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Fulvic acid disturbs processing of procollagen II in articular cartilage of embryonic chicken and may also cause Kashin-Beck disease

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Kashin-Beck disease is an endemic osteoarthropathy in China which may lead to skeletal deformation and dwarfism. We have analysed articular cartilage from two patients and found an accumulation of the precursor molecule, pro-pN-collagen II (pN, peptide atached at the amino-terminus) which was not present in extracts of control fetal cartilage. In addition, collagen II isolated from the same tissue by limited pepsin digestion had a decreased electrophoretic mobility, increased proline hydroxylation and decreased thermal stability. Previously, a genetic defect in pro-pN-collagen-I processing has been described in calf and sheep (dermatosparaxis) and man (Ehlers-Danlos, type VII) which caused an extreme fragility of the skin [Lenaers, A., Ansay, M., Nusgens, B. V. & Lapière, C. M. (1971) *Eur. J. Biochem. 23*, 533–541; Helle, O. & Nes, N. J. (1972) *Acta Vet. Scand. 13*, 443–445; Lichtenstein, J. R., Martin, G. R., Kohn, L. D., Byers, P. H. & McKusick, V. A. (1973) *Science 182*, 298–300]. Accordingly, one may assume that the impaired conversion of pro-pN-collagen II to collagen II and the structural alteration of collagen II, presumably caused by fulvic acid and other environmental factors, play an important role in the pathogenesis of Kashin-Beck disease.

Interstitial collagens, such as types I, II and III, are synthesized in a precursor form, procollagen, with additional peptides at both the amino-terminus (pN) and the carboxy-terminus of the triple-helical molecule [1]. It has also been shown that genetic defects in man, cattle and sheep are due to an incomplete conversion of pro-pN-collagen I to collagen I [2, 3].

Kashin-Beck disease is an endemic osteoarticular disorder with a high prevalence in a geographic area of China that extends obliquely from the north-eastern provinces to Tibet in the south-west. Some reports indicate that neighboring areas in the USSR, as well as some regions in Vietnam and Korea, may also be endemic for Kashin-Beck disease [4]. In China, a population of 50 million lives in the endemic area and about 3 million persons were found to be severely affected as the disease shows up in early adolescence. A clinical classification has been proposed, taking into account the progressive manifestation of clinical symptoms with painful and stiff joints in the beginning and complete dysfunction of grossly enlarged and disproportionate joints at later stages. Often, the short stature of patients is a characteristic clinical feature of the disease. There is convincing evidence that the disorder is acquired rather than inherited, blaming environmental factors for the disease [5]; in particular, low levels of selenium in nutrition [6], high levels of humic acids (e.g. fulvic acid) in drinking water [7] and high concentrations of mycotoxins elaborated by the mold *Fusarium oxysporum* on rotten grain (Yang, G. B., unpublished results). It is widely accepted that the pathophysiological process occurs mainly, if not exclusively, in the articular cartilage, where histological damage can be seen as a coagulative necrosis of hypertrophic chondrocytes in the epiphyseal growth plates [8].

In the present study we have analysed articular cartilage from two patients with Kashin-Beck disease and found an accumulation of pro-pN-collagen II, as well as a pepsin-solubilized collagen II which is structurally altered. Furthermore, we provide experimental evidence that fulvic acid, a presumptive cofactor of the disease, can inhibit the conversion of procollagen II to collagen II, after fulvic acid was injected into fertilised chicken eggs.

MATERIALS AND METHODS

Extraction and fractionation of collagen from cartilage by differential salt precipitation

All procedures were performed at 4°C. Articular cartilage from two adult patients with Kashin-Beck disease was obtained during orthopedic surgery and immediately frozen. In addition, articular cartilage from a human stillborn fetus was available for analysis. Cartilage specimens were homogenized, then extracted with 1 M NaCl and 0.05 M Tris, pH 7.4, containing 1 mM phenylmethylsulfonyl fluoride as a protease inhibitor. The insoluble material was collected by centrifugation and subjected to limited pepsin digestion (0.1 mg/ml, 24 h). Collagen types were separated from the pepsin extracts by differential salt precipitation [9].

