróza, Zeel comp., makroskopická morfológia, histopatológia, pyridinolín.

Histological evidence for cartilage degeneration was observed in the ACLT knees of both treated and untreated rabbits. In OA controls the articular cartilage showed degenerative changes, including: rough surface, loss of superficial layer, erosion, fissures, irregular arrangement and form of chondrocytes. In the osteoarthritic group treated with Zeel comp. the signs of cartilage degeneration of femoral condyles were limited. The mean global histopathological score in the Zeel comp. group was significantly lower in medial condyles (20.70±0.64, p<0.05) in comparison with saline treated OA group (23.40±0.54). Similar results were observed in lateral condyles.

Biochemical determinations showed significantly higher concentrations of urinary Pyr in the OA group compared to healthy rabbits during the whole period of the study (9 weeks), indicating a higher collagen degradation of cartilage and subchondral bone in the osteoarthritic animals. The urinary levels of rabbits treated with Zeel comp. was clearly lower compared to the OA controls.

***Conclusion*: The results of this study showed that, intraarticular Zeel comp. injections did not completely prevent the development of joint damage in the ACLT rabbit model of osteoarthrosis, however the results of our morphological and histological experiments as well as the biochemical findings demonstrate a significant and substantial decrease in severity of the damage caused in condyle cartilage and in chondrocytes.**

Key words: rabbit, osteoarthrosis, Zeel comp., gross morphology, histopathology, pyridinoline.

INTRODUCTION

Osteoarthrosis (OA) is one of the most common causes of physical disability in the civilised countries. It is characterised by a loss of cartilage, bone remodelling and a synovial reaction resulting in a narrowing of the joint space, subchondral sclerosis and formation of marginal osteophytes (1). The changes are easily recognised by x-ray but this parameter is only sufficient to study advanced stages of the disease. The method is too insensitive to detect early signs of osteoarthrosis or to follow the success of therapeutical measures.

Therefore, animal models as those used in this study, are frequently employed for a more exact evaluation of pathophysiology or the investigation of the efficacy of new drugs (7). Experimentally induced OA in animals has been employed for many years using a variety of techniques including immobilisation of the joint, surgical alteration and destabilisation of the joint architecture, as well as intra-articular injection of destructive agents (papain, chondroitinase) (2, 4, 8).

Zeel comp. is a mixture of homeopathic extracts prepared from: Rhus toxicodendron, Arnica montana, Solanum dulcamara, Sanguinaria canadensis and sulfur, used in antihomotoxic medicine of osteoarthrosis (produced by Biologische Heilmittel Heel GmbH, Germany). A clinical study with Zeel comp. in patients with gonarthrosis showed a pronounced beneficial effect (3).

The aim of this study was therefore the evaluation of the Zeel comp. effect on the articular cartilage of knee joint during development of OA in rabbits following anterior cruciate ligament (ACL) transsection using the model described by Yoshioka et al. (13).

The parameters tested were gross morphology, histology as well as urinary pyridinoline (as a marker of collagen degradation). Concerning the latter, Thompson et al. (11) and the others (9, 10) demonstrated that urinary levels of pyridinoline significantly correlate with cartilage damage and with the X-ray-based rating of osteoarthritic joints.

MATERIALS AND METHODS

Experimental design

A total of 12 New Zealand White (NZW) male rabbits weighing 2.53±0.09 kg each were used in this study. The rabbits were randomly divided into two groups of 6 animals after anterior cruciate ligament transsection (ACLT) of right knees and simple arthrotomy of their contra-lateral left knees. Group 1 — untreated rabbits (osteoarthritic, OA) received 0.3 ml intra-articular injection of sterile saline into the right ACLT knee and left knee (the latter serving OA — Sham control). Group 2 — treated animals (Zeel comp.) were injected in the same manner with 0.3 ml Zeel comp. into the right ACLT knee and 0.3 ml sterile saline into the left knee (Zeel comp. — Sham control). Zeel comp. or saline were injected intra-articularly twice a week, immediately after surgery until sacrifice at 9 weeks.

