Dietary vitamins and selenium diminish the development of mechanically induced osteoarthritis and increase the expression of antioxidative enzymes in the knee joint of STR/1N mice

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Summary

Objective: To study the influence of dietary vitamins and selenium on mechanically-induced osteoarthritis (OA) and the expression of antioxidative enzymes in male STR/1N and Balb/c mice. Male STR/1N mice are prone to develop OA caused by a varus deformity-induced mechanical overload of the medial tibial plateau.

Methods: After 12 months of feeding (special diet supplemented with the vitamins E, C, A, B6, B2, and selenium) serial histological sections of the knee joints were evaluated for development of osteoarthritic changes (grade 0–4). Serum glutathione peroxidase activity (GSH-px) was measured photometrically. Expression of antioxidative enzymes was demonstrated by immunohistochemistry.

Results: All control STR/1N mice showed OA lesions (grade 3–4) while the special diet decreased OA incidence significantly down to ~65% (mostly grade 2). Even in Balb/c mice the incidence was decreased by the special diet from ~21% (control animals; grade 1) to ~14%. Serum GSH-px activity increased diet-dependently in both mouse strains but was generally higher in Balb/c mice. In both mouse strains the special diet increased the expression of GSH-px and Cu/Zn-SOD in articular cartilage while there was no expression of Mn-SOD. There was also a special diet-dependent increase in expression of GSH-px in the synovium of both mouse strains while an increase in expression of Mn-SOD and Cu/Zn-SOD could only be seen in the synovium of STR/1N mice.

Conclusions: A diet supplemented with vitamins/selenium might be important in prevention or therapy of mechanically induced OA. We hypothesize that free oxygen radical species might be involved in the mechanical induction of OA. © 2002 OsteoArthritis Research Society International

Key words: Osteoarthritis, Diet, STR/1N mouse, Cartilage, Mechanical overload antioxidants, Vitamins, Selenium.

Introduction

The etiology of osteoarthritis (OA), a degenerative joint disease, is still not fully understood. Next to many other factors mechanical overload seems to be involved in the degeneration of articular cartilage1–3. Thus, certain in vivo and in vitro studies have been established, demonstrating a decrease in biosynthetic activity or viability of chondrocytes or alterations in the extracellular matrix after mechanical damage of cartilage tissue4–8. However, many of the molecular mediators involved in this degenerative process are still unknown.

In several diseases like cancer, cataract or coronary artery disease a curative or preventive potential has been shown for dietary vitamins and selenium9–12. Additionally, a selenium deficiency is known to induce degenerative processes in articular joints13. Thus, we hypothesized that a dietary supplemented cocktail containing different vitamins and selenium might reduce the mechanical induction of articular cartilage degeneration. Our hypothesis is supported by studies from Kaiki et al., demonstrating that a combination of free oxygen radical species and mechanical load induces OA-like changes in articular cartilage, as well as by studies by Miyagi et al. and Tiku et al., showing that static load of cartilage induces release of free radical mediators followed by matrix degradation14–16.

The influence of dietary vitamins or selenium on the etiology of OA has already been investigated in several studies17–23. However, the data are heterogeneous, probably due to the fact that OA is a multifactorial disease. Additionally, the studies were not evaluated with respect to mechanical induction of OA. Thus, the influence of dietary vitamins and trace elements on the etiology of mechanically induced OA still remains unknown.

STR/1N mice are an inbred strain whose male mice are genetically predisposed to develop a pronounced instability in the knee joint with a varus deformity followed by osteoarthritic lesions in the mechanically overloaded medial portion of the tibial plateau24.

In order to prove our hypothesis we fed male STR/1N mice in comparison with Balb/c mice with a control or vitamins/selenium supplemented diet and investigated diet-dependent morphological changes in the medial tibial plateau. Since it is known that vitamins or trace elements (vitamin E, selenium) might influence the antioxidative status of several tissues or blood serum we additionally looked for alterations in the expression or activity of antioxidative enzymes in the knee joint and blood serum25,26.