Electrophoresis

SDS/PAGE was performed on slab gels, using 6% polyacrylamide for the running and 4% for the stacking gels,

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Abbreviation. pN, amino-terminal peptide.

for the separation of collagen chains, while 12% and 9% running gels were used to separate the peptides resulting from BrCN cleavage and mammalian collagenase digestion, respectively [10].

Preparation of antiserum against human collagen II

An antiserum against the purified collagen II was raised in rabbits [11]. The antibody titer was determined by direct ELISA. Using immunohistochemistry, the antiserum specifically reacted with the cartilagenous portion in sections which also contained bone and perichondrium. No cross-reactivity was detected with collagen I, III, V, IX and XI, as assayed by immunoblotting, at a working dilution of 1:50 (data not shown).

Immunoblotting

Following electrophoretic separation, protein bands were blotted onto a nitrocellulose filter by wet or semi-dry electrotransfer. Filters were blocked with 15% non-fat dried milk in deionized water and incubated overnight at room temperature with the primary antiserum (1:50 in 0.1% bovine serum albumin). The filter was then incubated with alkaline-phosphatase-conjugated swine immunoglobulins against rabbit immunoglobulins (Dako Diagnostika, Denmark; diluted 1:3000) for 1 h at room temperature. 5-Bromo-4-chloro-3-indolyl phosphate (7.5 mg/50 ml; Serva, Heidelberg, FRG) and nitro-blue tetrazolium (15 mg/50 ml; Sigma, Munich, FRG) were used as substrates.

Preparation of fulvic acid

Local drinking water of a Kashin-Beck-disease-affected area was acidified with HCl then passed over a column (*3 cm × 60 cm*) packed with GDX-102 resin (Tianjing 2nd chemical factory, Peoples Republic of China). The water-soluble fraction, fulvic acid, was retained on the column because of its affinity for the resin. Fulvic acid was eluted from the resin by a solvent containing ethanol and amonia at a ratio of 1:2 [12]. The eluate was filtered and the filtrate containing fulvic acid was lyophilised and stored at 4 °C.

Cleavage of collagen II with mammalian collagenase

A 20-µl aliquot of a 0.1% collagen solution (5 mM $CaCl_2$, 0.3 M NaCl, 50 mM Tris, pH 7.5) was incubated for 2 h at 25 °C with 10 µl human-leukocyte-derived collagenase (a gift from Dr Tschesche, Institut für Biochemie, Universität Bielefeld, FRG) which had been activated with 1 mM $HgCl_2$ at 37 °C for 2 h [13]. The reaction was stopped with 5 µl 20% SDS. The collagen digest was analysed by SDS/PAGE. The migration of peptides was densitometrically analyzed.

Cleavage of collagen II with BrCN

Isolated collagen II was cleaved with BrCN in 70% formic acid at room temperature overnight [14]. The resulting peptides were identified by SDS/PAGE. The migratory positions of the peptides was checked by densitometric analysis (Optoquant, Biometra, Göttingen, FRG).

Circular dichroism and transition profiles

CD spectra were recorded on a Jasco J-500 A spectropolarimeter, equipped with a temperature-controlled quartz cell of 1 cm path length (Gilford). The molar ellipticity was calculated on the basis of a mean residue molar mass of 98 g/mol. 100% conversion corresponds to the ellipticity for totally denatured collagen. Thermal-transition curves were recorded at a fixed wavelength (221 nm) by raising the temperature linearly at a rate of 30 °C/h using a Gilford temperature programmer. The sample concentration was 20 µg/ml. The sample buffer was 0.05% acetic acid. A change in the thermal stability due to radiation damage could not be detected.

Electron microscopy

Electron micrographs of collagen II were performed by rotary-shadowing techniques as described previously [15].

Amino acid analysis

Protein samples were hydrolyzed with 6 M HCl for 24 h at 110 °C under nitrogen. Amino acid analysis was performed on a Beckman system 6300 using ninhydrin for the post-column colour reaction.