Surgical procedure

All rabbits were anaesthetised by intramuscular injection of ketamine (100 mg/kg) and xylazine (16mg/kg). Following the anaesthetises, both knees were shaved and disinfected with betadine solution. A medial parapatellar incision was made on the skin followed by arthrotomy. The patella was dislocated laterally and the knee placed in full flexion.

The ACL was then transsected. After transsection the joint was irrigated with sterile saline and closed. The capsule and the synovium were closed with a running suture of 2—0 silon. The skin was closed in the same manner with additional interrupted sutures of 3—0 silon. The sham controls received the same treatment except for the transsection of the ACL.

Post-operatively the animals were permitted cage (60x50x40 cm) activity. The animals were closely monitored for infections and other complications. The average weight of the rabbits at surgery was 2.53±0.09 kg, and at death 3.37±0.13 kg.

Gross morphology

Both knees (ACLT and sham control) were inspected and gross morphological changes of the medial condyles were rated according to modified criteria published by Yoshioka et al. (13): grade 1 — intact surface; grade 2 — slightly rough surface, minimal erosions; grade 3 — markedly rough surface and hypertrophic cartilage with moderate erosions; grade 4 — severe erosions, loss of cartilage exposing the underlying bone.

Histological preparations

Both medial and lateral femoral condyles of the right and left knees were used for histological preparation and assessment. The tissues were fixed in 10 % formaldehyde solution, decalcificated at room temperature in EDTA at pH 7.6. After decalcification, the femoral condyles were cut along the sagittal plane and both medial and lateral condyles were embedded in paraffin. Five micron sections were cut with a Reichter—Jung microtome and stained with haematoxyline, eosin and safranin O.

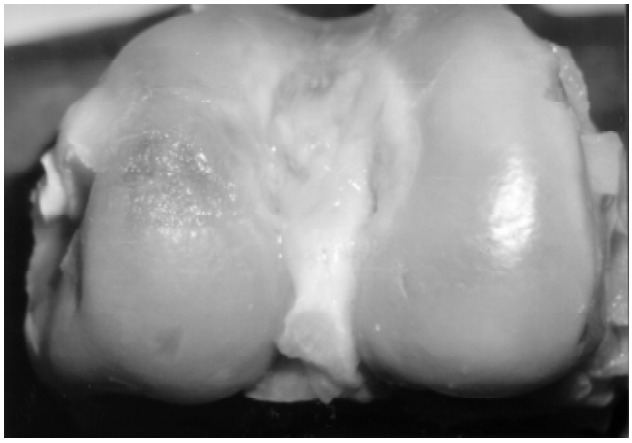
The conditions of articular cartilage were characterised histologically based on regressive changes of cartilage such as: loss of superficial layer, superficial erosion, fibrillation and fissures of cartilage, loss of proteoglycan, disorganisation of chondrocytes, loss of chondrocytes, exposure of subchondral bone, cluster formation obtained in Table 1 according to Kikuchi et al. (5).

Biochemical determination

24-hour urine samples were collected from the rabbits in the metabolic cage during the 9 weeks and stored at –60 °C until analysis. Pyridinoline (Pyr) was determined in the pooled urinary samples from three rabbits. The urinary samples were supplemented by 6 samples of healthy rabbits without surgery (normal values). The samples were hydrolysed at 110 °C in 6 M HCl for 18 hours. Urinary Pyr corrected for creatine was then measured by high performance ion-exchange chromatography as described elsewhere (6) with some slight modifications. Small solid phase extraction (SPE) columns filled with microparticulate cellulose (approx. 1 ml) were conditioned with 12 ml of mobile phase (n-butanol:acetic acid:water = 4:1:1). Following the conditioning, 0.5 ml of hydrolysate was mixed with 0.5 ml of glacial acetic acid and 2 ml of n-butanol and applied to the SPE columns. After washing with the mobile phase (3x4 ml), pyridinoline crosslinks were eluted from the columns by 1.8 ml of mobile phase used for the subsequent high performance liquid chromatography (HPLC) analysis on HEMA BIO 1000 SB 250x4 mm columns (Tessek, Czech Republic). The mobile phase was prepared by mixing 0.45 M sodium sulphate and 0.3 M

Tab. 1. Method of histopathological evaluation of cartilage degeneration.