Our data show that a vitamins/selenium-enriched diet reduces mechanical induction of articular cartilage degeneration and increases the expression of antioxidative enzymes.
Differences in the contents of vitamins and selenium in control and special diet

<table>
<thead>
<tr>
<th>Substance</th>
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<tbody>
<tr>
<td>Vitamin E (mg/kg diet)</td>
<td>300</td>
<td>120</td>
</tr>
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<td>Vitamin C (mg/kg diet)</td>
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</tr>
<tr>
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<td>30</td>
<td>12</td>
</tr>
<tr>
<td>Vitamin B2 (mg/kg diet)</td>
<td>40</td>
<td>25</td>
</tr>
<tr>
<td>Selenium (Na2SeO3 mg/kg diet)</td>
<td>2</td>
<td>0.3</td>
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</tbody>
</table>

Diets were from SSNIF, Soest, Germany.

enzymes in the knee joint. Based on our data we hypothesize that free oxygen-radical species might be involved in the mechanical degeneration of articular cartilage.

Materials and methods

ANIMALS AND DIETS

Six weeks after birth 72 male STR/1N mice (NIH, Bethesda, USA) and 44 male Balb/c mice were divided into two groups each and fed for 12 months with a control diet or a special diet (Table I).

HISTOLOGY AND EVALUATION OF ARTICULAR CARTILAGE DEGENERATION

After physical examination and subsequent ether narcosis ~1 ml blood was isolated from the left heart ventricle of each mouse for measurement of serum glutathione peroxidase activity. Then the knee joints were isolated for paraffin embedding. For paraffin embedding knee joints were incubated for 48 h in 4% paraform aldehyde and then decalcified in 20% EDTA (pH 7.2) at 37°C (decalcification was evaluated by radiography). Samples were dehydrated and embedded in paraplast. Serial frontal sections (8 μm thickness) were made from each joint and stained with toluidine blue. Histological evaluation of cartilage degeneration was made by grading morphological osteoarthritic changes in the medial tibial plateau according to a previously described classification scheme (Fig. 1)27.

GLUTATHIONE PEROXIDASE ACTIVITY

Glutathione peroxidase (GSHpx) activity in serum was assayed using a modified method described elsewhere by following the oxidation of NADPH at 334 nm in the presence of glutathione reductase which catalyzes the reduction of GSSG formed by the peroxidase at 37°C38. The standard reaction mixture contained PBS (pH 7.0), 0.16 mM NADPH (Boehringer Mannheim 107824, Germany), 1 mM GSH (Boehringer Mannheim 127736, Germany), 1.2 mM dimethyl-benzyl hydroperoxide (Merck 820502, Germany), 0.24 U/ml glutathione reductase (Boehringer Mannheim 105678, Germany) and 50 μl sample in a total volume of 1 ml. The reaction was started by addition of dimethyl-benzyl hydroperoxide. Enzyme activity was expressed as μmol NADPH oxidized/min/g protein (= U/g protein). Protein concentration was determined using the method from Bradford29.

IMMUNOHISTOCHEMISTRY

For cryofixation the knee joints were incubated in 20% saccharose for 30 min, transferred into a 3% gelatine solution and frozen in liquid nitrogen. 8 μm thick cryosections were made and rinsed 3x with TRIS-buffered saline (0.14 M NaCl in 20 mM TRIS/HCL buffer, pH 7.4; TBS). The sections were blocked in 0.6% H2O2-methanol for 30 min, rinsed again in TBS and incubated for 45 min with the primary antibody at room temperature. After washing with TBS the sections were incubated for 30 min with the second antibody, washed again and marked with a third antibody for 30 min. The immunoreactivity was visualized by DAB staining. Sections were rinsed again with aqua dest, nuclei were counterstained with Meyer’s hemalum (diluted 1:1 with aqua dest) and mounted with aqueous medium Aquatex (Merck, Germany); (for antibodies used, see Table II).

STATISTICS

The significance of differences between incidences of OA in control diet and special diet groups were calculated for STR/1N mice and Balb/c mice using the χ2-test (for α=0.05). For the glutathione peroxidase activity each calculated value was expressed as means±S.D., and the significance of differences between groups was analysed using the two-tailed Student’s t-test.

Results

MORPHOLOGICAL CHANGES IN THE KNEE JOINT

Degeneration of articular cartilage in the medial portion of the tibial plateau was classified by grading of morphological changes (grade 0–4; Fig. 1).

After 12 months all male STR/1N mice fed with a control diet showed osteoarthritic degeneration in the medial portion of the tibial plateau (Fig. 2) with grades 3 (~19%) or mostly 4 (~81%). Mice fed with the special diet had a significant lower level of osteoarthritic changes than control animals (incidence ~65%) and most of the osteoarthritic joints were grade 2 (~38%). Only ~18% of the special diet animals showed morphological changes of grade 4 in comparison with 81% in the control group.