Treatment of fertilized chicken eggs with fulvic acid

Fertilized eggs were incubated at 37.8 °C for 9 days, then injected with 50 µl 1 mg/ml fulvic acid in 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4 and 1.4 mM KH_2PO_4, pH 7.3 (buffer A). The site of the injection onto the egg shell was sterilized with 75% ethanol and sealed with sterilized 1.5% agarose after the injection. The control eggs were injected with buffer in the same manner. Incubation was continued up to day 16, when chicken embryos were recovered from the eggs. Excess liquid was dried with filter paper. The mass of the embryos was determined and their femora were carefully isolated. The epiphysis was separated from the metaphysis and diaphysis for separate analysis.

Determination of the mineral content, DNA and protein concentration

The difference between the dry mass before and after demineralization in 0.2 M EDTA was used to calculate the mineral content [16]. The total protein content in the metaphysis and diaphysis was determined by the method of Lowry [17]. DNA content was measured using the method of Burton [18].

RESULTS

Specimens of articular cartilage from two Kashin-Beck disease patients were available for biochemical analysis. Since the experimental observations were virtually identical for both persons, the data from one patient only is presented.

Analysis of neutral salt extracts

Neutral salt extracts were analysed by PAGE and compared with the migration pattern of cartilage extracts of human fetal cartilage. More material was extracted from the patients' cartilage (3%) than from the control (< 1%) and the predominant protein band visualised after Coomassie-blue staining migrated slower than that of collagen II from the control (Fig. 1a, lanes A, B). Immunoblot analysis of a similar electropherogram clearly demonstrated that the slower-mi-

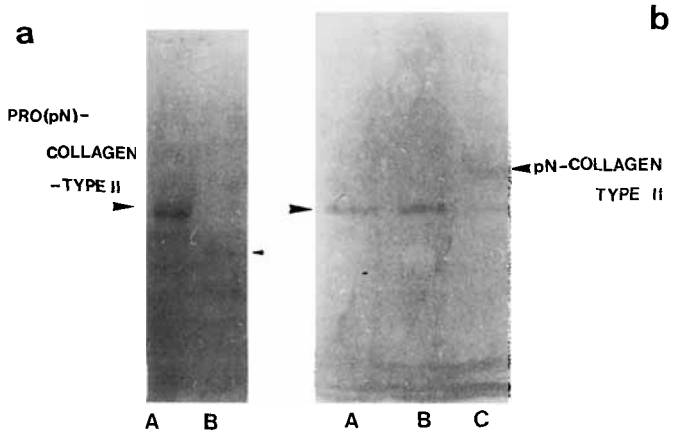

Fig. 1. Separation of collagen chains by SDS/PAGE. (a) Neutral salt extract of articular cartilage from severely affected patients with Kashin-Beck disease (A); collagen II from human fetal cartilage (B). After electrophoretic separation, gels were stained with Coomassie blue. (b) Immunoblot analysis using anti-collagen II antibodies. Human type II collagen (A); neutral salt extract of articular cartilage from a patient with Kashin-Beck disease after limited pepsin digestion (B); neutral salt extract, as in B, however prior to pepsin digestion (C).

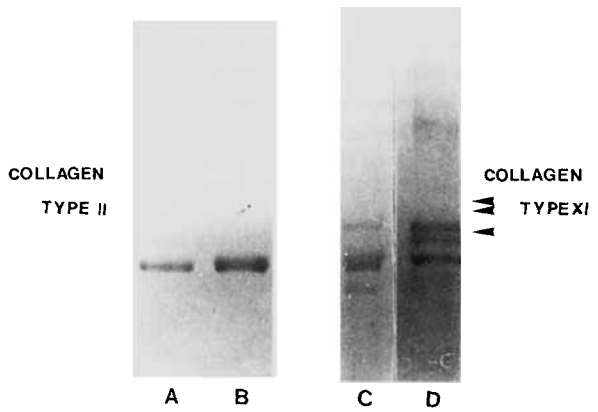

Fig. 2. SDS/PAGE of pepsin-solubilized fractions from normal (A, D) and Kashin-Beck-disease-affected human cartilage (B, C). The extracts obtained by pepsin digestion were fractionated by salt precipitation in 0.9 M NaCl (A, B) and 1.2 M NaCl (C, D) with 0.5 M acetic acid. After electrophoretic separation, polyacrylamide gels were stained with Coomassie blue.