	+1	+2	+3	+4
Loss of superficial layer	<Slight	Moderate	Focally severe	Extensively severe
Erosion of cartilage	<Detectable	Moderate	Focally severe	Extensively severe
Fibrillation and/or fissures	<Noticeable (<1 very small)	Moderate (1 small)	Marked (2 small or 1 medium)	Extensive (3 small, 2 medium or 1 large)
Loss of proteoglycan	<Paler stain than control	Moderate loss of safraninophilia	Marked loss of safraninophilia	Total loss of safraninophilia
Disorganization of chondrocytes	Noticeable	Moderate with some loss of columns	Marked loss of columns	No recognizable organization
Loss of chondrocytes	<Noticeable decrease in cells	Moderate decrease in cells	Marked decrease in cells	Very extensive decrease in cells
Exposure of subchondral bone	<Focal exposure of bone	Moderate exposure of bone	Fairly extensive exposure of bone	Very extensive exposure of bone
Cluster formation	<3-4 small, or 1-2 medium	5-6 small, 3-4 medium or 1-2 large	7 or more medium or 5-6 large	7 or more small, 5-6 medium or 3-4 large



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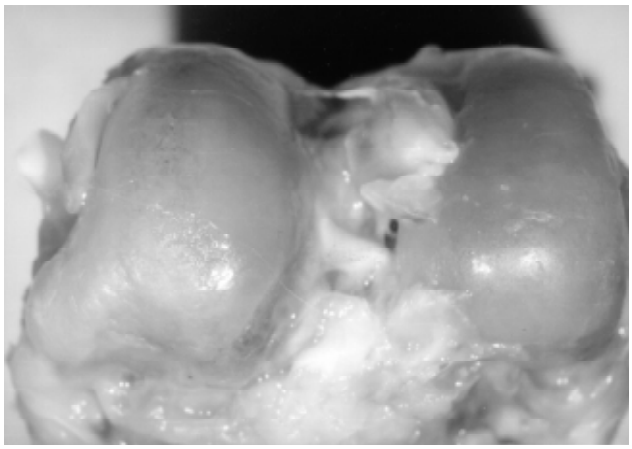

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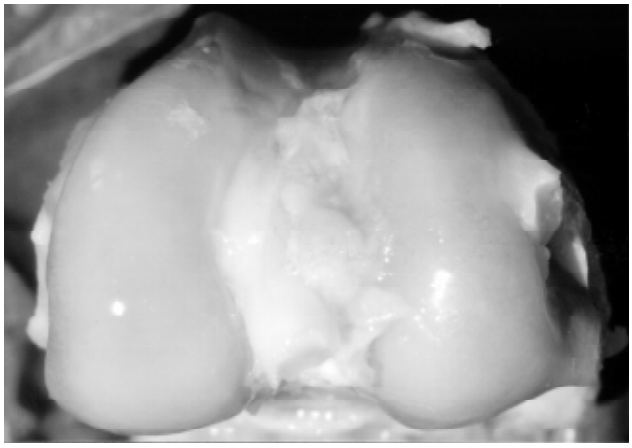

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Fig. 1. Gross morphology of femoral condyles. (a) Untreated osteoarthritic knee. Articular cartilage was rough, hypertrophic with severe erosions. (b) Osteoarthritic knee treated with Zeel comp. Articular cartilage was less rough, hypertrophic with moderate erosions. (c) Contralateral sham control. Smooth cartilage surface showed normal appearance.

acetate buffer, pH 3.0 at a ratio of 9:22. Analysis was performed isocratically at a flow rate of 0.8 ml/min. A fluorescence detector was used for the analyses (excitation at 295 nm and detection at 400 nm).

