Effects of ethane-1-hydroxy-1,1-diphosphonate on ossification of the posterior longitudinal ligament in Zucker fatty rats

K Yamamoto, A Imakiire, T Shishido, T Masaoka
Department of Orthopedic Surgery, Tokyo Medical University, Tokyo, Japan

ABSTRACT

Purpose. Using Zucker fatty rats as an animal model, we evaluate the effectiveness of ethane-1-hydroxy-1,1-diphosphonate on ossification of the posterior longitudinal ligament by histopathologically investigating the prodromal, early, and advanced stages of ossification of the spinal ligaments.

Methods. 73 Zucker fatty rats were allocated to the ethane-1-hydroxy-1,1-diphosphonate group (n=33) and the control group (n=40). The former group was fed ethane-1-hydroxy-1,1-diphosphonate daily. The feed was given starting 2 months after birth and continued until the rats were killed at 3 to 18 months later. Chemical analysis of the blood, radiographic tests, and histopathological examination were then conducted for both groups.

Results. The results showed that ossification of the spinal ligaments involved excessive cartilage cell proliferation around areas affected by enthesitis; enlargement of the fibrocartilage tissue layer; ligament thickening; calcification of the matrix around the cartilage cells; and ossification of the spinal ligaments through enchondral ossification. Radiographic examinations showed that osteoproliferation in vertebral bodies in rats receiving ethane-1-hydroxy-1,1-diphosphonate was generally suppressed compared with controls, whereas histopathological examinations found no clear difference in cartilage cell proliferation in areas affected by enthesitis between the two groups, indicating the absence of calcification or osteoproliferation in areas affected by enthesitis for the rats receiving ethane-1-hydroxy-1,1-diphosphonate.

Conclusion. Ethane-1-hydroxy-1,1-diphosphonate is effective in suppressing progressive ligament ossification.

Key words: etidronic acid; longitudinal ligament; ossification; rats, Zucker; spinal diseases

Address correspondence and reprint requests to: Dr Kengo Yamamoto, Department of Orthopedic Surgery, Tokyo Medical University, 6-7-1 Nishishinjuku, Shinjuku-ku, Tokyo, 1600023, Japan E-mail: kengo-y@tkg.att.ne.jp
INTRODUCTION

Ossification of the spinal ligaments mainly affects the elderly population. However, because the condition advances slowly, it usually goes unnoticed; and by the time clinical symptoms become evident, ossification is often nearly completed. Hence, it is difficult to track chronological changes during the ossification of spinal ligaments and to determine the pathology of the disease.

The use of animal models is thus essential in investigations of ligament ossification. Various studies have identified associated local factors, such as continuous mechanical stress or structural properties of the spinal cord, as well as systemic factors such as abnormal glycolipid metabolism, abnormal endocrine system, obesity, and genetic abnormality. Because Zucker fatty rats (ZFRs) naturally develop abnormal glycolipid metabolism, non–insulin-dependent diabetes, and ligament ossification, we have been using them to study the pathology of ligament ossification, and to determine the pathology of the disease.

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MATERIALS AND METHODS

ZFRs were first described by Zucker and Zucker in 1961. The cause of these rats’ hereditary obesity is the so-called ‘fatty mutation’ (fa), which exhibits recessive Mendelian inheritance. Whereas rats with the genotype fafa are obese, those with the Fafa or FaFa genotypes are lean. Because fafa rats are sterile, they are produced (at a one in four probability) by mating Fafa rats. The ZFRs begin to exhibit obesity at about 5 weeks after birth, when they are visually distinguishable from FaFa or Fafa rats. These double-recessive ZFRs exhibit symptoms that are associated with human obesity or early adult-onset diabetes, such as overeating, obesity, hyperinsulinaemia, and mild reduced glucose tolerance.

73 ZFRs (age range, 5–20 months) were used in this study. 33 rats received EHDP (the EHDP group), while the remaining 40 did not (the control group). For the EHDP group, a rat feed (CE2; Nihon Crea, Tokyo, Japan) was made into pellets containing 1% (w/w) EHDP. Ingestion of this feed was allowed ad libitum. The mean daily amount of feed consumed was approximately 20 g. The feed was given starting 2 months after birth and continued until the rats were killed 3, 6, 9, 12, 15, or 18 months later.