Balb/c mice fed with the control diet showed osteoarthritic changes of grade 1 with an incidence of ~21% (Fig. 3). In Balb/c mice fed with the special diet the incidence for

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Table I

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Fig. 1. Histological grading of articular cartilage degeneration in the medial tibial plateau of male STR/1N mice (toluidine blue-stained paraffine sections). (a) Frontal section of a knee joint. F=femur, T=tibia, m=meniscus, arrows=medial tibial plateau with osteoarthritic lesion. Bar=60 μm. (b) Superficial fibrillation (arrows) of the tissue without any loss of cartilage (grade 1). Bar=10 μm. (c) Fissuring and cracking of the tissue down to the tidemark (small arrows) with small punched out defects (large arrows) and significant loss of tissue (grade 2). Bar=10 μm. (d) Calcified cartilage forms the articular surface at the level of the tidemark (arrows, grade 3). Bar=20 μm. (e) At least 60–70% of the articular surface in the medial tibial plateau consists of exposed subchondral bone (arrows). Bar=20 μm.
osteoarthritic lesions was slightly but not significantly decreased (∼14% of the joints showed degeneration of articular cartilage, grade 1).

SERUM GLUTATHIONE PEROXIDASE ACTIVITY

Serum glutathione peroxidase (GSH-px) activities in Balb/c mice were in general ∼2.3-fold higher than in STR/1N mice (Fig. 4). In both Balb/c and STR/1N mice the special diet induced a ∼1.5-fold increase in enzyme activities in comparison with corresponding control serums. In the Balb/c group the special diet raised serum GSH-px activity from ∼46.2 (control group; U/g serum protein; mean values) up to ∼68.1 and in the STR/1N group from ∼20.2 (control group) up to ∼29.5.

IMMUNOHISTOCHEMICAL DETECTION OF ANTIOXIDATIVE ENZYMES

We evaluated expression of the antioxidative enzymes Mn-SOD, Cu/Zn-SOD and GSH-px in articular cartilage and synovium of knee joints (data shown in Table III and Figs 5 and 6). Immunostaining of these enzymes (excluding Mn-SOD) was in general stronger in animals fed with the special diet than in control animals.

In detail, there was no immunoreactivity for Mn-SOD in articular cartilage in any of the animal groups. In contrast, Balb/c mice showed a strong staining for Mn-SOD in the synovium independent of the diet. In STR/1N mice the control group had a weak staining for Mn-SOD in the synovium but a strong signal after feeding the supplemented diet.

There was a weak staining for Cu/Zn-SOD in articular cartilage of control Balb/c mice and no staining in cartilage of control STR/1N mice. The special diet highly increased the expression so that most of the cells in the cartilage were positive for Cu/Zn-SOD in both Balb/c and STR/1N mice. Corresponding immunostaining in the synovium demonstrated a strong expression of Cu/Zn-SOD in all four experimental groups.

Table II

<table>
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<tr>
<th>Antigene</th>
<th>1. Antibody (in TBS)</th>
<th>2. Antibody (PAP-conjugated; in TBS, 10% rat serum)</th>
<th>3. Antibody (PAP-conjugated; in TBS, 10% rat serum)</th>
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<tr>
<td>Mn-SOD</td>
<td>Anti-human Mn-SOD (Bender Med. Systems, Vienna, Clone MnS-1; 1:100)</td>
<td>Rabbit-anti-mouse (DAKO, Germany; 1:200)</td>
<td>Goat-anti-rabbit (DAKO, Germany; 1:150)</td>
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<tr>
<td>Glutathione peroxidase</td>
<td>Anti-bovine GSH-px (Biogenesis, Germany 4690-4004; 1:100)</td>
<td>Rabbit-anti-sheep (Jackson ImmunoResearch, Lab. Inc; 1:150)</td>
<td>Sheep-anti-rabbit (Jackson ImmunoResearch, Lab. Inc; 1:150)</td>
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</tbody>
</table>

Fig. 2. Influence of control and special diet (vitamins/selenium-supplemented) on the OA incidence and OA grades in STR/1N mice after 12 months of feeding (measured by evaluation of histological serial sections of the medial tibial plateau). Each bar corresponds to a certain amount of sections and is given in % of all sections (the incidence is the sum of grades 1–4). The difference between the incidence of control (■) and special diet (□) is significant ($\chi^2$-test: for $\alpha=0.05$; $\chi^2=12.25>3.84146$ critical value).

