EFFECTS OF EXCESSIVE FLUORIDE INGESTION IN RATS ON DIFFERENTIAL EXPRESSION OF COLLAGEN TYPES AND CHONDROCYTE DIFFERENTIATION IN CARTILAGE

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SUMMARY: To investigate the effect of excessive fluoride ingestion (EFI) on the differential expression of collagen phenotypes and chondrocyte differentiation in cartilage, 60 Spraque-Dawley rats, 4-6 weeks old, were divided into three groups and exposed to 0 (control), 50, and 100 ppm fluoride (from NaF) in drinking water for 3 and 5 months. Correlating with the increased F in the serum and bone in the two groups exposed to EFI, chondrocytes in the proliferative and hypertrophic zones of growth plate cartilage (GPC) and rib cartilage (RC) were retained and formed a mixture tissue cartilage peninsulas or islands with ossified islands in the deep zone of GPC and RC. Staining reactions for types II and X collagen were increased in the over-thickened region of GPC and were decreased in RC. Type X collagen staining reactions were also increased in the upper and middle zones in articular cartilage (AC) of tibia in the higher fluoride (EFI-II) group. Abnormal collagen expression in rats of the EFI groups resulted from abnormal differentiation of chondrocytes in proliferative and hypertrophic zones in GPC and RC and from degenerative changes in AC of rats in the EFI groups. Four types of abnormal chondrocyte differentiation in hyaline cartilage of rats with skeletal fluorosis (SF) suggested two pathogenic mechanisms for early stage of SF: osteomalacia-like changes in GPC and RC coupled with delayed mineralization and degenerative changes in AC.

Keywords: Cartilage, Chondrocyte differentiation, Collagen phenotype, Fluoride and collagen, Fluoride in rats, Pathogenic mechanisms.

INTRODUCTION

Skeletal Fluorosis (SF) is a chronic endemic osteoarthropathy. It develops mainly in the young and in middle age and results from the effects of fluoride on the normal skeletal metabolic processes including osteogenesis, osteoclast activity, and mineralization. The major symptoms are osteopetrosis, osteoporosis, and osteomalacia. In advanced cases, the larger joints become bent or crooked, muscle tension increases, and joint movement is limited, resulting in limb deformity and even loss of mobility. Excessive fluoride ingestion (EFI) impairs the development of cartilage and bone. The growth of children in fluorosis areas is significantly slower than that in fluorosis-free areas. In animal experiments, EFI leads to necrosis and an impaired maturation of chondrocytes. Ream has observed that the thickness of growth plate cartilage (GPC) was increased in the femur of rats drinking water containing NaF at a concentration of 120 mg/L for 4 weeks. EFI also resulted
in increased thickness of the hypertrophic zone in GPC of rats, characterized by pathologic lesions similar to rickets. Moreover, chondrocyte maturation was impaired in GPC in rats treated with 500 ppm NaF over three months. In young dogs, EFI resulted in atrophy, degeneration, necrosis, and impaired maturation of chondrocytes as well as delayed mineralization of AC. In rats, endochondral ossification and mineralization were delayed, and the ratio of newly formed spongy bone was increased, and a single and/or multi chondrocyte necrosis appeared.

In the 1970s Susheela suggested that collagen protein was a target damaged by EFI. Since then, disturbance of collagen metabolism in experimental animals affected by EFI has been noted in studies showing that fluoride decreased the biosynthesis of collagen protein and increased the catabolism of collagen and the urinary excretion of hydroxyproline. EFI promoted the \( \alpha_1 (II) \) collagen chain molecular weight, decomposed an insoluble collagen, and decreased the matrix of rib cartilage. Therefore, EFI can affect the collagen metabolism of cartilage and bone, but little is known about the effects of EFI on collagen phenotypic expression in abnormal chondrocytic differentiation and delayed matrix mineralization in the developmental impairment of cartilage and bone.

Types II, IX, X, and XI collagen are the main collagen proteins in cartilage, and their phenotypic expression has an important role in chondrocyte differentiation and matrix mineralization. However, little is known about the modes of impaired endochondral ossification in children with fluorosis and how it can be prevented and cured. The present study was therefore undertaken to investigate the effect of EFI on the differential expression of collagen types and chondrocyte differentiation in cartilage of rats.