grating band reacted with antibodies against collagen II (Fig. 1b, lane C). When a parallel sample was treated with pepsin prior to its electrophoretic separation the slower-migrating band disappeared and was shifted to a strong immunoreactive band which was at a position to which collagen II migrated (Fig. 1b, lanes A, B). It is known that the precursor forms of interstitial collagens are converted to collagen molecules by limited pepsin digestion. Since the precursor form of collagen II which accumulated in the affected tissue migrated as a larger, yet single chain under non-reducing conditions, one can conclude that the carboxy-terminal procollagen peptides are cleaved regularly. The carboxy-terminal procollagen peptides are known to form otherwise disulfide bridges between procollagen chains. Thus, the apparently larger molecular mass of the collagen α-chains

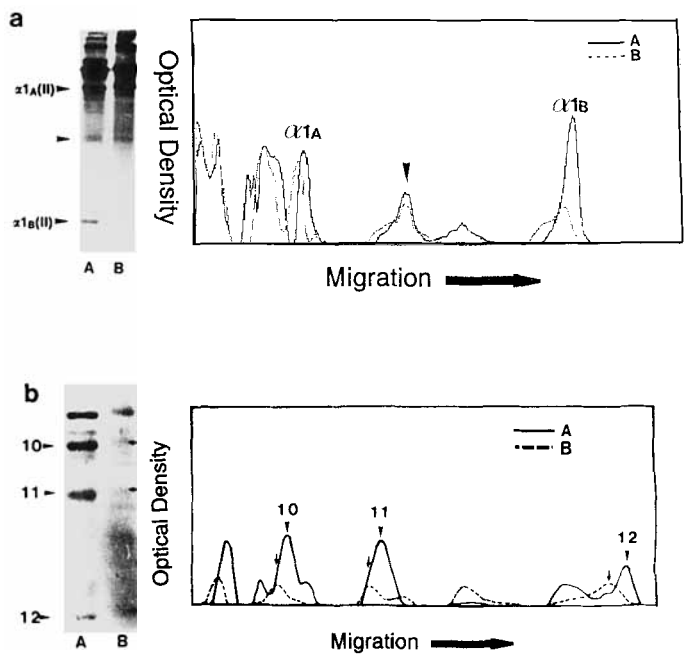

Fig. 3. Electropherograms of cleavage products of collagen II from Kashin-Beck disease patients (A) and control (B) by BrCN (b) and vertebrate collagenase (a). After electrophoretic separation, gels were stained with Coomassie blue. The relative position of individual bands was determined by two-dimensional scanning.

Table 1. Hydroxylation of proline in collagen II

Source of collagen II	Hydroxyproline/proline
	mol/mol
Patient 1	0.870 ± 0.030
Patient 2	0.883 ± 0.011
Control fetal cartilage	0.824 ± 0.006
[9]	0.825

is solely due to the presence of the pN-aminopropeptides, which do not form interchain disulfide bridges [14].

Analysis of pepsin-solubilized material

The higher extractability of affected cartilage was most obvious when limited pepsin digestion was used to solubilize the remaining cartilage tissue following neutral salt extraction. The cartilage specimen of the patient was almost completely solubilized by pepsin, while the control was only partially soluble by the same limited proteolysis. Following differential salt precipitation, one large pool of collagen was obtained which consisted of pure collagen II (Fig. 2, lane A) and another which contained pure collagen XI in the case of the control (Fig. 2, lane D). In the case of the affected cartilage, one collagen II pool was obtained in 0.9 M NaCl and 0.5 M acetic acid (Fig. 2, lane B) and a fraction which was collected in 1.2 M NaCl and 0.5 M acetic acid as expected for collagen XI, although this material showed a migration pattern different from the control collagen XI (Fig. 2, lane C). No immunoreaction with either anti-(collagen I) nor anti-(collagen II) antibodies was seen in this pool (data not shown). While no further material was available for detailed analysis