Three tests were conducted—namely, chemical analysis of the blood, radiographic tests, and histopathological examination. For the blood tests, the abdomen was opened under general anaesthesia, and 3–4 mL of blood per 100 g of body weight was collected from the posterior vena cava. To ascertain the effects of EHDP on calcium metabolism, we measured the levels of calcium, ionised calcium, inorganic phosphorus, and alkaline phosphatase.

For the X-ray examination, we removed the ribs and soft tissue around the spinal column, extracted the entire column in one piece, and subjected it to X-ray examination using a Softex-type ESM (Softex Inc, Tokyo, Japan). For the histopathological examination, the spinal column was fixed in a solution of 0.5% (v/v) cetylpyridinium chloride and 10% (v/v) neutral formalin immediately after the X-ray analysis, and it
was divided into 3 segments: the cervical spine, thoracic spine, and lumbar spine. These segments were demineralised in 10% (v/v) EDTA solution, then embedded in paraffin, and sliced into consecutive 4-µm sagittal sections. Staining was performed using haematoxylin-eosin, Azan-Mallory, elastica van Gieson, and toluidine blue (pH, 4.1).

We analysed data with the Mann-Whitney U test, using a significance level of p<0.05.

RESULTS

Haematological findings

For the control group, the mean level of serum calcium was 5.32 mEq/L (2.66 mmol/L), that of ionised calcium at 37°C and pH 7.4 was 2.62 mEq/L (1.31 mmol/L), that of inorganic phosphorus was 6.53 mg/dL (2.11 mmol/L), and that of alkaline phosphatase was 24.7 K-A. The mean level of inorganic phosphorus for the EHDP group was higher than that for the control group at any age However, no marked differences in levels of calcium, ionised calcium, or alkaline phosphatase were observed between the study groups (Table).

Radiographic findings

Degenerative changes, such as intervertebral space narrowing, and the formation of small labial osteophytes at the anterior angle of vertebral bodies near the rearward curvature of the superior lumbar vertebrae appeared to start at about 8 months of age in the control group. With time, osteophytes increased in size in the shape of dykes, and after the age of 15 months, these lesions grew to one third to one half of the anteroposterior diameter of the vertebral bodies.

To compare this type of hyperostotic change between the control and EHDP groups, we used the X-ray scans to measure the anteroposterior diameter of vertebral bodies at the centre of the second thoracic vertebra (where bone growth is active) and the seventh thoracic vertebra (where bone growth is relatively slow). In the control group, the anteroposterior diameter of the second and seventh thoracic vertebrae increased until the age of 17 months, and then decreased at 20 months. In contrast, the anteroposterior diameters in the EHDP group were smaller than those of the controls at any age, and the increment in the anteroposterior diameter was also lower (Mann-Whitney U test; second vertebra: p<0.001 at 14 and 17 months, and p<0.05 at 20 months; seventh vertebra: p<0.001 at 11, 14, and 17 months, and p<0.05 at 20 months) [Fig. 1].

Histopathological findings

There was a difference in osteoproliferative development in vertebral bodies between the 2 groups. In the control group, osteoproliferative changes appeared in the anterior section of vertebral bodies near the peak of the rearward curvature of the superior thoracic vertebrae. Starting at about 8 months, we observed osteoproliferative changes in the angle of the
superior and inferior vertebral bodies, along with the irregularity of the collagen fibre of the anterior intervertebral fibrous ring. At 20 months, these changes became exacerbated. In the EHDP group, osteoproliferative changes at the angle of vertebral bodies were mild (protruding $<0.5$ mm from the normal vertical plane) at all ages, and the degree of thickening of the anterior cortical bone was also mild.

In the control group, anterior swelling and cracking of the intervertebral annulus fibrosus were seen starting at about 8 months, and cartilage cell proliferation was seen in the enthesoperidiscal region of the superior and inferior vertebral bodies. At 11 months, the anterior cortex of vertebral bodies thickened and anteroposterior diameters increased. As a result, the fibrous structure of the anterior longitudinal ligament was lost. In some rats, osteoproliferation was in the shape of a candle flame, the anterior longitudinal ligament had disappeared, and cartilage cell proliferation in the enthesoperidiscal region was marked. After 14 months, ossification further advanced and showed bonding and bridge structures. These changes expanded from the superior thoracic vertebrae with time. In contrast, lesions in the EHDP group were mild and mostly localised within
the superior thoracic vertebrae; these lesions did not form bridges (Figs. 2 and 3).