MATERIALS AND METHODS

**Experimental animals:** Sixty Sprague-Dawley rats (from the Experimental Animal Center of Xi’an Jiaotong University, Xi’an) aged 4 to 5 weeks old with body weights of 50-90 g were randomly divided into the control group (Control), EFI group I (EFI-I, 50 ppm F ion from 110.5 mg NaF per liter of distilled water), and EFI group II (EFI-II, 100 ppm F ion from 221.0 mg NaF per liter of distilled water). The number of rats and the fluoride (F) concentration of the drinking water in the different groups are shown in Table 1. All rats were fed the same prepared diet consisting of 25% flour, 52% corn, 20% bran, 1% yeast, 1% bone dust, and 0.1% NaCl.

**F content assay:** Blood was collected from the celiac artery under general anesthesia with 1.0 mL of 20% Nembutal/100g body weight per rat at the 3rd and 5th month of the experimental period. The serum was separated by centrifugation, and stored at -20°C. The F contents in the serum and in the wet-digested (HNO\(_3\)) tibia were assayed by the fluoride-ion electrode method.
Table 1. Number of rats and experimental conditions in different groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of rats</th>
<th>Experimental conditions隋 in drinking water</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>3 Months</td>
<td>5 Months</td>
</tr>
<tr>
<td>Control</td>
<td>19</td>
<td>9</td>
</tr>
<tr>
<td>EFI - I</td>
<td>20</td>
<td>12</td>
</tr>
<tr>
<td>EFI – II</td>
<td>21</td>
<td>11</td>
</tr>
<tr>
<td>Total</td>
<td>60</td>
<td>32</td>
</tr>
</tbody>
</table>

Tibia bone measurements: The diameter at the middle and the transverse length at the proximal and the middle points of the left tibia were measured with a micro ruler.

Tissue preparation: Growth plate cartilage (GPC), articular cartilage (AC) in tibia, and rib cartilage (RC) with some bone tissues from the right side were fixed in 4% paraformaldehyde for 24 hr. The samples were decalcified for 6 weeks in 0.3% EDTA, and embedded in paraffin wax after a series of dehydrations. Paraffin sections (5-8 μm) were cut, mounted on slides pre-treated with 10% poly-L-Lysine, and stored at room temperature until used.

Histochemistry: Representative sections of all cartilage samples of GPC, AC, and RC were stained by hematoxylin-eosin (H-E), and 0.1% toluidine blue to estimate the content of proteoglycan in parallel sections.

Immunohistochemistry: Deparaffinized sections were incubated with testicular hyaluronidase (2 mg/mL, PBS [phosphate buffered saline], pH 5 for 30 min at room temperature). For detection of type II collagen, pretreatment with pronase (1 mg/mL, PBS, pH 7.3 for 30 min at room temperature) was performed according to Aigner. For type X collagen staining, sections were pretreated with type XIV protease (Sigma P 8038, 0.02 mg/mL, PBS, pH 7.3 for 30 min at room temperature). Primary antibodies were incubated overnight at 4°C and visualized with alkaline-phosphatase-labeled secondary antibodies. Color development was continued for 30 min at room temperature using 3-hydroxy-2-naphthoic acid 2,4-dimethylanilide as a substrate. Finally, nuclei were counterstained with hematoxylin.

Collagen antibodies: A monoclonal antibody (E8) specific for collagen type II was kindly provided by Dr R Holmdahl. Mouse monoclonal antibodies against human recombinant type X collagen (X-53 and X-34) were obtained from the laboratory of one of us (Prof K von der Mark). The specificity of all antibodies was tested by ELISA (Enzyme-linked Immunosorbent Assay), Western blotting, and by immunostaining on test tissues.
Statistical analysis: All data were analyzed by SPSS 10 for Windows software. Data entries are means with plus or minus standard deviation (\(\bar{x} \pm s\)). Differences were considered significant if \(P < 0.05\).

RESULTS

Changes of F content in serum and tibia of rats in different groups: Tables 2 and 3 show that the F content in serum and tibia of rats in the EFI-I and EFI-II groups was significantly higher than that of the control group at the end of the 3rd and the 5th month, except for the serum F in the EFI-I group at the end of the 5th month. A positive correlation of the F content between serum and tibia bone was 0.68 in the 3rd month (\(P < 0.01\)) and 0.91 in the 5th month (\(P < 0.01\)) of the experiment.