Statistical analyses of histological results were carried out using statistical method, one-way analysis of variance (ANOVA) for paired data sets with a level of significance at $p<0.05$.

RESULTS

Gross morphology

The changes in osteoarthritic group without treatment were seen on both, medial and lateral region, but markedly on medial condyle of the ACLT right knee. The articular cartilage was characterised by a rough and hypertrophic appearance with severe erosions (Fig. 1a). The severity of cartilage damage was generally lower in Zeel comp. group (Fig. 1b) in comparison with OA group. The gross morphological examination of contralateral sham controls (left knee) revealed very little or no changes (Fig. 1c). Gross morphological grading of cartilage damage was performed on the medial femoral condyles and the results are illustrated in Fig. 2. Only one contra-lateral condyle (sham control) in OA group showed signs of cartilage damage on grade 2. The cartilage damage in Zeel comp. group was significantly lower compared to OA group. While in the OA ACLT right knees 3 out of 6 condyles demonstrated grade 4, in the Zeel comp. treated group grade 4 was not observed.

Histological evaluation

Histological evidence for cartilage degeneration was observed in the ACLT knees of both treated and untreated rabbits. The surface of articular cartilage of sham controls (contralateral knees) was smooth without erosions, fibrillation and fissures with a mostly normal histological appearance. The matrix was intact and the arrangement of chondrocytes was regular (Figs 3a, 3b, 3c — medial condyle). In OA controls the articular cartilage showed degenerative changes, including: rough surface, loss of superficial layer, erosion, fibrillation and/or fissures, irregular arrangement of chondrocytes. Necrotic chondrocytes (without nuclear staining) were rarely seen, and were sporadically mixed with hypertrophic (enlarged) and hyperchromic (hyperfunctional) chondrocytes (Figs 4 a, 4b, 4c — medial condyle). In the osteoarthritic group treated with Zeel comp. the signs of cartilage degeneration of femoral condyles were limited (Fig. 5a, 5b, 5c — medial condyle).

Histopathological score

The mean global histopathological score in the Zeel comp. group was significantly lower in medial condyles (20.70 ± 0.64, $p<0.05$) in comparison with saline treated OA

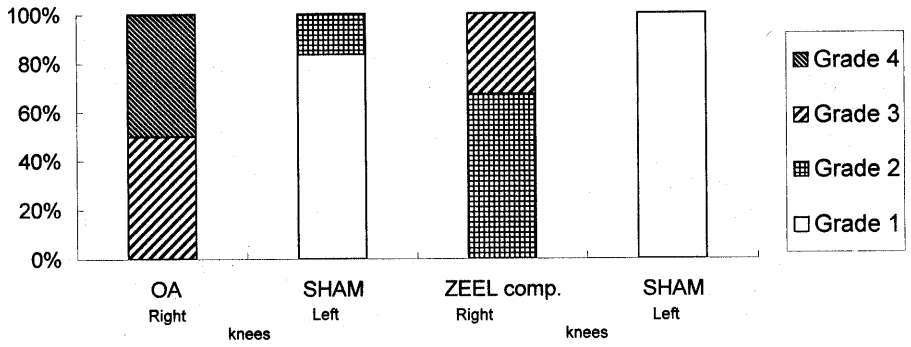
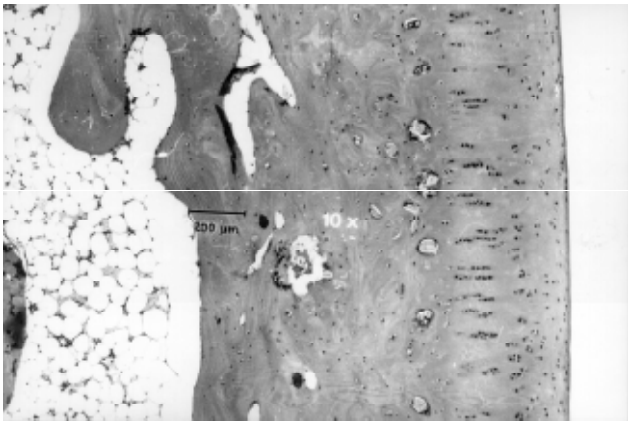


