Combined effects of estrogen deficiency and cadmium exposure on calcified hard tissues: Animal model relating to itai-itai disease in postmenopausal women

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Abstract: Using ovariectomized rats as a model of postmenopausal women, we studied the effects of estrogen (Es) deficiency and in combination with cadmium (Cd) exposure on the calcified hard tissues related to the development of itai-itai disease. Es deficiency suppressed the synthesis of carbonic anhydrase required for the crystal nucleation process, causing the crystal structure defects in the tooth enamel. Regarding the combined effects of Es deficiency and Cd exposure on the bone, in which rats were given drinking water containing Cd ions, soft X-ray radiography revealed a development of labyrinthine pattern in the calvaria, and micro-computed tomography demonstrated the declining trabecular architecture of the tibia, suggesting Cd–induced osteoporotic change. Further, electron microscopy showed the increase of amorphous minerals in the calvaria. In conclusion, the combined effects of Es deficiency and Cd exposure can be responsible for accelerating the declining bone strength together with the crystal structure defects resulting in the preferential occurrence of itai-itai disease in postmenopausal women.

Keywords: estrogen deficiency, cadmium exposure, soft X-ray radiography, micro-computed tomography, electron microscopy, itai-itai disease

Introduction

Itai-itai (ouch-ouch) disease caused by cadmium (Cd) exposure is considered a type of osteomalacia, and is characterized by increased osteoid formation, with low-grade bone mineralization.[1)] Animal experiments have shown that Cd exposure leads to the development of osteoporosis,[2)] thereby implying that this disease results in both osteomalacia and osteoporosis. According to Nomiyama,[3)] osteoporotic changes are the most common feature of long-term Cd exposure, and osteomalacia may develop under poor nutritional conditions, though Cd exposure is now clearly defined as one of risk factors for developing osteoporosis.[4)–6)] The development of itai-itai disease has generally been attributed to the renal tubular dysfunction induced by Cd exposure.[4),7)–14)] Some researchers hold that Cd ions might themselves engage in osteoid formation[15)] or act directly on bone and thereby aggravate the postmenopausal loss of bone minerals, although the underlying mechanism remains unclear;[2),13),16),17)] however, evidence of bone mineral loss in terms of increased osteoclast numbers have not yet been obtained.[2)] These conflicting views have led to a lack of clarity in the mechanism underlying bone mineral loss in this disease. Further, the occurrence of itai-itai disease has been reported to be at particularly high risk in postmenopausal women, not in younger women, men, or children.[14)] The reasons for this preferential distribution of the disease are yet to be substantiated despite the speculation that the combined effects of Es deficiency and Cd exposure might cause Cd-induced osteoporosis.[14)–16),18)]

Among the various risk factors for the development of postmenopausal osteoporosis, estrogen (Es) deficiency is a commonly known factor.[19)–22)] As

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Abbreviations: CA: Carbonic anhydrase; Es: Estrogen; μCT: Microcomputed tomography; CDL: Central dark line.

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models of Es deficiency, ovariectomized animals are used since they exhibit skeletal demineralization which characteristics similar to those of osteoporosis observed in postmenopausal women.[23)–26)] Regarding the development of osteoporosis including itai-itai disease, many researchers still consider that the bone mineral loss mainly occurs due to bone resorption by osteoclasts. On the other hand, we have recently proposed that crystal structure defects might be related to the bone mineral loss as well.[27)] Carbonate ions supplied by carbonic anhydrase (CA) activity are indispensable to initiate the nucleation process of apatite crystal by binding to magnesium (Mg) ions,[27)–37)] which are thought to inhibit the mineralization process.[35)] Following the formation of Mg-CO_3 compound "huntite" at the calcification front,[38)] the crystal nucleation takes place. Exposure to Cd ions, for example, caused crystal structure defects in developing rat tooth enamel due to the reduction in the enzymatic activity of CA by replacing Zn with Cd ions.[27)] In this respect, the crystal structure defects are expected to be closely associated with the decline of CA activity. Therefore, we can hypothesize that the failure of crystal formation, rather than renal tubular dysfunction, might be one of the factors contributing to the development of itai-itai disease. However, Cd exposure alone would be insufficient to explain the preferential occurrence of the disease in postmenopausal women. Besides Cd exposure, Es deficiency is expected to contribute to the development of Cd-induced osteoporosis because Es may regulate the osteoblast differentiation.[39)] However, information on the precise effect of Es deficiency on crystal formation is scarce at present. This prompted us to investigate the effect of Es deficiency on the crystal formation and how the bone formation might be affected by the combination with Cd exposure. Thus, the present study was designated to assess the preferential development of itai-itai disease after menopause.