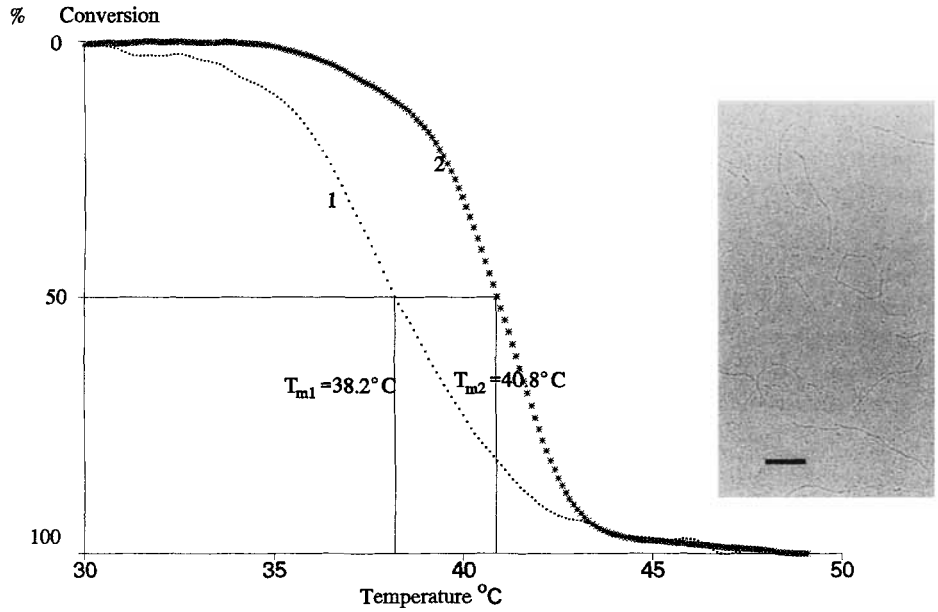

Fig. 4. Melting curves of collagen II from Kashin-Beck disease patients (1) and control (2). The inset shows an electron micrograph of collagen II from the cartilage of a Kashin-Beck disease patient after rotary shadowing. The bar represents 100 nm.

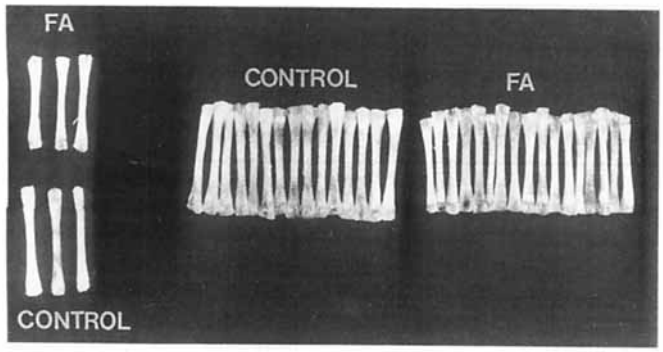

Fig. 5. The femurs of chick embryos treated with fulvic acid (FA) and from controls.

of this pool, the collagen-II-containing pool was subjected to a detailed analysis.

Cleavage *with mammalian* collagenase and BrCN

Pepsin-solubilized collagen II from the affected cartilage migrated somewhat slower than control collagen II (Fig. 2, lanes A, B). The difference in migration was more obvious when collagen fragments were analysed which were obtained either by cleavage with mammalian collagenase (Fig. 3a) or by cleavage with BrCN (Fig. 3b). Since all collagen II peptides from the cartilage of the patients migrate slower than the respective control peptides, one can conclude that the difference in migration arises from molecular alterations along the entire molecule. The amino acid analysis was typical for human collagen II with a slightly increased molar ratio of hydroxyproline/proline for collagen II from the patients (Table 1).

Thermal stability and electron micrographs of collagen II

Since the thermal stability of the collagen triple helical molecule is a sensitive parameter for the physiological functioning of a connective tissue, we determined the melting curve of collagen II from cartilage from the patients (Fig. 4). Clearly, the melting point of collagen II from the patients is about 2–3 °C lower than that of control collagen II, a value similar to that published previously [19]. Electron microscopy shows that the collagen II from cartilage of the patients forms thread-like molecules similar to those seen in normal human collagen II (Fig. 4, inset).