Fig. 2. Gross morphological assessment of the femoral condyles from osteoarthritic and Zeel comp. treated groups (n=6) with appropriate sham controls of contralateral left knees. The extent and grade of cartilage damage in the condyles from the ACTL knees in Zeel comp. treated group was less severe than in untreated osteoarthritic group (expressed in percentage).



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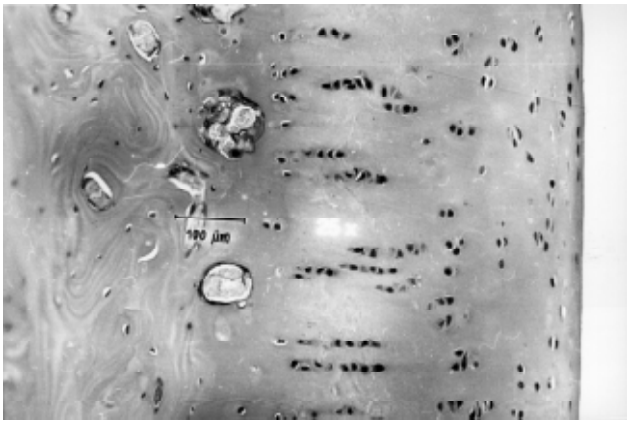

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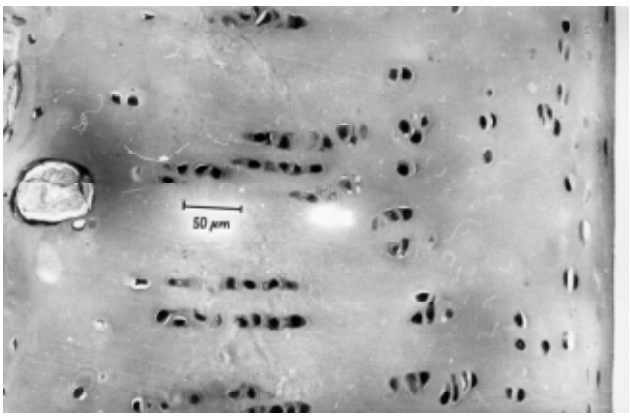

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Fig. 3. Histological evaluation (Indian line) of femoral medial condyle specimen of contralateral sham control knee (lines in the figures represent: a — 200 µm, b — 100 µm, c — 50 µm). Normal articular cartilage. Surface was smooth. Chondrocytes arranged regularly.

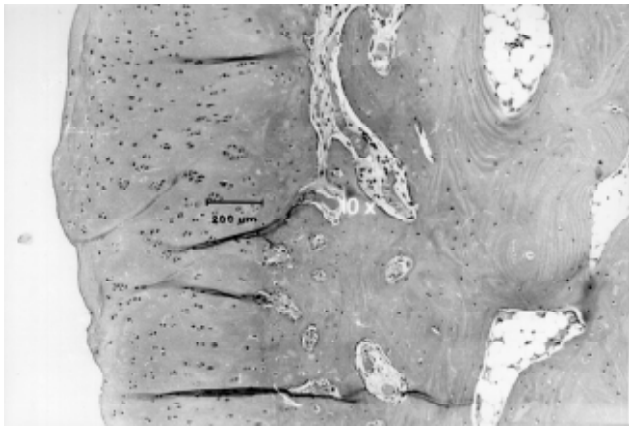
group (23.40±0.54) (Tab. 2). Similar results were observed in lateral condyles. The items of the histopathological score in the Zeel comp. treated rabbits were usually lower than in OA controls. In the treated group 2 items of the condyle score decreased significally: disorganisation of chondrocytes and cluster formation.