Table 2. F contents (mg/L) in serum of rats in all groups in the 3rd and the 5th month

<table>
<thead>
<tr>
<th>Group</th>
<th>No.</th>
<th>3rd month  (\bar{x} \pm s)</th>
<th>5th month  (\bar{x} \pm s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8</td>
<td>0.61 ± 0.02(^a)</td>
<td>7</td>
</tr>
<tr>
<td>EFI-I</td>
<td>8</td>
<td>1.13 ± 0.06(^b)</td>
<td>4</td>
</tr>
<tr>
<td>EFI-II</td>
<td>10</td>
<td>3.32 ± 0.46(^c)</td>
<td>9</td>
</tr>
</tbody>
</table>

\(P < 0.05\) if letters are different and \(P > 0.05\) if letters are the same among groups.

Table 3. F content (mg/g) in tibia of rats in all groups in the 3rd and the 5th month

<table>
<thead>
<tr>
<th>Group</th>
<th>No.</th>
<th>3rd month  (\bar{x} \pm s)</th>
<th>5th month  (\bar{x} \pm s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8</td>
<td>0.68 ± 0.04(^a)</td>
<td>9</td>
</tr>
<tr>
<td>EFI-I</td>
<td>12</td>
<td>4.56 ± 0.16(^b)</td>
<td>5</td>
</tr>
<tr>
<td>EFI-II</td>
<td>7</td>
<td>7.06 ± 0.58(^c)</td>
<td>10</td>
</tr>
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</table>

\(P < 0.05\) if letters are different and \(P > 0.05\) if letters are the same among groups.

Comparison of tibia growth of rats in the different groups: Table 4 shows that the width and vertical length of the tibia in the EFI-I and EFI-II groups were significantly lower than in the control group.
Table 4. Comparison of the mean width (diameter) and mean vertical length of the rat tibia among three groups in the 3rd month of the experiment (\(\bar{x} \pm s \text{ mm}\))

<table>
<thead>
<tr>
<th>Group</th>
<th>No.</th>
<th>Vertical length</th>
<th>Width of tibia 1</th>
<th>Width of tibia 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7</td>
<td>38.33 ± 1.90a</td>
<td>6.99 ± 0.27a</td>
<td>2.44 ± 0.13a</td>
</tr>
<tr>
<td>EFI-I</td>
<td>12</td>
<td>36.47 ± 1.16b</td>
<td>6.88 ± 0.40b</td>
<td>2.38 ± 0.21b</td>
</tr>
<tr>
<td>EFI-II</td>
<td>10</td>
<td>32.26 ± 0.94c</td>
<td>6.16 ± 0.52b</td>
<td>2.18 ± 0.14c</td>
</tr>
</tbody>
</table>

Width of tibia 1: width of transverse diameter at proximal point of tibia.
Width of tibia 2: width of transverse diameter at middle point of tibia.
P < 0.05 if letters are different and P > 0.05 if letters are the same among groups.

Morphological changes in cartilage of rats treated with EFI: In the control group, the edges of growth plate cartilage (GPC) and articular cartilage (AC) in tibia, and the rib cartilage (RC) were normal and arranged in an orderly pattern. The chondrocyte columns had 6.23 ± 0.95 layers in the proliferative zone and 3.97 ± 0.71 in the hypertrophic zone with an orderly arrangement and no chondrocyte necrosis.

In the EFI groups, GPC in the tibia and rib cartilage was significantly crooked (Figure 1), but was not in AC. Chondrocyte columns ranged from 7.96 ± 1.01 layers in the EFI-I group to 8.53 ± 1.24 layers in the EFI-I group in the proliferative zone and from 5.00 ± 0.76 layers in the EFI-I group to 6.11 ± 1.43 layers in the EFI-II group in the hypertrophic zone of the GPC in the 3rd month (Table 5).

Table 5. Comparison of growth plate cartilage (GPC) thickness inibia of rats in the different groups

<table>
<thead>
<tr>
<th>Group</th>
<th>No.</th>
<th>Months</th>
<th>Thickness of GPC (in µm)</th>
<th>Layers of chondrocyte in proliferative zone</th>
<th>Layers of chondrocyte in hypertrophic zone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8</td>
<td>3</td>
<td>585.75 ± 92.50a</td>
<td>6.23 ± 0.95a</td>
<td>3.97 ± 0.71a</td>
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<tr>
<td>EFI-I</td>
<td>12</td>
<td>3</td>
<td>702.00 ± 172.50b</td>
<td>7.96 ± 1.01b</td>
<td>5.00 ± 0.76b</td>
</tr>
<tr>
<td>EFI-II</td>
<td>10</td>
<td>3</td>
<td>785.00 ± 241.75c</td>
<td>8.53 ± 1.24c</td>
<td>6.11 ± 1.43c</td>
</tr>
<tr>
<td>Control</td>
<td>9</td>
<td>5</td>
<td>375.00 ± 89.57a</td>
<td>5.38 ± 0.86a</td>
<td>3.48 ± 0.87a</td>
</tr>
<tr>
<td>EFI-I</td>
<td>8</td>
<td>5</td>
<td>425.00 ± 45.25b</td>
<td>5.98 ± 1.05b</td>
<td>4.20 ± 0.68b</td>
</tr>
<tr>
<td>EFI-II</td>
<td>10</td>
<td>5</td>
<td>507.50 ± 167.54c</td>
<td>6.56 ± 1.13c</td>
<td>5.07 ± 1.23c</td>
</tr>
</tbody>
</table>

