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Selenium deficiency and fulvic acid supplementation induces fibrosis of cartilage and disturbs subchondral ossification in knee joints of mice: an animal model study of Kashin-Beck disease

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Abstract. Kashin-Beck disease is an acquired, chronic and degenerative osteoarticular disorder. Selenium deficiency and fulvic acid in drinking water have been implicated in the cause of this disease. Pathologically, chondronecrosis of the growth plate and articular cartilage and subconsequent disturbance of ossification were observed in the joints. In this animal model study, mice were fed with a selenium deficient diet and fulvic acid supplemented drinking water for two generations. In undecalcified histological preparations of bone we carried out histological staining to detect mineralized and unmineralized bone and cartilage. The results revealed that selenium deficiency and fulvic acid supplementation induced degeneration of the articular cartilage in the knee joints of mice. Dynamic fluorescent labelling of ossification, enzyme histochemical detection of alkaline phosphatase activity in osteoblasts and a typical immunohistochemical localization of collagens type I and II indicated the development of fibrocartilage at the articular surface of knee joints, resembling the early stages of osteoarthrosis. This became obvious by disturbed development of the articular space and meniscus, markedly impaired formation of subchondral bone and early differentiation failure during enchondral ossification. This animal model provides an approach to study the molecular pathogenesis of Kashin-Beck disease.

Key words: Kashin-Beck disease – Selenium deficiency – Fulvic acid – Methyl methacrylate-embedding method – Undecalcified bone preparation

Introduction

Kashin-Beck disease (KBD) which occurs mainly in China, is an endemic, degenerative, osteoarticular disorder associated with severe skeletal deformation and dwarfism. Multiple degenerative and necrotic lesions within the articular cartilage and the growth plate represent the initial pathological changes which are associated with a disturbed mineralization and disfiguration of joints (Yang et al. 1990b). Furthermore, morphological studies on affected tissues show a distored deposition of collagen fibres in cartilage. Recently we described a molecular defect in collagen II from the articular cartilage of two patients (Yang et al. 1991). Specifically, we showed an overmodification and an impaired conversion of PN-collagen II to functional collagen II (Yang et al. 1993).

The epidemiology of the KBD provides convincing evidence that selenium deficiency in food and a high content of fulvic acid in drinking water were the main causative factors of the disease in China (Jiang and Xu 1989). In China, dietary supplementation of selenium to populations in the endemic areas and a better quality of drinking water have substantially reduced the number of new cases of KBD (Li 1989). Selenium is part of the active sites of glutathione peroxidase (GSH-px) and type I iodothyronine deiodinase. There is general agreement that both enzymes play crucial roles in vivo since glutathione peroxidase can protect tissues against reactive oxygen damage and iodothyronine deiodinase can convert thyroxine to the biologically active form, 3,5,3′-triiodothyronine (Peng et al. 1988). In selenium deficiency, a variety of tissues and cells could become less resistant to injury. Accordingly, a variety of pathological symptoms related to selenium deficiency are known including KBD and Keshan-disease (Zhaohan et al. 1990). Fulvic acid, occuring naturally in water, soil and peat as a fraction soluble in water and aqueous acidified solutions, is produced by chemical and microbial decomposition of plants and animals. It contains many reactive groups including carboxyls, hydroxyls, carbonyls, phenols and quinones. It is possible that the combination of selenium deficiency and high intake of fulvic acid causes the onset of KBD.

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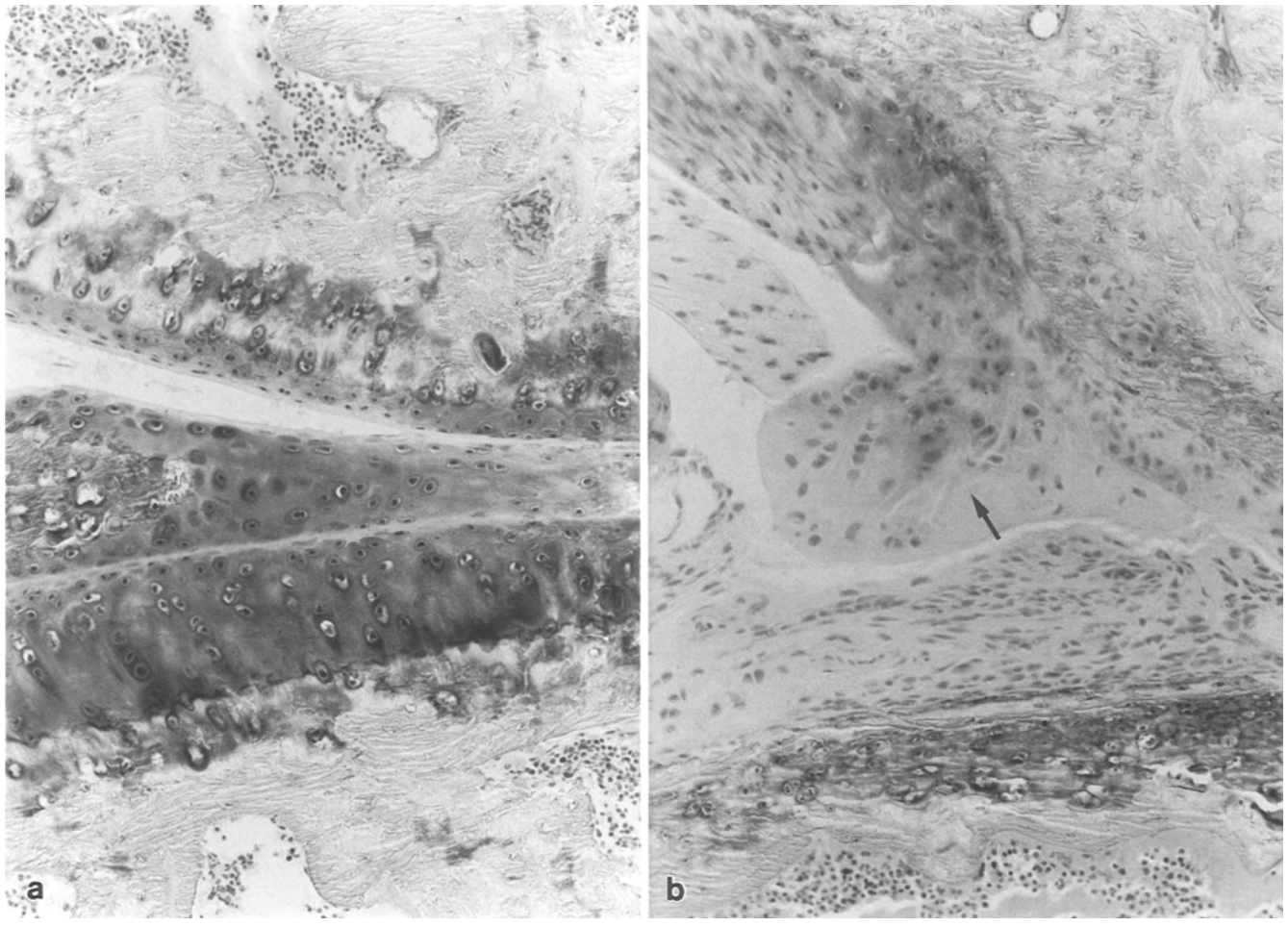