In vivo impact of fulvic acid on fertilized chicken eggs

Since humic acid or chemically related agents, either found in drinking water or present on contaminated grain, presumably trigger Kashin-Beck disease, 50 µl fulvic acid (1 mg/ml in buffer A; the water-soluble portion of humic acid is usually found at a concentration of 1 µg/ml in drinking water in Kashin-Beck-disease-affected areas) from the endemic area were injected into fertilized chicken eggs on day 9 of embryonic development. The chicken embryos were dissected on day 16. The long bones of fulvic-acid-treated embryos were shorter and slightly thinner than controls (25% reduction in mass; Fig. 5); they had a lower mineral content (a decrease of about 15%) and the total protein and collagen DNA contents were reduced (Table 2). Neutral salt extracts were prepared from various tissues and analysed by PAGE and subsequent immunoblotting. Precursor forms of collagen II were found in articular cartilage of fulvic-acid-treated embryos (Fig. 6A). Interestingly, neutral salt extracts of sternal cartilage did not show any slower-migrating collagen band (Fig. 6E). Furthermore, collagen precursor could not be identified in control tissues from untreated chicken eggs (Fig. 6B, F). In pepsin extracts of the epiphysis, type I, II, IX, and XI were identified from both groups following differential salt precipitation and SDS/PAGE. No difference was noticed in the electrophoretic migration of whole collagen chains or BrCN-derived peptides of collagen II from both groups (Fig. 7).

DISCUSSION

Kashin-Beck disease is virtually unknown outside China and some far-eastern areas of the Soviet Union. Epidemiologi-

cal surveys indicate that environmental rather than genetic factors may be responsible for the onset of this degenerative disease of articular cartilage. In particular, fulvic acid, the water-soluble portion of humic acid, along with selenium deficiency may represent major disease factors. Tissue specimen of articular cartilage from two patients affected with Kashin-Beck disease were available for analysis and could be compared with human control tissue. Most remarkable was the extractability of the affected tissue by neutral salt solutions and limited pepsin digestion, the presence of pN-collagen II, (even though the tissue was not stored under special conditions), an altered migratory pattern of pepsin-solubilized collagen II, and finally the lower thermal stability of collagen II.

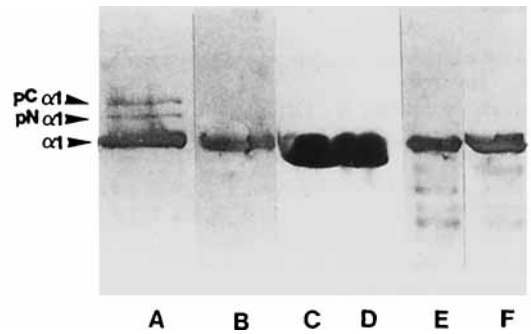

Fig. 6. Immunoblot analysis of neutral salt extracts of articular cartilage (A, B) and sternal cartilage (E, F) from fulvic-acid-treated embryos (A, E) and from controls (B, F). Lanes C and D are aliquots of samples shown in lane A and B; however after limited pepsin digestion.

The presence of pN-collagen II in the neutral salt extracts of the affected cartilage indicates that the conversion of pN-collagen II in cartilage of the patients is impaired. This observation is reminiscent of genetic disease in cattle and sheep, in which it was shown that pN-collagen I had accumulated in the skin of the affected animals [2, 20]. The defect was attributed to a deficiency in the enzyme pN-propeptidase I which cleaves the pro-pN-peptides from the procollagen I molecule [21]. The defect in Kashin-Beck disease is not due to a genetic defect, but is rather caused by fulvic acid and other environmental factors known from the endemic disease area. This notion is supported by the observation that fulvic acid injected into fertilised eggs induces a tissue-specific accumulation of procollagen II in the articular cartilage and not in the sternal cartilage.