The chronological changes in the posterior longitudinal ligament observed in the control group were classified into 4 stages as described below. Changes in areas affected by enthesitis were marked, and ligament thickening accompanied by cartilage cell proliferation was also observed.

**Stage 0 (no clear changes in areas affected by enthesitis)**

In the areas affected by enthesitis, small vessels were seen between the shallow and deep layers of the ligament, and in the enthesoperidiscal region; small round cells were present in the boundary region between bone and ligament. Toluidine blue staining identified enthesitis as a metachromatic area (Fig. 4).

**Stage 1 (cartilage cell proliferation in areas affected by enthesitis)**

Ligament was slightly thickened, and cartilage cell proliferation was seen in the deep layer. Toluidine blue staining clearly identified enthesitis as a metachromatic area (Fig. 5).

**Stage 2 (formation of calcified cartilage tissue in areas affected by enthesitis)**

Ligament thickening was marked, and proliferation of calcified cartilage-like tissue was seen. The front of calcification was seen in the transition area with the ligament (Fig. 6).

**Stage 3 (osteoproliferation in areas affected by enthesitis)**

Ossified lesions formed in areas affected by enthesitis. Toluidine blue staining clearly identified the enthesoperidiscal region as a metachromatic area. In addition, mild metachromatic reactions were seen around ossified lesions (Fig. 7).

Although there were no marked differences between the 2 groups in the location and severity of histological findings, such as ligament thickening and cartilage cell proliferation, osteoproliferation and ligament ossification in areas affected by...
enthesitis were not observed in the EHDP group. In the control group, stage-1 changes started to appear at about the age of 5 months, whereas stage-2 changes gradually appeared after 11 months. On the other hand, in the EHDP group, stage-1 changes accounted for about half of the changes observed at the age of 20 months, and stage-3 changes were undetectable (Figs. 8 and 9).

DISCUSSION

Spinal ligament findings

In the control group, osteoproliferation in the spinal ligaments was observed in the anterior section of vertebral bodies; in the anterior longitudinal ligament, cartilage cell proliferation was marked in the enthesoperidiscal region; and in the posterior longitudinal ligament, prodromal changes—namely, cartilage cell proliferation, formation of fibrous cartilage tissue and ligament thickening—were observed mainly around areas affected by enthesitis. Following calcification of the matrix around cartilage cells, the spinal ligaments ossified predominantly through enchondral ossification. Hence, our results agreed with those from other studies that suggest that enthesitis has an important role in the pathology of the early stages of ligament ossification.

In the control group, although cartilage cell proliferation and ligament thickening were detected in the same areas affected by enthesitis in the EHDP group, these changes appeared at a later stage of development. Osteoproliferative changes in vertebral bodies were not visible on the X-rays, and ligament ossification or calcification around cellular proliferation was undetectable in histological studies. These ossification processes were therefore suppressed.

Action mechanisms of ethane-1-hydroxy-1,1-diphosphonate

Bisphosphonates are compounds with 2 phosphorus atoms covalently attached to a carbon atom and are chemical analogues of pyrophosphate, which has an oxygen atom instead of a carbon atom. Changing the side chains of the central carbon atom can result in various analogues. The effects of bisphosphonates such...
as etidronate, clodronate, pamidronate, and alendronate on bones have been investigated. Of these, etidronate (i.e. EHDP) is one of the drugs often used in experiments. Whereas the oxygenphosphorus bond in pyrophosphate can be broken easily by alkaline phosphatase, the carbonphosphorus bond in bisphosphonates is stable against heat and many chemicals, and also resistant to enzymatic hydrolysis.7

The physiochemical actions of EHDP are similar to those of pyrophosphate; EHDP not only suppresses the formation of calcium phosphate crystals and delays the aggregation of these crystals, but it also blocks the dissolution of these crystals.8,9 Such actions clarify that EHDP has a strong affinity towards the solid phase of calcium phosphate,10 and this underlies the ability of EHDP to suppress calcification in soft tissue. As far as its inhibitory action on the calcification of bone and cartilage is concerned, EHDP has been reported to inhibit the activities of alkaline phosphatase; block the intramembranous transportation of calcium; act as a surfactant for matrix vesicles; inhibit the synthesis of collagen or glycosaminoglycan11; and suppress the decomposition of proteoglycan.