Fig. 1. **a** Undecalcified section. Knee joint of mouse (control group). Articular cartilage with meniscus. Mouse of the second generation at the age of 49 days. Toluidine blue staining, ×160. **b** Undecalcified section. Knee joint of mouse with disturbed articular cavity (study group). Collagen fibres with small islands of hyaline cartilage (→). Toluidine blue staining. ×160

Materials and methods

Drinking water from a KBD affected area was acidified with hydrochloric acid and subsequently passed over a GDX-102 resin (Tianjin 2nd Chemical Factory, China). The water soluble fulvic acid was absorbed on the resin and eluted by a solvent containing ethanol and ammonia in a ratio of 1:2. From 1000 l of drinking water, about 300 mg of fulvic acid was collected and stored at +4° C until use.

A study group (nine animals) of two male and seven female NMRI mice, at the age of 50 days, were fed a selenium deficient diet and fulvic acid supplemented drinking water. The fulvic acid supplemented drinking water contained 20 ppb fulvic acid. The selenium deficient feed (C 1045 Selen-arm, Altromin, Germany) had a concentration of 31 ppb selenium, while the control group obtained a feed with a selenium content of 314 ppb. The mice were each fed with their special diet and water for 2 weeks. Afterwards, the male and female animals were kept together for another week. Gravid female mice and the second generation of mice, which were used for these studies, received the same diet. A control group of two male and seven female mice were maintained under normal diet from which also fulvic acid was omitted. Second generation mice of each group were fed up to an age of 21 days. Afterwards, an aqueous solution of xylenol orange (90 mg/kg body weight) was administrated at first and then calcein (20 mg/kg body weight) was administered (Rahn 1976; Wolf et al. 1982). Both fluorochromes were twice injected intra-peritoneally at an interval of 7 days (day 21, 28: xylenol orange; day 35, 42: calcein). At the age of 49 days mice were sacrificed for morphological evaluation. Ten mice of each group were used in the investigation.

The hind legs of the mice were dissected and the skin, muscle and tendons were separated from the joints. The joints were fixed in 1.4% paraformaldehyde with 5% sucrose in 0.02 M phosphate buffer solution (pH 7.4) for 24 h at +4° C. The dehydration, immersion, embedding in methacrylate at −15° C and undecalcified sectioning were performed as described previously (Wolf et al. 1992). Unstained and undecalcified sections of 10 µm thickness were investigated with a fluorescence microscope. The histomorphology of cartilage and calcified tissue was analysed by toluidine blue and von Kossa staining.

Antisera against mouse collagen I and II were raised in rabbits as described (Yang et al. 1991). The antibody titre was determined by direct enzyme-linked immunosorbent assay and the specificity of the antiserum was checked by immunohistological staining of suitable tissue sections and by immunoblotting.

To ensure adhesion through the entire immuno- and enzyme histochemical procedures, sections were dried overnight or longer at 40° C. A complete removal of resin was achieved using 2 × xylene for 20 min and 1 × methyl glycol acetate for 20 min. Rehydrated sections were transfered in 0.1 M phosphate buffer for histochemical demonstration of alkaline and acid phosphatase by a recently described procedure (Wolf et al. 1992). For the immunological investigation of collagens pretreatment of sections it is necessary