P < 0.05 if letters are different and P > 0.05 if letters are the same among groups.
Chondrocyte columns were disordered, and extended to the precalcification zone of metaphysis; cartilage residues within bone trabeculae were significantly greater than in the control group. Moreover, some focus over-thickness areas appeared in cartilage with upward of 15 chondrocyte layers in the proliferated zone and upward of 20 layers in the hypertrophic zone of GPC. The surface of AC in tibia of some rats in the EFI-II group was eroded. Morphological changes in cartilage of rats in the EFI-II group were more serious than in the EFI-I group. After 5 months, a focus chondrocyte necrosis in GPC had appeared and was gradually intensified, but the thickness of GPC and the chondrocyte layers in both the proliferative zone and the hypertrophic zone were decreased. A focus of thickened GPC was observed in both EFI groups and co-appeared with cartilage peninsula, cartilage islands, and protofibrils in the EFI-II group.

Location of types II and X collagen in cartilage: GPC in tibia: Peri- and extracellular staining reactions of type II collagen occurred in both the normal and the thickened areas of GPC of rats in all three groups (Figure 2) by immunohistochemistry. Peri- and extracellular staining reactions of type X collagen were distributed especially in the hypertrophic zone including the normal and the over-thickened regions, but not in the rest and the proliferative zones of GPC in the control and EFI groups (Figure 3).
Figure 2. Type II collagen staining in all cartilage zones of growth plate cartilage and cartilage residues of rats in the control (2A), EFI-I (2B), and EFI-II group (2C). (100X).

Figure 3. Type X collagen staining in the hypertrophic cartilage zone (3A) and the abnormal thickened cartilage (3B-C) and its residues within bone trabeculae of rats in the control (3A), EFI-I (3B), and EFI-II (3C) group. (100X).
Cartilage residues within bone trabeculae zone: Peri-and extracellular staining reactions of type II and X collagen were still seen in cartilage residues within bone trabeculae, but not in epiphyseal nuclei and tibia bone in all groups (Figures 2 and 3). Type X collagen staining reaction was diffused in the end plate zone of epiphyseal nuclei, among chondrocyte clusters in the hypertrophic zone, and around cartilage peninsula and cartilage islands, which extended into the precalcification zone of metaphysis, but not into epiphyseal nuclei, bone peninsulas, and islands in GPC of rats with EFI in the 5th month (Figure 4).

Rib cartilage: Peri-and extracellular staining reactions of type II collagen were noticeably absent in RC of rats in the EFI groups (Figures 5 and 6).

Articular cartilage in tibia: Peri-and extracellular staining reactions of type II collagen were located in all cartilage of AC (Figures 7A-B) in the three groups. Type X collagen staining reactions occurred in the upper and middle zones of AC of tibia in the EFI groups (Figure 8B-C), but hardly at all in the control group (Figure 8A).

Proteoglycan staining in GPC: Toluidine blue staining occurred in both the normal and the abnormal regions in GPC (Figures 9A-C) and AC of tibia and RC among the three groups. In some rats of the EFI-II group, the staining was absent in RC (Figure 10C) and in the upper zone of AC (Figure 7C).
Figure 5. Pathological changes of rib cartilage of rats in the control (5A), EFI-I (5B), and EFI-II group (5C). (H-E 100X).

Figure 6. Loss of peri- and extracellular staining of type II collagen in rib cartilage of rats in the EFI-II group (6C) and slight loss in the EFI-I group (6B), in contrast to the control (6A). (100X).
**Figure 7.** Normal type II collagen staining in articular cartilage of rats in the control (7A) in contrast to staining loss in local regions of the EFI-I group (7B). Note loss of toluidine blue staining in upper zone in EFI-II group (7C). (100X).