Biochemical determinations

Concentrations of urinary Pyr were significantly higher in the OA group compared to healthy rabbits during the whole period of the study (9 weeks), indicating a higher collagen degradation of cartilage and subchondral bone in the osteoarthritic animals (Fig. 6). The urinary levels of rabbits treated with Zeel comp. was clearly lower compared to the OA controls (except the first week in Zeel comp. group).

DISCUSSION

Joint destabilisation secondary to a complete transection of the ACL is known to cause a breakdown of articular cartilage with the resulting loss of joint function. 9 weeks after surgery, inspection of the joints revealed an extensive dama-



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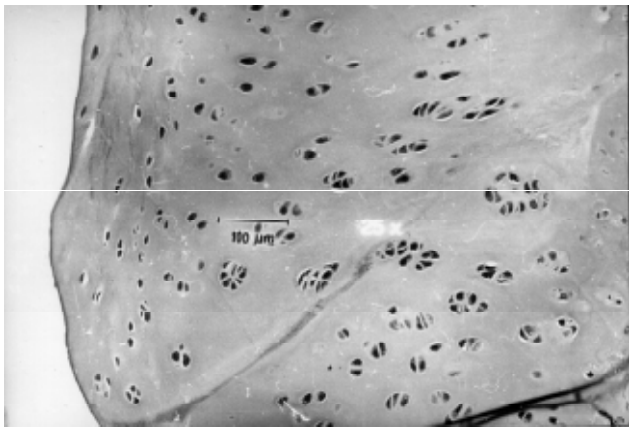

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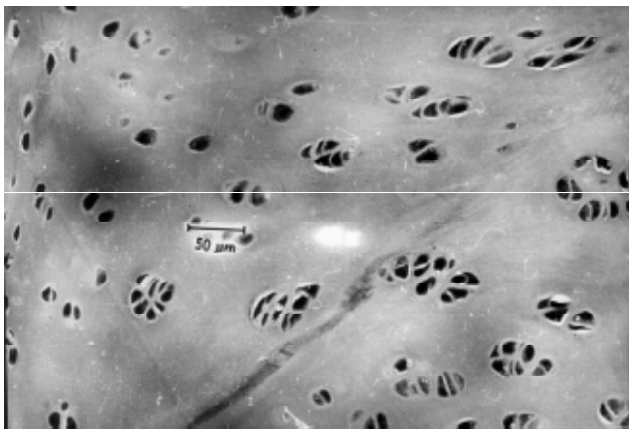

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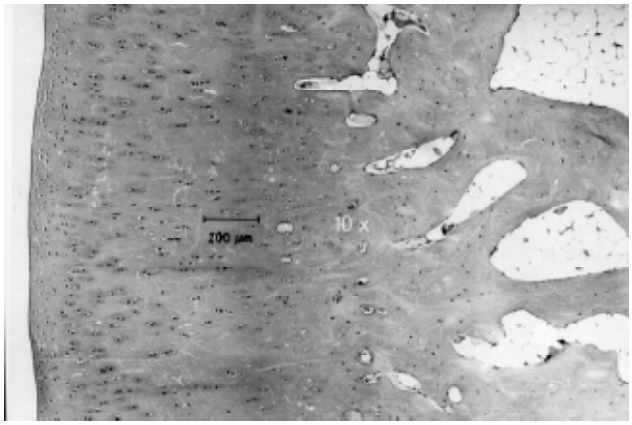
Fig. 4. Histological evaluation (Indian line) of femoral medial condyle specimen of untreated osteoarthritic knee (lines in the figures represent: a — 200 µm, b — 100 µm, c — 50 µm). Degenerated articular cartilage, with rough surface and fissures. Chondrocytes were arranged in groups. Subchondral cortex was perforated by arterioles.