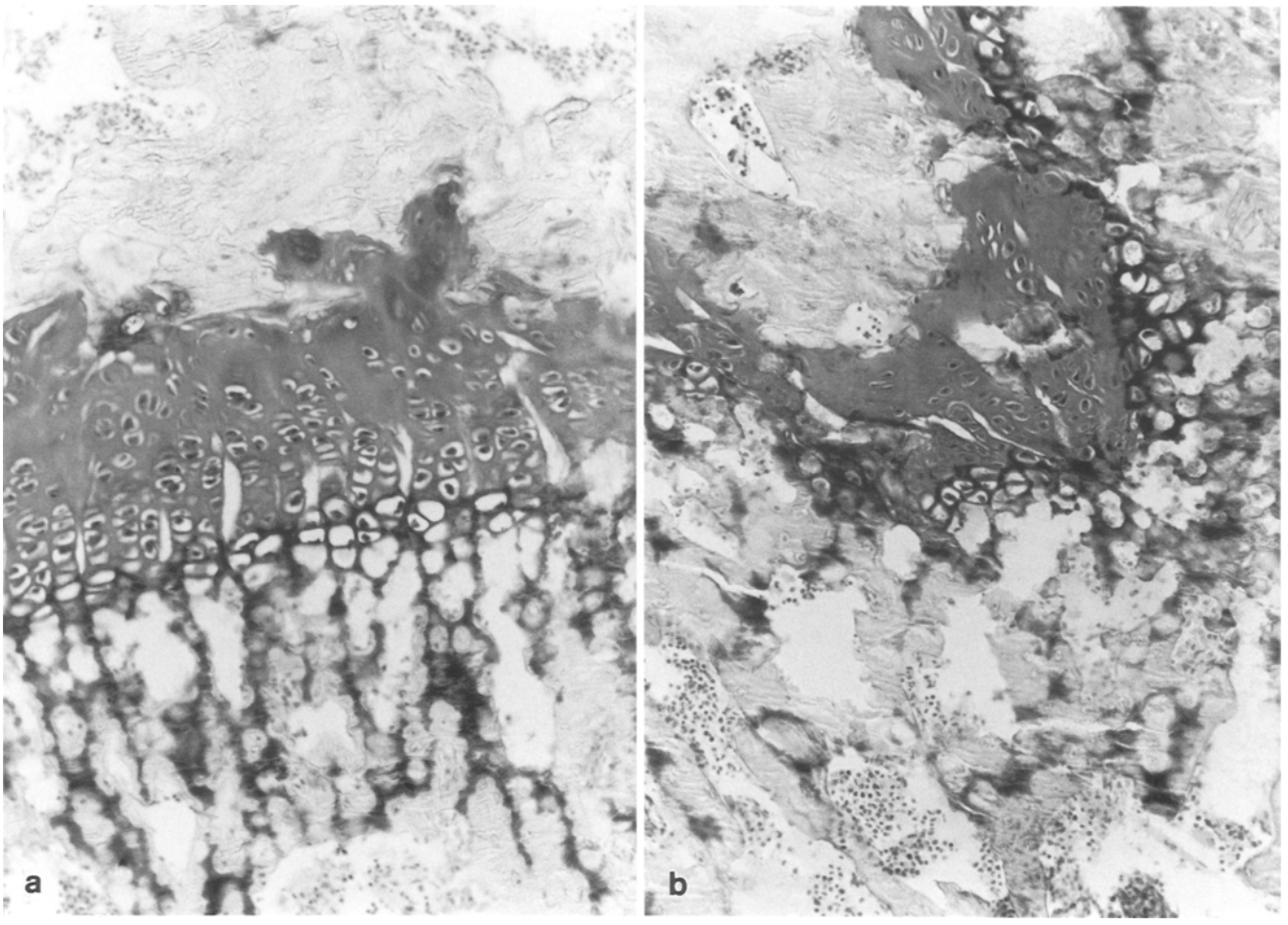

Fig. 2. **a** Undecalcified section. Control group. The enchondral growth plate of tibial bone shows a regular zonal arrangement. Toluidine blue staining. ×160. **b** Undecalcified section. Study group. The enchondral growth plate is slightly irregular in their zonal arrangement. Mineralized bone and cartilage of the hypertrophic zone are dark coloured. Toluidine blue staining. ×160

to remove calcium salts and proteoglycanes, as well as to reverse antigen masking caused by formaldehyde fixation. Rehydrated sections were decalcified in 10% EDTA, 0.1 M TRIS-buffer (pH 7.0–7.2) for 3 × 20 min. Afterwards, the sections were treated with 0.1% hyaluronidase (Sigma, Deisenhofen, Germany) and trypsin (Sigma) to expose the epitopes of collagen. A pre-incubation of slides was then carried out with 10% normal swine serum (Dako code no. Z 180) and 4% bovine serum albumin (BSA) in 0.01 M phosphate buffer (pH 7.4). All immunohistochemical investigations were performed with the alkaline phosphatase-anti-alkaline phosphatase (APAAP) technique according to the manufacturer's instructions. Rabbit antisera against collagens type I and II were produced (see above). Primary antibodies were diluted in 0.01 M phosphate buffer (pH 7.4) with 4% BSA and incubated at +4° C overnight. For APAAP sandwich assays, swine anti-rabbit immunoglobulin (Dako, code no. Z 400) as secondary antibody was combined with rabbit anti-rat immunoglobulin (Dako, code no. Z 455) as tertiary antibody and a monoclonal rat APAAP complex (Dako, code no. D 488). The immunohistochemical reaction was visualized by a modified system using naphthol-AS-TR-phosphate as substrate and fast blue as azo-colouring agent for conjugation. Levamisol was added to block endogenous alkaline phosphatase. Negative and positive (cartilage tissue) controls were processed for immunostaining in accordance with Bourne (1983).

All slides were examined by means of an Axioskop light microscope (Zeiss, Oberkochen, Germany) with additional equipment for fluorescence microscopy (HBO 50 violet excitation) and a 35 mm camera with automatic exposure timer to take the light microscopy pictures (Zeiss). The films for light and fluorescence microscopy were Agfa CT 100, 100 ASA and AGFAPAN, APX 25 (Agfa, Germany); exposure times were between 60–90 s for fluorescence microscopy.