**Figure 8.** Immunohistochemistry staining of type X collagen in articular cartilage of tibia of the control (8A) and in peri- and extracellular regions in the upper and middle zone of cartilage in the EFI-I and EFI-II groups (8B and C) showing eroded cartilage surface in EFI-II group (8C). (100X).
Figure 9. Proglycan stained by toluidine blue showing distribution of cartilage and cartilage residues within bone of rats in the control (9A), EFI-I (9B), and EFI-II group (9C). (100X).

Figure 10. Proglycan stained by toluidine blue in cartilage and cartilage residues within bone (10A-B), and loss in RC of rats in the EFI-II group (10C). (100X).
Toluidine blue staining occurred in cartilage residues within bone trabeculae of GPC (Figure 9), but not in epiphyseal nuclei in cartilage and ossified regions. Staining appeared at the edge of cartilage peninsulas and cartilage islands extended into metaphysis, but was not seen in the ossified regions in cartilage peninsulas and cartilage islands in the 5th month of the experiment (Figure 4D).

DISCUSSION

In the rats exposed to excessive fluoride ingestion (EFI), impaired chondrocyte differentiation and abnormal mineralization occurred along with increased fluoride in serum and bones. Pathologically, these changes involved over-thickening and crooked edges in the growth plate cartilage (GPC) and rib cartilage (RC), disordered chondrocyte columns, stored chondrocytes in proliferate zones and hypertrophic zones, mixed tissue in cartilage peninsulas or cartilage with ossified islands in the deep zone of the GPC and RC (especially in the EFI-II group), protofibrils and chondrocyte necrosis in GPC in the EFI-II group, and impaired tibia growth. These changes are similar to those of rickets in humans and osteomalacia in rats exposed to EFI.2-4

The expression pattern of types II and X collagen in rats exposed to EFI can be generalized into four types:

1 Types II and X collagen staining reactions were expressed in the over-thickened region of GPC and RC. The results shows that the predominance of type II collagen was predominantly synthesized in the differentiated chondrocyte in hyaline cartilage, and type X collagen was synthesized mainly in hypertrophic zone and from hypertrophic chondrocytes to osteoblast-like stages in calcified zones.8-10 This modulation pattern is a normal collagen expression pattern of chondrocyte differentiation in rats with EFI.

2 Type X collagen staining reactions were expressed in the edge of cartilage peninsulas and cartilage islands extending into precalcification zones, but they were not stained in the osteoid regions in cartilage peninsulas and cartilage islands. In some rats in the EFI groups, type X collagen staining reactions were diffused in chondrocytes close to the end plate zone of epiphyseal nuclei and chondrocyte clusters in the deep zone of GPC. Since types II and X collagen were deposed in matrices of over-thickened regions of GPC and RC, and in cartilage residues within bone trabeculae, normal mineralization of cartilage and bone was impaired in rats with EFI. Therefore, a definitive pathology was impaired mineralization of cartilage or osteomalacic in GPC and RC of rats with skeletal fluorosis (SF).
Type X collagen staining reactions were expressed in upper and middle zones in AC of tibia in the EFI-II group. This result is similar to the premature chondrocyte differentiation of hypertrophic cells of type X collagen in upper and middle zone in AC of osteoarthritis. Necrotic and apoptosis chondrocytes reduced the synthesis of type II collagen and proteoglycan in the local regions of RC and GPC. In contrast to GPC and AC, the reduction of type II collagen staining reactions in RC of rats with the EFI groups was consistent with the decreased $\alpha_1$ (II) collagen chain molecular weight, and the decreased the matrix of rib cartilage in EFI rats. Meanwhile, proteoglycan staining with toluidine blue in GPC, AC, and RC cartilage samples coexisted with type II collagen staining reactions.

Our results demonstrated that osteomalacic changes occurred in rats with EFI in GPC and RC along with osteomalacia and degeneration in AC. Thus, impairment of mineralization in GPC and bone, together with osteoarthritis in AC, are the two main pathological changes in the early stage of SF. However, further investigation is needed to determine whether there is abnormal expression of types II and X collagen in the serum of patients with SF, and if it can be an index to diagnose early cartilage damage in children with SF.

Thus, expression patterns of types II and X collagen showed that there were four types of abnormal differentiation in chondrocytes of hyaline cartilage with SF, i.e., the abnormal differentiated chondrocyte in proliferative and hypertrophic zones in GPC and RC, premature chondrocyte differentiation to hypertrophic cells in AC, and cartilage residues within bone trabeculae. Therefore, osteomalacia and degenerative changes are seen as two main pathogenic mechanisms for SF.

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