Collagen II contained in pepsin extracts of the cartilage of patients shows a reduced electrophoretic migration of whole α-chains as well as of fragments after cleavage with mammalian collagenase or BrCN. This observation implies an alteration of the entire molecule. The higher content of hydroxyproline of collagen II from the affected cartilage probably cannot explain the difference in migration. The excess hydroxylation, however, could be responsible for the lower thermal stability of collagen II. Although hydroxylation of proline is essential for the stabilization of the triple helix of collagen, it could be that there is an optimal degree of hydroxylation of proline beyond which the thermal stability of the triple helix decreases (Notbohm, H., unpublished observation). Further biochemical analysis of collagen II from Kashin-Beck disease cartilage may lead to a better understanding of this altered properties.

Since collagen II of fulvic acid treated embryos appears to be normal upon electrophoretic separation, we may have

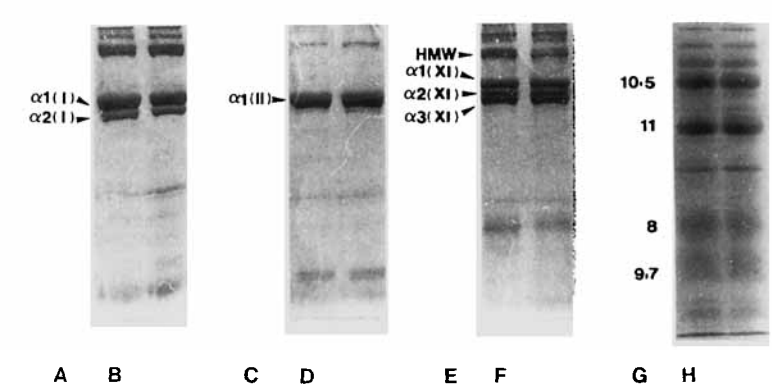

Fig. 7. Collagen I (lanes A, B), collagen II (lanes C, D) and collagen XI (lanes E, F) of pepsin-solubilised and salt-fractionated material from epiphyseal cartilage after electrophoretic separation and Coomassie blue staining (lanes G, H). BrCN-derived peptides of pepsin-solubilised collagen II after electrophoresis separation and Coomassie blue staining. (A, C, E, G) Samples from cartilage of fulvic-acid-treated embryos; (B, D, F, H) samples from control cartilage.

Table 2. Effect of fulvic acid (FA) on the development of chick embryos. Hyp, hydroxyproline.

Sample	Body mass	Femur mass	Mineral content	Protein/DNA		Hyp/Pro	
				epiphysis	metaphysis/ diaohysis	epiphysis	metaphysis/ diaphysis
	g	mg	%	mol/mol			
Control (n = 30)	20.4	60.0	50.0	25.5	26.0	0.516	0.621
FA group (n = 28)	18.3	47.0	43.0	24.0	19.8	0.472	0.446

observed species specific differences or differences due to developmental states: embryonic chicken versus adolescent individuals. This may also underline that fulvic acid is not the sole factor causing Kashin-Beck disease.

A molecular model of how an impaired conversion of pN-collagen II disturbs the fiber formation has been proposed [22]. Accordingly, one may assume that, in Kashin-Beck disease, the fiber formation is greatly disturbed by the presence of pN-collagen II as well as by the thermally labile and overhydroxylated collagen II. This clearly would result in a disturbed macromolecular organisation.

There are a variety of mechanisms by which fulvic acid may exert its damaging effect, none of which has been experimentally proven. Since fulvic acid consists of a mixture of poorly defined organic compounds with a wide variety of reactive groups, it is conceivable that fulvic acid reacts directly in the tissue with either collagen or procollagen peptidase, leading to insufficient procollagen conversion. It is also known that fulvic acid can generate reactive oxygen radicals which may indirectly interfere with procollagen processing. Taking into account that selenium as a scavenger of free radicals seems to be deficient in Kashin-Beck-endemic areas, this trace element could be missed for its protective role in a situation of high levels of free radicals generated by fulvic acid [23].

In China, Kashin-Beck disease has been declared a national health priority problem. Recently, the number of affected persons has apparently fallen, presumably due to improved nutritional conditions, higher standards in the quality of drinking water and selenium supplementation in nutrition. Yet, there is considerable interest in the underlying molecular defect(s) which could provide new perspectives in understanding of the initial causative events in other osteoarticular disorders.

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