Results

An epidemiological survey in China has shown that KBD mainly involves children, in early childhood. Thus, in an attempt to establish an animal model of KBD, mice were treated with the presumptive causative factors for two generations. Ten mice of the second generation were used in the investigation at the age of 49 days.

At this stage of life, the development and differentiation of joints was not completed (Fig. 1 a). The articular cavity, which is bordered by synovial membranes and by the articular surface, could not be identified in all sections. The synovial membrane provides a connection from vascularized connective tissue to menisci. Structurally, the menisci were composed of bilateral hyaline cartilage orientated in axial direction. A calcified core of the meniscus was observed, presumably at the site of

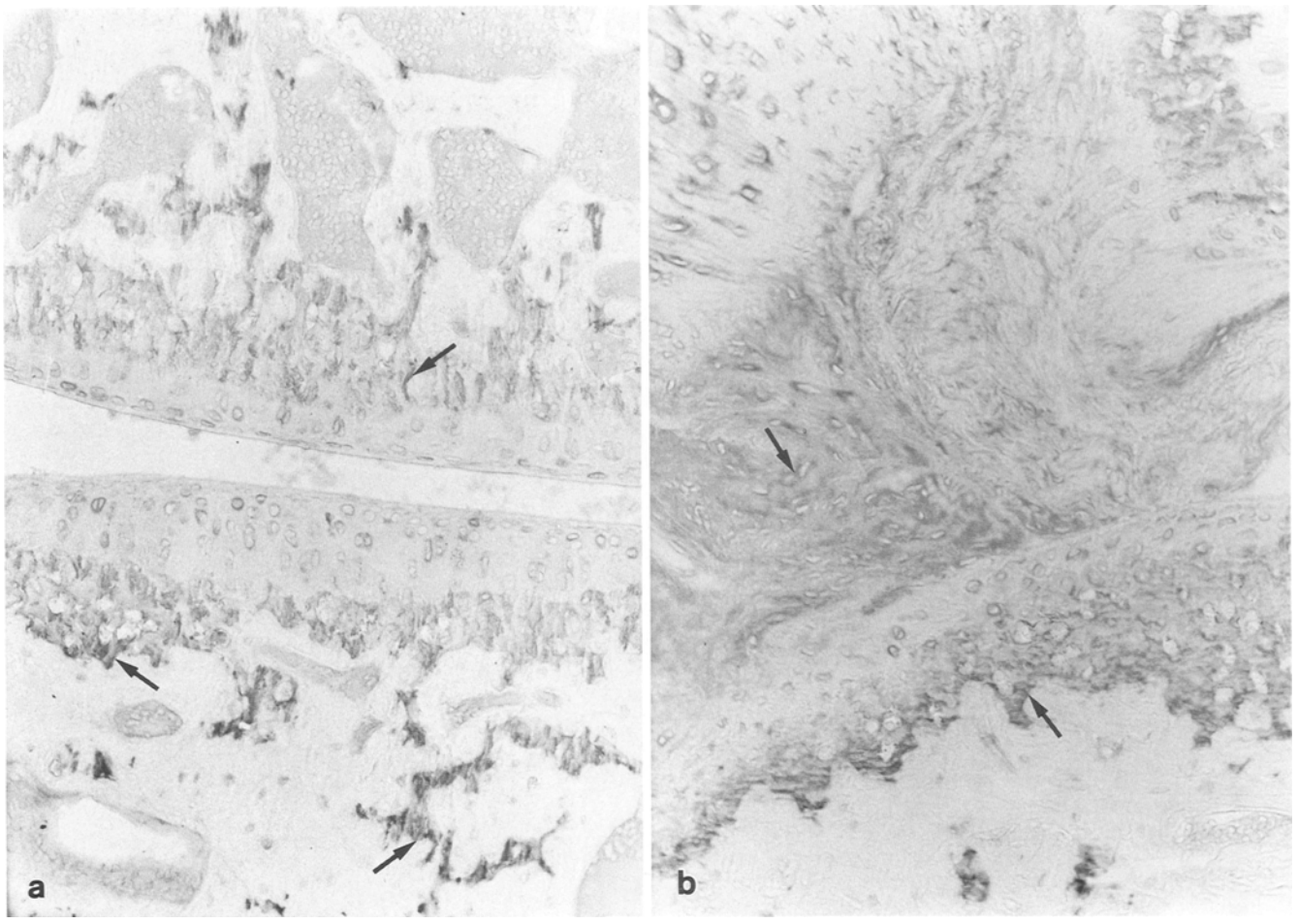

Fig. 3. a Immunohistochemistry of collagen type II. Enchondral growth plate (control group). Hyaline cartilage of the hypertrophic zone and the central region of the newly formed trabecular bone (epi-, subchondral) show a positive staining (→). Polyclonal anti-collagen type II antibody. Undecalcified section. ×160. **b** Immunohistochemistry of collagen type II. Disturbed articular cavity (study group; see Fig. 1b). Positive staining (→) is observed in the collagenous fibres of the articular cavity and in the articular hyaline cartilage. Polyclonal anti-collagen type II antibody. Undecalcified section. ×160

maximal compression. An ossified patella was covered by a layer of cartilage perichondrium which was fixed to the synovial membrane. The exterior patella was connected to the perichondrium of tibia and femur by collagenous fibres. The articular surface of tibia and femur were covered by hyaline cartilage, which reached its widest extention in the area facing the menisci, where one can expect the major weight loading of joints. Fibrocartilage could be observed at the central site of the surface of tibia and femur, while interchondral ligaments could not be observed. A connection between tibia, femur and menisci by collagenous tissue was found. These morphological observations suggest that the development of the joint is still occuring and an opening of the articular space is not yet completed.