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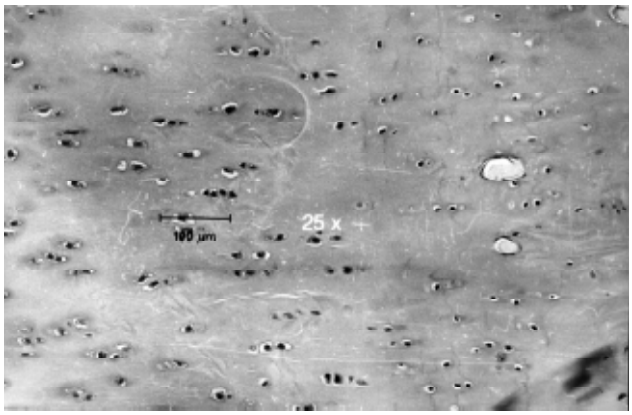

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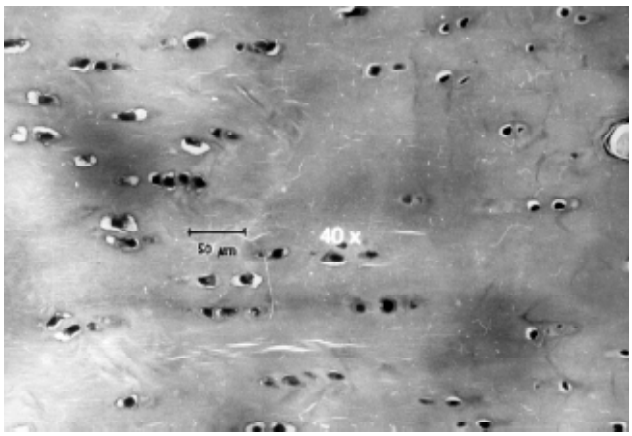

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Fig. 5. Histological evaluation (Indian line) of femoral medial condyle specimen of osteoarthritic knee treated with Zeel comp. (lines in the figures represent: a — 200 µm, b — 100 µm, c — 50 µm). Moderately degenerated articular cartilage, with nearly smooth surface. The chondrocytes were hyperplastic (increased in number), and hyperchrome. The arrangement of chondrocytes was linear, nearly regular. Subchondral cortex was perforated by arterioles.

Tab. 2. Histopathological scores in OA and Zeel comp. groups of rabbits.

	OA	Zeel comp.
Femur — medial condyle		
Global score	23.4±0.54	20.7±0.64*
Loss of superficial layer	3.2±0.41	3.0±0.89
Erosion of cartilage	3.0±0.89	3.0±0.63
Fibrillation and/or fissures	3.2±0.41	2.8±0.75
Loss of proteoglycan	3.3±0.82	3.0±0.89
Disorganization of chondrocytes	3.5±0.55	2.8±0.75*
Loss of chondrocytes	3.0±0.63	2.7±0.52
Exposure of subchondral bone	2.2±0.41	2.2±0.41
Cluster formation	2.0±0.00	1.2±0.41**
Femur — lateral condyle		
Global score	18.8±0.60	16.0±0.51*
Loss of superficial layer	2.2±0.41	2.2±0.75
Erosion of cartilage	2.1±0.65	1.7±0.52
Fibrillation and/or fissures	2.2±0.75	1.5±0.55
Loss of proteoglycan	3.1±0.55	2.8±0.41
Disorganization of chondrocytes	3.2±0.75	2.5±0.55*
Loss of chondrocytes	2.5±0.55	2.2±0.41
Exposure of subchondral bone	2.2±0.75	1.8±0.41
Cluster formation	1.3±0.52	1.3±0.52

* Significantly different from OA control, $p<0.05$
** Significantly different from OA control, $p<0.01$