Materials and methods

Experimental animals. In order to obtain the Es deficient animals, 5-week-old Sprague-Dawley rats that were overiectomized at 3-week-old rats and the 5-week-old normal female rats were purchased from Tokyo Laboratory Animals Science Co., Ltd. The overiectomized rats were divided into two groups; the Es deficient and the combination of Es deficiency and Cd exposure groups. Also the normal rats were divided into two groups; the Cd exposure and the control groups. For studying the Cd exposure and the combination groups, animals were provided free access to drinking water containing 100 mg/L Cd ions ($CdCl_2$), while the Es deficient and the control animals were provided tap water. Three months later, the rats (4-month-old) were examined by soft X-ray radiography, micro-computed tomography (μCT), and electron microscopy. The rats were anaesthetized with ether, and the samples of tooth enamel, were excised. The animal protocol was approved by the Animal Care and Use Committee of Meikai University.

Western blot analysis of CA. The immature enamel collected from the 4-month-old rat incisors was also prepared for biochemical analysis. After removal of adhering blood and surrounding soft tissue, the incisors were briefly rinsed with a cold saline solution. Immature enamel tissues were scraped from the incisors by using razor blades. They were directly homogenized with an electrophoretic sample buffer in order to extract matrix proteins. The supernatant containing the enamel matrix proteins was briefly separated by centrifugation at 12,000 × g for 1 min, and protein content was measured by the Bradford method.[40)] Equal amounts of protein (30 μg) from each sample were subjected to electrophoresis. After electrophoretic blotting on a nitrocellulose membrane (BA 85; Schleicher & Schuell, Dassel, Germany), the membrane was subjected to amido black staining and immunological detection to assess the effect of Es deficiency on CA. The electrophoretic blotting and immunological detection of CA on the nitrocellulose membrane were performed by the method described by Towbin *et al.*[41)] Antibodies against CA were prepared as described previously.[27)]

Soft X-ray radiography and micro-computed tomography (μCT). Rat calvarial tissues were examined using a soft X-ray radiography device (Sofron SRO-M50, Softex Co., Ltd., Japan) in order to compare the degree of mineralization under different experimental conditions. The samples were placed on the X-ray film cassette, and exposed to soft X-rays. Soft X-ray radiography was performed at 25 kV and 3 mA, for 5 min. After developing the photographic papers, each image was processed using a photo scanner (GT-9800F, Seiko Epson Co., Ltd., Japan). To examine the effects of the combination on the trabecular architecture, samples of tibiae were subjected to μCT scanning (Skyscan 1172, Skyscan, Kontich, Belgium). After removal of surrounding soft tissues, each sample was freeze-dried and fixed on a wax bed. The μCT was operated at 100 kV, 100 μA,