Subchondral ossification results in lamellar bone formation which further differentiates into Haver's cortical and cancellous bone with bone marrow. Both femur and tibia showed a typical bone architecture. The growth plates of femur and tibia showed a regular zonal arrangement, clearly distinguished in resting zone, proliferation zone and hypertrophic zone (Fig. 2a). Normal cancellous bone formation with active modelling of bone surface was observed, characterized by a layer of polyhedral osteoblasts at the surface of osteoid bone and multinucleated osteoclasts in the resorption lacunae in enchondral ossification. The central region of these trabecular bones showed a strong reactivity with antibodies against collagen type II (Fig. 3a). The structure of cortical diaphyseal bones was lamellar with a predominant periosteal and endosteal bone formation. In most cases, the metaphyseal cortical bone formation showed Haversian remodelling with wide Haversian canals and active bone formation. In osteoblasts from areas with subchondral and enchondral ossification strong activity of alkaline phosphatase was observed by enzyme histochemistry (Fig. 4a). The newly formed bone was dynamically labelled by fluorochromes. Woven bone exhibits a diffuse labelling pattern whereas lamellar bone exhibits a linear labelling pattern (Figs. 5a, 6a, 7a). Two to three weeks after labelling in normal bone tissue the lamellar formation of cancellous bone showed a more distinct

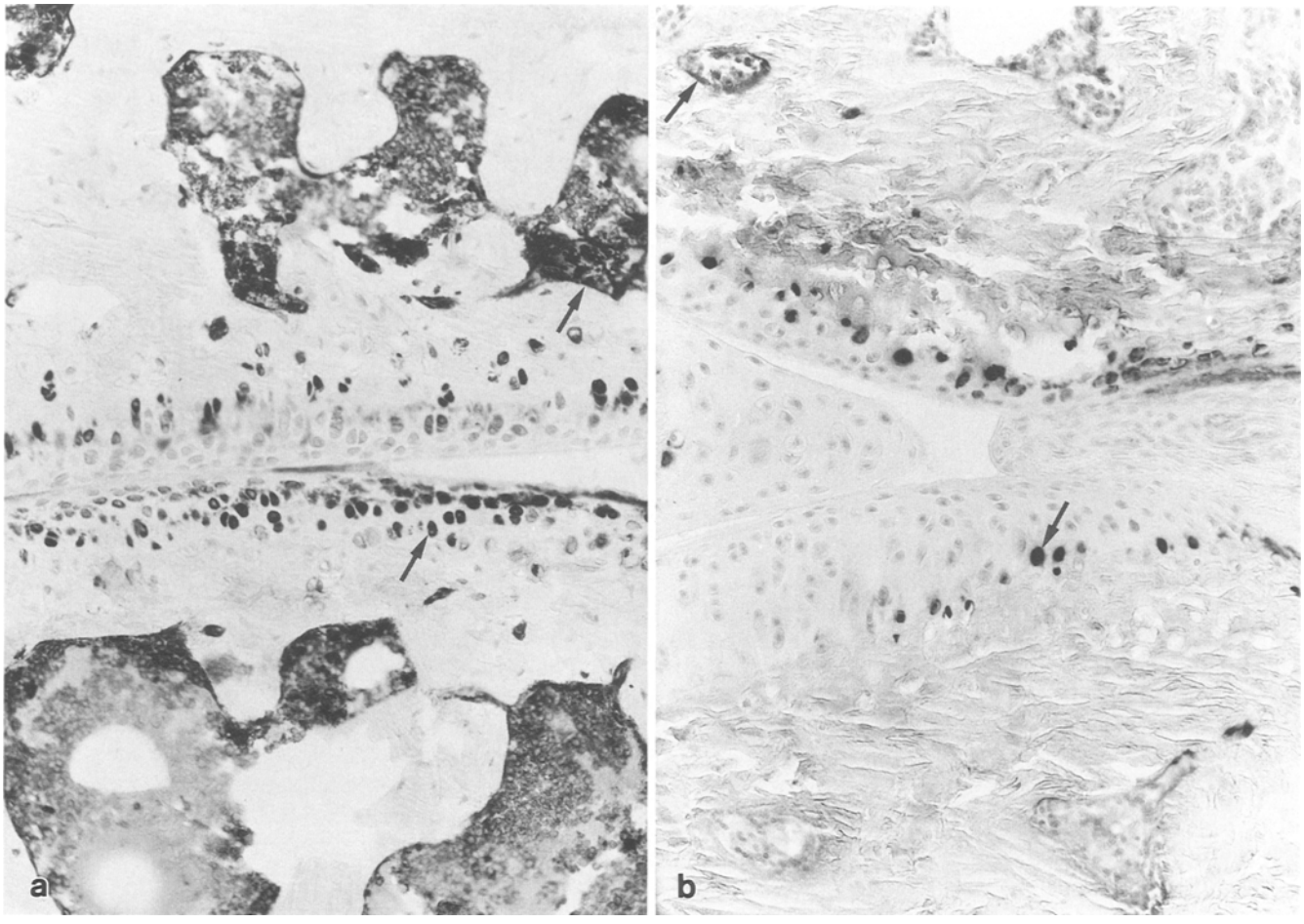

Fig. 4. **a** Enzyme histochemistry of bone alkaline phosphatase activity. Articular cartilage (control group). Osteoblasts, chain-like arranged in the subchondral zone, hypertrophic chondrocytes of the articular cartilage and fibroblasts in the bone marrow show a strong reactivity (→). Nuclear counterstaining with methyl-green. Undecalcified section. ×160. **b** Enzyme histochemistry of bone alkaline phosphatase activity. Articular cartilage (study group). Irregularly distributed osteoblasts have low activity (→). Undecalcified section. ×160

linear fluorescent pattern than that seen during early growth periods, where the formation of woven bone can also be observed.