Fig. 3. Influence of control and special diet (vitamins/selenium-supplemented) on the OA incidence and OA grades in Balb/c mice after 12 months of feeding (measured by evaluation of histological serial sections of the medial tibial plateau). Each bar corresponds to a certain amount of sections and is given in % of all sections (the incidence is the sum of grades 1–4). There is no significant difference between the incidence of control and special diet ($\chi^2$-test: for $\alpha=0.05$; $\chi^2=0.49<3.84146$ critical value).

Fig. 4. The influence of control (C) and special diet (supplemented with vitamins/selenium; S) on glutathione peroxidase serum activity (GSH-px) in Balb/c and STR/1N mice after 12 months of feeding (mean values±s.d.; $N=10$; differences between all groups are significant, $P<0.01$).
experimental groups although staining was a little weaker in the STR/1N control group.

Immunoreactivity for GSH-px was diet-dependent in articular cartilage and synovium of both STR/1N and Balb/c mice. While there was no staining in articular cartilage of control animals, there was a weak staining in the synovium. Both tissues demonstrated a strong expression of GSH-px in both strains of mice after feeding the supplemented diet.

Table III

<table>
<thead>
<tr>
<th></th>
<th>STR/1N Special diet</th>
<th>STR/1N Control diet</th>
<th>Balb/c Special diet</th>
<th>Balb/c Control diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mn-SOD Articular cartilage</td>
<td>Ø</td>
<td>Ø</td>
<td>Ø</td>
<td>Ø</td>
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<tr>
<td>Synovium</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Cu/Zn-SOD Articular cartilage</td>
<td>+++</td>
<td>Ø</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Synovium</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
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<tr>
<td>GSH-px Articular cartilage</td>
<td>+++</td>
<td>Ø</td>
<td>+++</td>
<td>Ø</td>
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<tr>
<td>Synovium</td>
<td>+++</td>
<td>+</td>
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</table>

Ø=no staining; ++=few positive cells; +++=about half of the cells are positive; ++++=most of the cells are positive.

Fig. 5. The influence of control and special diet (supplemented with vitamins/selenium) on expression of immunoreactive antioxidative enzymes in cryostat sections of knee joints of STR/1N mice after 12 months of feeding. sb=subchondral bone. (a) No immunoreactivity for glutathione peroxidase (GSH-px) in articular cartilage of control animals. Arrows=cartilage surface. Bar=10 μm. (b) Cell associated immunoreactivity for GSH-px (long arrows) in articular cartilage of the special diet group. Short arrows=cartilage surface. Bar=6 μm. (c) No immunoreactivity for Cu/Zn-superoxide dismutase in articular cartilage of control animals. F=femur, T=tibia, arrow=joint cavity. Bar=30 μm. (d) Cell associated immunoreactivity for Cu/Zn-superoxide dismutase in articular cartilage of the special diet group (arrows). Bar=30 μm.
Discussion

In our study we investigated the effect of dietary vitamins and selenium on the mechanical induction of OA and the expression of antioxidative enzymes in the knec joint. Male STR/1N mice are genetically prone to develop a varus deformity. As a consequence there is a mechanical overload of the medial tibial plateau followed by OA-like degenerative changes in the articular cartilage starting with slight fibrillation in the superficial layer followed by deep fissures and subsequent loss of uncalcified and finally calcified cartilage tissue. Thus, STR/1N mice are a useful model for the investigation of mechanical overload of articular cartilage. However, many of the molecular mechanisms responsible for this degeneration are still unknown.

We show for the first time that the antioxidative status of male STR/1N mice is reduced in comparison to Balb/c mice. Thus, we hypothesize that the level of ROS in the joint might be increased and involved in the mechanical induction of cartilage degeneration in this animal model. The fact that STR/1N mice have generally low ROS scavenger potential and that we found a diet-dependent increase in expression and activity of antioxidative molecules parallel to a decrease in mechanical induction of OA deserves more detailed investigation. It raises the question of whether mechanical overload is responsible for generation of reactive oxygen species (ROS) in articular cartilage. The hypothesis that mechanical stress leads to accumulation of free radicals and subsequent degeneration of articular cartilage has already been postulated by Milam et al. with respect to degenerative temporomandibular joint diseases. Tiku et al. also postulate that an increased oxidative activity in chondrocytes might be an important factor in the etiology of osteoarthritis. Thus, higher expression of antioxidative enzymes (which we found in our study) would lead to protection against development of ROS-induced OA.