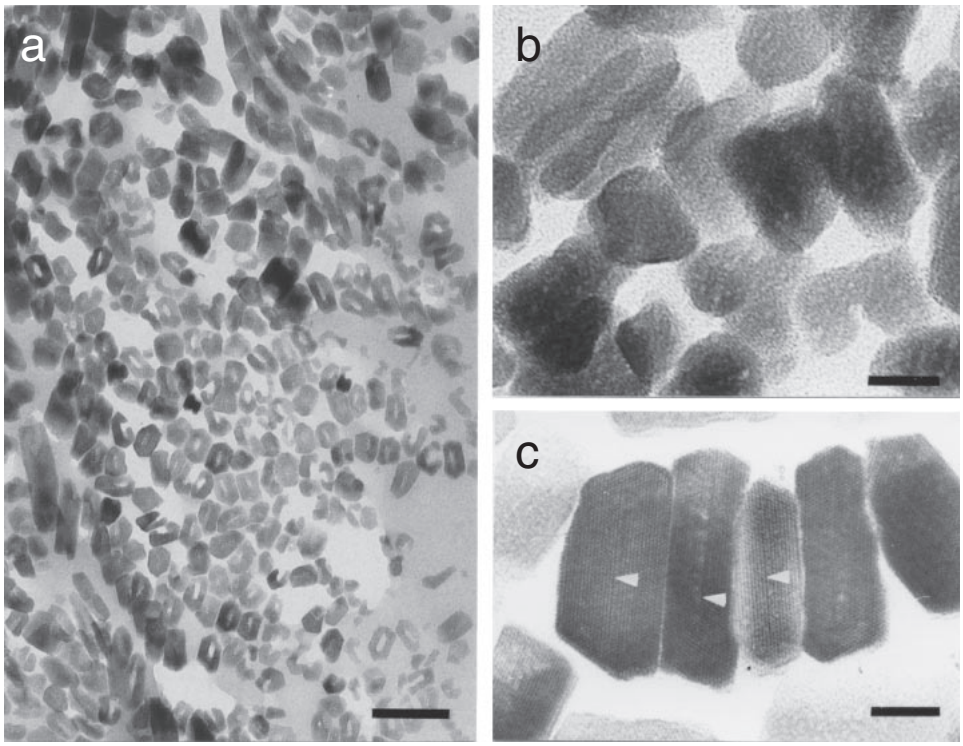

Fig. 1. Electron micrographs of enamel crystals (a and b) affected by estrogen (Es) deficiency, and those (c) of the control rat. Low magnification demonstrated, in part, the appearance of perforated crystals (a) showing voids at their centers, and higher magnification reveals an increase of amorphous minerals (b) without proper lattice fringes in the developing tooth enamel. In the control rat enamel crystals (c) possess the central dark lines in their centers. Arrowheads indicate the central dark lines observed in enamel crystals of the control rat. (a): bar = 100 nm, (b and c) bars = 15 nm.

and rotation step 0.2 degree, with a 0.5 mm-thick aluminum filter. At the end of μCT acquisition, the images obtained for each sample were reconstructed using a software program (NRecon, Skyscan).

Transmission electron microscopy. The developing tooth enamel obtained from ovariectomized rats and calvaria affected by the combined effects were dissected into small pieces by using razor blades. The samples were fixed with 2% glutaraldehyde in a 0.1 M cacodylate buffer (pH 7.4) for 1 h at 5°C, post-fixed with 1% osmium tetroxide in the same buffer for 2 h at 5°C, dehydrated by passage through a series of ascending ethanol concentrations, and embedded in Araldite 502 resin. Thin sections were obtained using a Porter-Blum MT2-B ultramicrotome (Sorvall, U.S.A.) equipped with a diamond knife. Thin sections were floated on water saturated with crystal minerals to prevent crystal dissolution. Unstained sections were examined under a JEM 100CX transmission electron microscope (JEOL Ltd. Tokyo, Japan) at an accelerating voltage of 80 kV.

Results

Effect of Es deficiency on the developing tooth enamel. Electron micrographs of the enamel crystals obtained at the nearly mature stage demonstrated that Es deficiency caused partial defects in the crystal structure, such as crystal perforations (Fig. 1a). This resulted in the formation of voids in the center of these aberrant crystals. Furthermore, higher magnification revealed an increase in the amount of amorphous minerals, which was observed as a loss of lattice fringes (Fig. 1b). These crystal structure defects were apparently formed due to the failure of crystal nucleation–the central dark lines (CDLs)–and an increase of amorphous minerals (Fig. 1b). On the contrary, it is clearly that the CDLs were observed in the crystals of the control (Fig. 1c).

Next, we performed biological analysis of CA in enamel matrix proteins in order to assess the effect of Es deficiency on CA production during the process of crystal nucleation. Amido black staining resulted in

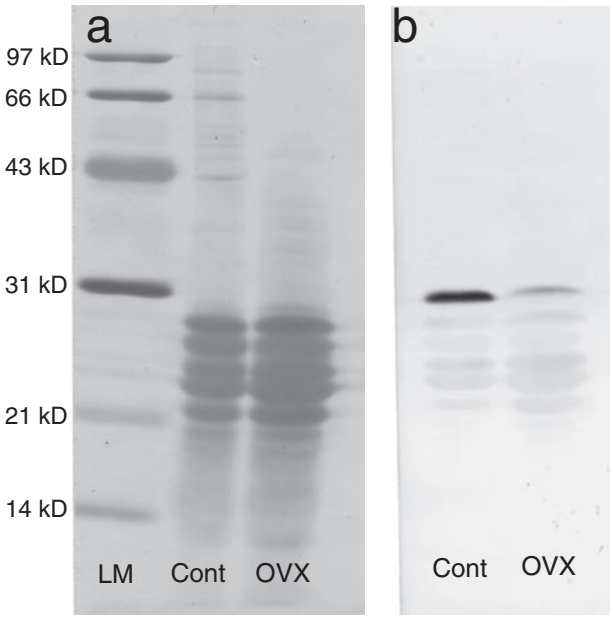

Fig. 2. Electrophoretic patterns and immunoblot analysis of the immature enamel matrix proteins. (a), Amido black staining; (b), Immunoblot analysis. The synthesis of carbonic anhydrase is adversely affected by Es deficiency in enamel forming cells (b). (LM), Low–molecular–weight standards; (Cont), the sample of enamel matrix proteins obtained from the control rats; (OVX), the sample of enamel matrix proteins obtained from the Es deficient rats.