In the study group the animals (ten mice) exposed to fulvic acid supplementation and selenium deficiency showed changes in the articular cartilage similar to those seen in the early stages of osteoarthrosis. The development of the articular space was disturbed (Fig. 1b) and a slightly irregular shape and stratification of the enchondral growth plate was observed (Fig. 2b). Conventional light microscopy did not reveal marked changes of stratification of hyaline cartilage, of the primary spongiosum, secondary spongiosum or in cancellous bone formation of the enchondral growth plate. Immunohistochemistry of these zones demonstrated the typical distribution of collagen II fibres located in the centre of newly formed trabecula, which were successively degraded in the course of further differentiation. The collagen II expression pattern correlates with that of the control group (Fig. 3a). By fluorescence microscopy a moderately disturbed differentiation of bone was demonstrated. The proportion of woven bone was increased in cancellous bone formation of the diaphysis as well as in the surrounding cortical bone. The pathological changes were mainly found in the articular space and articular hyaline cartilage of femoral and tibial bone. In some cases, virtually no typical meniscus was seen; it was replaced by a cord of fibrocartilage which formed a connection with the articular cartilage (Fig. 1b). In most cases fibrosis of articular cartilage could be observed. In the fibrocartilage, chondrocytes had a chain like morphology and still synthesized collagen II which was deposited pericellularly (Fig. 3b). In sections stained with toluidine blue, these fibres showed a distinctive metachromasia.

Under the fibrocartilage subchondral ossification of the articular region had ceased, as revealed by the low activity of alkaline phosphatase (Fig. 4b) and the virtual absence of linear label with fluorochromes (Fig. 5b). In some other areas, a disturbance of subchondral ossification could also be observed, as demonstrated by the diffuse fluorescent labelling caused by woven bone formation (Figs. 6b, 7b).

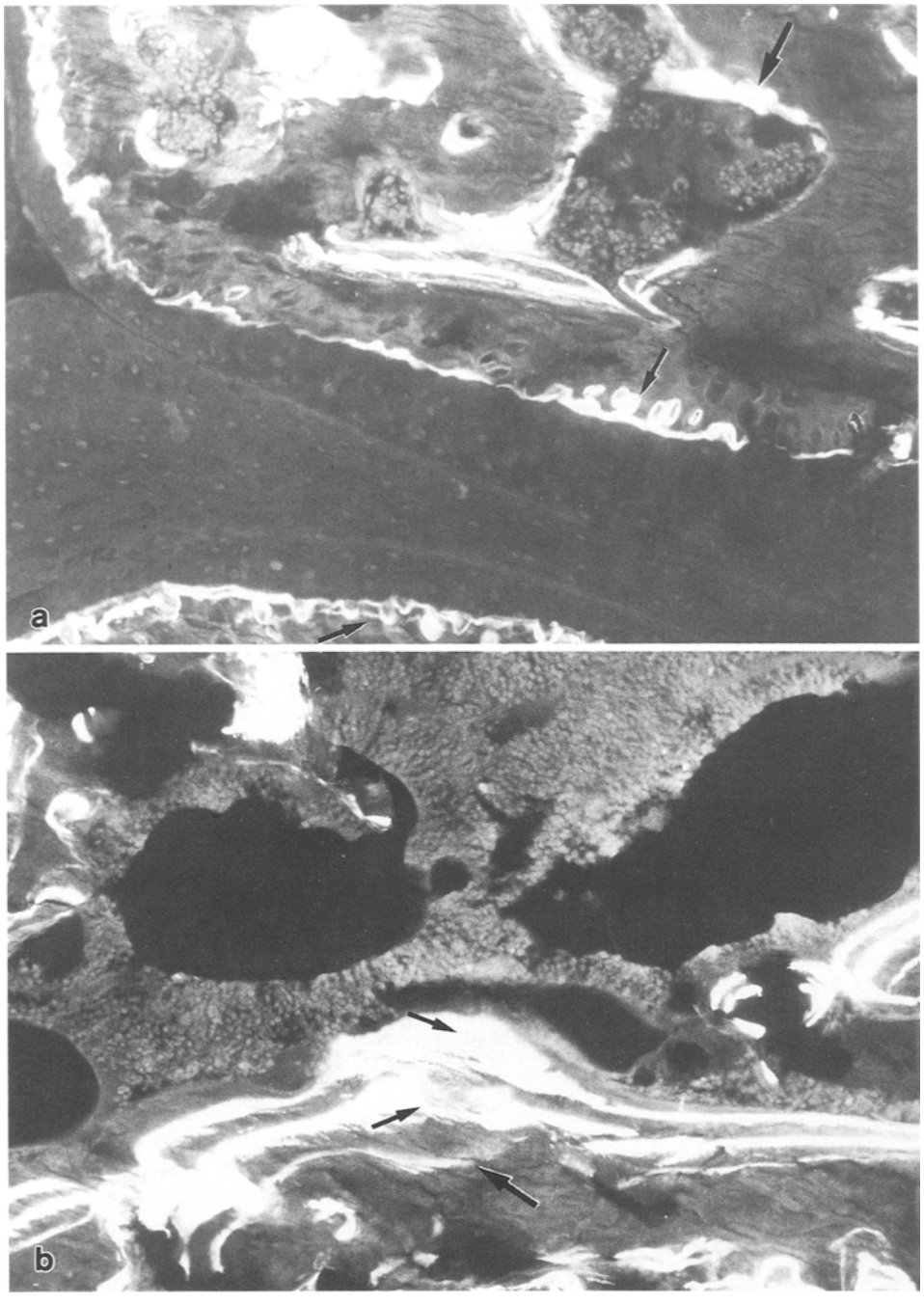

Fig. 5. a Fluorescence labelling of bone. Articular cartilage (control group). The subchondral zone shows a regular bone formation (linear label). UV-light. Calcein: green (→). Xylenolorange: orange to yellow (→). Undecalcified section. ×160. **b** Fluorescence labelling of bone. Articular cartilage (study group). The subchondral region demonstrates the disturbed bone formation and a diffuse label of the newly formed woven bone. UV-light. Calcein: green (→). Xylenol-orange: orange to yellow (→). Undecalcified section. ×160