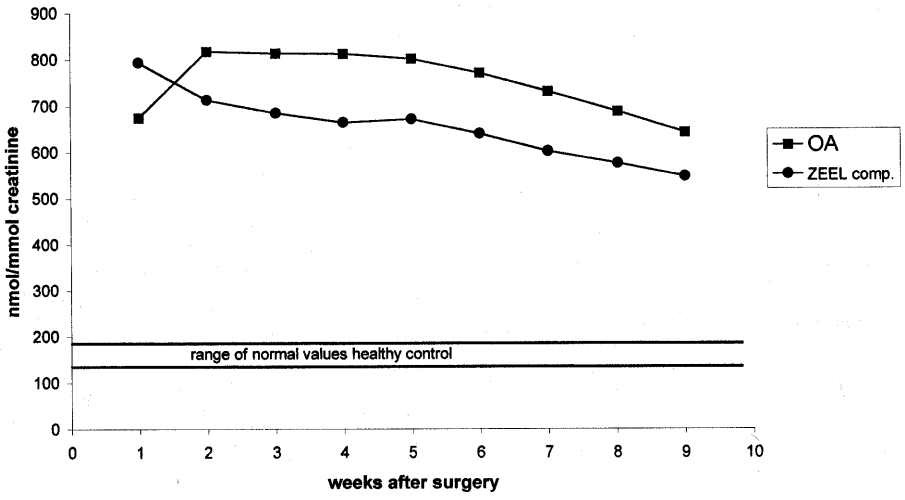

Fig. 6. Urinary concentrations of pyridinoline in healthy control rabbits, untreated OA group and rabbits treated with Zeel comp. are expressed every week during 9 weeks after surgery.

ge in the ACLT knees of all animals receiving saline as the vehicle. All tested parameters indicated severe osteoarthrosis: roughness of the cartilage surface, the thickness of the cartilage, number and distribution of chondrocytes as well as the morphology of these cells. Morphological grading of the femoral condyles and the histological scores in ACLT knees revealed a significantly lower extent and severity of cartilage damage in Zeel comp. — treated rabbits compared to the saline received OA control animals. Urinary pyridinoline levels in the ACL transected animals were higher in control OA group without treatment (see Fig. 6) supporting on a biochemical basis the view of cartilage protection by Zeel comp.

Zeel comp. is a mixed solution of different ingredients and the substance contents of his preparation is quite low. Nevertheless, our results show a substantional protective effect of Zeel comp. on the experimentally induced damage of rabbit joints. This corresponds well with its beneficial effect on clinical symptoms observed in patients with gonarthrosis (3). Since our present study was primarily designed to evaluate the effects of the drug on morphological and histological features of the disease rather than to investigate the pharmacology of this substance. It is difficult to derive any such conclusions from these experiments. However, data of in vitro investigations on the activities of individual components of Zeel comp. on the immune system showed that Rhus toxicodendron and Arnica montana inhibited the respiratory burst of polymorphonuclear granulocytes (12). Rhus toxicodendron also markedly inhibited IL-6 release and moderately stimulated the synthesis of TGF-beta in human whole-blood cultures.

Thus we suggest that at least one type of pharmacological mechanisms of Zeel comp. could work via the cytokine communication of cells regulating the homeostasis of cartilage turnover.

In conclusion, intraarticular Zeel comp. injections did not completely prevent the development of joint damage in the ACLT rabbit model of osteoarthritis, however the results of our morphological and histological experiments as well as the biochemical findings demonstrate a significant and substantial decrease in severity of the damage caused in condyle cartilage and in chondrocytes.

Further investigations will have to show, which cell types are the targets of the active principles in Zeel comp. and how the cells respond to improve the state of the disease.*

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Address for correspondence: M. Stančíková, PhD, Research Institute of Rheumatic Diseases, Nábr. I. Krasku 4, 921 01 Piešťany, Slovakia.