Chondrocytes are generally able to produce ROS like superoxide anions, hydrogen peroxide and hydroxyl radicals. Exogenous hydrogen peroxide is known to damage cultured chondrocytes and is thought to be involved in cartilage degradation. Catalase and glutathione peroxidase play an important role in cartilage protection against hydrogen peroxide dependent inhibition of proteoglycan biosynthesis. Additionally, the hypothesis that ROS are involved in mechanical induction of cartilage degeneration is supported by the fact that intraarticular injections of hydrogen peroxide together with running load induce OA in rats which can be inhibited by simultaneous injection of vitamin E.

Synoviocytes express several ROS scavengers like superoxide dismutase, glutathione peroxidase or catalase. We show in this study, that this expression can be increased by a diet supplemented with vitamins and selenium. In contrast, ROS defence mechanisms in articular chondrocytes are generally weak probably due to the fact that articular cartilage normally exists in a low oxygen tension environment. However, chondrocytes have the potential to increase their antioxidant status and in the present study we were also able to increase the expression of antioxidative enzymes in articular cartilage of both STR/1N and Balb/c mice.

The influence of dietary vitamins or selenium on the etiology of OA has already been investigated in some studies. Riboflavin inhibits degeneration of cartilage in C57 black mice which develop OA spontaneously. Ascorbic acid reduces or increases the development of spontaneous or experimentally induced lesions in articular cartilage of guinea pigs. In contrast, a Framingham knee OA study did not show clear evidence for a correlation between incidence of OA and habits of eating with respect to antioxidative vitamins. Additionally, a controlled double-blind trial of selenium combined with the vitamins A, C, and E in OA also failed to show any improvement. Alpha-tocopherol, on the other hand, showed an analgesic effect in OA in human. The data in the literature are heterogeneous, probably due to the fact that OA is a multifactorial disease. Thus, the importance of vitamins and trace elements in the etiology of OA especially with respect to mechanical induction of cartilage degeneration still remains unclear. We show now that there is a connection between dietary intake of vitamins and selenium and the pathogenesis of mechanically-induced OA.

However, a number of problems add complexity to our study. There are no assays measuring the antioxidative...
activity in the joint and little is known about the tissue distribution and bio-availability of dietary supplemented antioxidant molecules within joint tissues. Thus, a correlation between mechanical overload and the induction of ROS in cartilage tissue still remains a hypothesis and has to be investigated further. Additionally, one has to keep in mind that some of the substances might have functions other or additional to antioxidative mechanisms that might be responsible for the reduction of mechanically induced OA in our study. Vitamin E is known to decrease collagen synthesis in fibroblast-like cells\(^41\). However, we measured the influence of vitamin E on collagen synthesis or proline incorporation in explant or cell cultures of bovine cartilage and we did not see any significant alterations (data not shown). Vitamin C is an important co-factor for the lysyl and prolyl hydroxylase\(^42\). These enzymes enable collagen molecules to form cross-links. Thus, vitamin C is important for the stability of the collagen network. We decided to add vitamin C to the supplemented diet although it is known that mice normally are able to produce vitamin C on their own. Vitamin C is an important co-worker of vitamin E and thus, these substances enhance each other in their antioxidative function. Pyridoxal phosphate within \(\beta\)2HPL supports the formation of lysyl oxidase, an enzyme initiating cross-link formation in collagens\(^43\). Besides, pyridoxal deficiency is known to induce alterations in proteoglycan metabolism\(^44\). Thus, a vitamin B6-enriched diet might increase the resistance of cartilage against mechanical degeneration.

Based on our data we conclude that the combination of dietary vitamins and selenium used in this study diminishes the mechanical induction of OA in STR/1N mice. Since reduction of OA was accompanied by an increase in expression and activity of antioxidative enzymes we hypothesize that ROS might be involved in the mechanical degeneration of articular cartilage. Which supplemented nutrient had the biggest influence in our study, and whether some of the substances have to act together as co-workers remains unknown and has to be investigated in future studies. Additionally, there might be species-dependent differences in the response to these nutrients and it remains unclear whether such a diet works for humans too. Nevertheless, our data encourage us to proceed in the investigation of preventive or even therapeutic potentials of dietary vitamins and trace elements with respect to the mechanical induction of OA.

Acknowledgments

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References