Discussion

Patients with KBD are found in geographic regions of China naturally deficient of selenium. Fulvic acid concentration in local drinking water is higher than in normal areas and it has some unique chemical properties. Both selenium deficiency and fulvic acid were proposed to be the factors crucial for the initiation of the disease.

Previously, we found that selenium deficiency and fulvic acid supplementation could induce modification of collagen I in bone and collagen II in cartilage (Yang et al. 1993). Consequently, the collagen molecules have a lower thermal stability and the bones have a reduced mechanical strength. In this morphological investigation, it was found that the mice fed with selenium deficient feed and fulvic acid supplemented drinking water developed fibrocartilge at the articular surface of knee joints. The articular space of knee joints of treated mice showed a pattern similar to that in developing osteoarthrosis. This included a disturbed development of the articular space and meniscus, a markedly impaired formation of subchondral bone tissue adjacent to the joint and

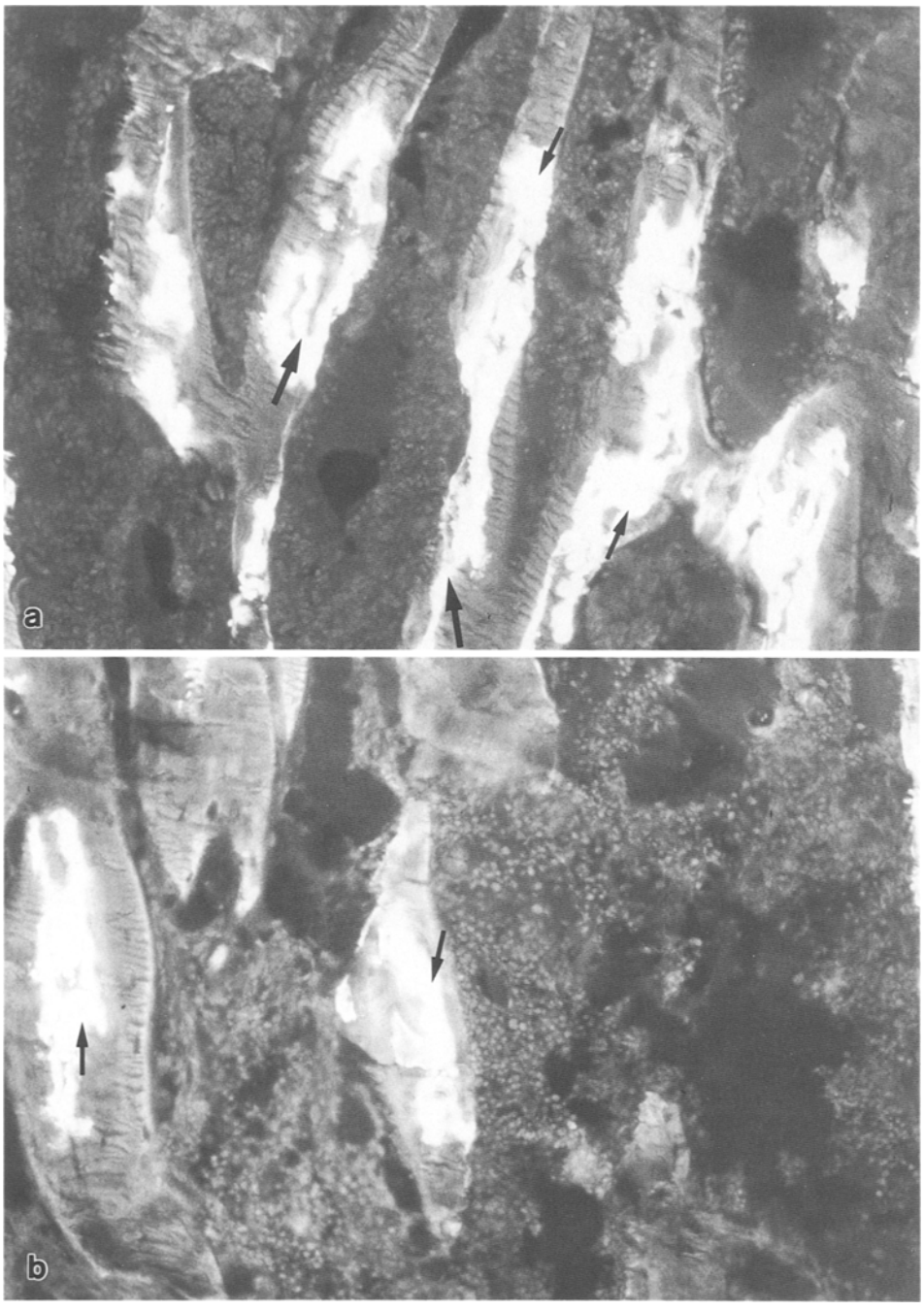

Fig. 6. a Fluorescence labelling of bone. Tibial epiphysis (control group). The enchondral ossification zone with the newly formed trabecular bone shows a diffuse and linear labelling (regular, woven (→) and lamellar (→) bone). UV-light. Undecalcified section. ×160. **b** Fluorescence labelling of bone. Tibeal epiphysis (study group). The enchondral ossification zone with the newly formed trabecular bone shows a diffuse (woven bone) labelling (→). UV-light. Undecalcified section. ×160

an early disturbance of differentiation in enchondral ossification. Fibrosis of the cartilage subsequently reduced subchondral ossification. Severe deformation of joints were not observed, but these changes are found only in late lesions in KBD with necrosis of the bone (Yang et al. 1990b).