Several experimental studies have demonstrated the suppression of calcification by EHDP. Shiota et al.12 administered a large quantity of EHDP (>40 mg/kg per day) to juvenile rats and documented severe calcification disorders. Uno et al.13 administered EHDP to rats with femoral fractures, and reported that healing was delayed because EHDP interfered with the absorption of cartilage cells in the enchondral callus; they also observed the calcification of fibrous callus. Kato14 administered a large quantity of EHDP to juvenile rats and observed changes associated with rickets, such as bone calcification disorders in the metaphysis and the matrix of the epiphyseal plate. These effects of EHDP could be cellular, not just physiochemical—the results of an in vitro study conducted by Khokher and Dandona15 showed that EHDP suppressed alkaline phosphatase activity and cellular proliferation in human osteoblasts.

These studies show that EHDP can suppress the growth and differentiation of cartilage cells and structural cells of the bone. Furthermore, one of the biological actions of EHDP is the suppression of bone resorption. Because of its high affinity to minerals, EHDP is deposited in bones; the activities of osteoclasts
are then suppressed when these cells envelope bone containing EHDP. This inhibitory action of EHDP on osteoclasts is seen with a relatively low dose; a higher dose suppresses the calcification of bones.  

When bisphosphonates are administered orally, their bioavailability is low (1%–10%). The majority of the drug is absorbed through the intestine, and 50% to 60% of absorbed drug is deposited in bones, while the remainder is excreted in the urine. Bisphosphonates can be deposited in areas other than bones, where they form complexes with metals, or aggregate in high-dose administrations. The half-life of bisphosphonates in human blood is approximately 30 to 120 minutes, whereas that in rat blood is shorter, at less than 60 minutes. The half-life in bones is greatly dependent on metabolic turnover, and that in rat bones is thought to range between 3 and 12 months.

Fleisch reported that the level of serum inorganic phosphate increased in a dose-dependent manner after EHDP administration in adult humans, and concluded that the kidney was involved in this process. Smith et al. administered EHDP to Wistar rats and reported that levels of serum inorganic phosphorus decreased. However, with ZFRs, there were no marked changes in the levels of serum calcium and alkaline phosphatase, but the level of serum inorganic phosphorus was higher for the EHDP group. This observation suggests that the kidneys of ZFRs, but not of Wistar rats, are structurally similar to those of human beings; furthermore, the level of serum inorganic phosphorus in ZFRs can serve as an indicator of EHDP absorption.

Study limitations

Three limitations of our study are the administration method used, the timing of administration, and post-administration follow-up. Our study only estimated the amount of EHDP consumed, and, because EHDP was mixed with the feed, the rate at which EHDP was
absorbed by the stomach could have been lowered. Despite these shortcomings, we decided to give EHDP orally because EHDP is administered orally to humans, and repeated subcutaneous injection would be stressful to rats. In addition, there are species differences in the rate of blood transfer for EHDP. Although the level of blood EHDP was not measured in this study, we estimated (on the basis of the amount of EHDP consumed in the feed and the results of past rat studies) that at least 15 mg/kg of EHDP was present in the blood of ZFRs. Strates et al.\textsuperscript{22} reported that a concentration of more than 15 mg/kg of EHDP in the blood suppressed calcification.

In this study, the administration of EHDP to ZFRs was initiated during the juvenile phase, at the age of 2 months, but because ligament ossification begins to appear after the age of 11 months in ZFRs, the EHDP regimen used could be considered preventive. In addition, the results of radiographic and histological examinations showed that EHDP administration suppressed the onset of ligament ossification and osteoproliferative changes in vertebral bodies. However, because the spinal column and spinal ligaments were not observed after the end of the EHDP administration, the long-term effects of EHDP administration could not be ascertained. As a result, when using EHDP for the suppression of ligament ossification, researchers need to investigate the timing and duration of EHDP administration.

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure8.png}
\caption{Radiological and histopathological findings of the posterior longitudinal ligament.}
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\begin{figure}[h]
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\includegraphics[width=0.5\textwidth]{figure9.png}
\caption{Stage classification of the histopathological findings of areas affected by enthesitis in the posterior longitudinal ligament.}
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REFERENCES