One can assume that selenium deficiency and fulvic acid supplementation cause a disturbance of cellular function and have an impact on the organized formation of the extracellular matrix and the tissue architecture. Selenium deficiency can interfere with the activity of GSH-px, which is a scavenger of reactive oxygen, while fulvic acid may accumulate in the skeletal system and induce a production of superoxide via its semiquinone radicals. An excess of reactive oxygen from fulvic acid cannot be removed in selenium deficiency and this high level of reactive oxygen may bring about an alteration of tissue structure and the extracellular matrix in the mice described here and in clinical KBD.

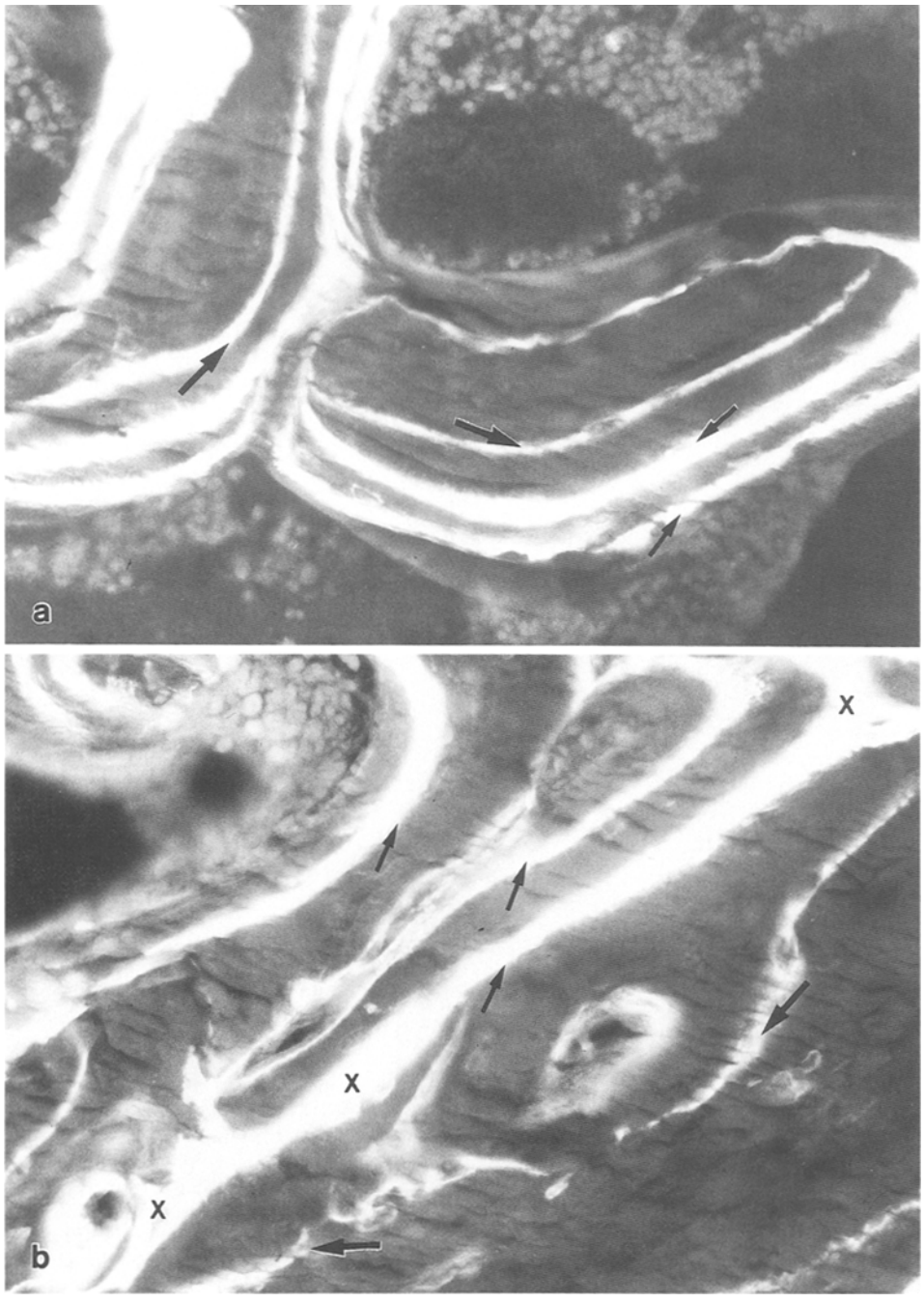

Fig. 7. a Fluorescence labelling of bone. Cortical bone of the tibial diaphysis (control group) shows a regular lamellar bone formation (linear label). UV-light. Calcein: green (→). Xylenolorange: orange to yellow (→). Undecalcified section. ×160. **b** Fluorescence labelling of bone. Cortical bone of the tibial diaphysis (study group). The newly formed cortical bone shows a linear (regular bone) and a diffuse (x) (woven bone) labelling. UV-light. Calcein: green (→). Xylenolorange: orange to yellow (→). Undecalcified section. ×160

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