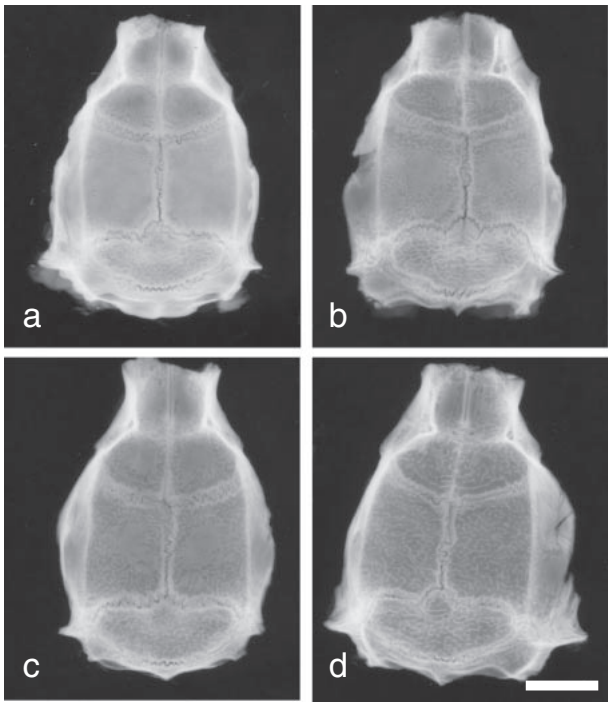

Fig. 3. Soft X-ray radiographs of rat calvariae obtained from the control and other experimental rats. (a), Control; (b), Cd exposure; (c), Es deficiency; (d), Combination of Es deficiency and Cd exposure. The combination of Es deficiency and Cd exposure rats clearly demonstrates the increase of the radiolucent area in the rat calvaria, resulting in a labyrinthine pattern of the calvarial feature (d), while the control rat does not create such a labyrinthine pattern (a). (a–d), same scale; bar = 0.5 cm.

appearance of smear protein bands corresponding to molecular weights of 31–43 kD and disappearance of the bands corresponding to some high-molecular-weight proteins (Fig. 2a). Western blot analysis using an anti-rat CA antibody revealed that the immunological reaction was weaker in the Es deficiency experimental group than in the control group indicating that Es deficiency suppressed the synthesis of this enzyme in enamel-forming cells (Fig. 2b).

Combined effects of Es deficiency and Cd exposure on the bone formation. To easily assess the morphological changes of calvarial tissue, the samples were analyzed by soft X-ray radiography. Soft X-ray radiographs revealed that the combined effects of Es deficiency and Cd exposure significantly altered the features of calvaria as compared to that of the control, resulting in the appearance of a labyrinthine pattern as shown in Fig. 3. Similarly, on μCT analysis of the tibia the rats subjected to both factors showed significantly greater distortion of the trabecular architecture than other experimental groups, indicative of osteoporotic changes (Fig. 4).

TEM observation. To assess the crystal structure of the labyrinthine pattern more precisely, we employed electron microscopy. Electron microscopy showed that the radiolucent area of calvarial tissue, which corresponds to the unmineralized area, was filled with a large amount of fine needle-shaped minerals; the radiopaque area contained relatively thick minerals, as shown in Fig. 5. Although fine needle-shaped minerals showed relatively electron-dense features at low magnification, a higher magnification revealed that most of these minerals had indistinct features and lacked the central dark lines or lattice fringes commonly observed in sound crystals (Fig. 6).

Further, we did not obtain any evidence for bone resorption by osteoclasts around the radiolucent area or an increase in osteoclast number, because we could not observe Howship's lacunae in the increased radiolucent area in the thick sections in the combined animal group (data not shown).

Discussion

Recently, we have proposed the mechanism underlying the development of crystal structure defects and the increase of amorphous minerals in calcified hard tissues and can be explained as follows: Each crystal develops within the organic envelope structure based on our previous findings and current study results,[27)–34)] which consists of an inner mineral zone, comprising of calcium, phosphate, and magnesium ions,[29),30)] and a surrounding thin outer organic layer. As mentioned before, carbonate ions released by the action of CA initiate the crystal nucleation by binding to Mg ions at the initial stage of crystal development.[27)–37)] This event may conducive to the development of the first lattice line by reactivated calcium and phosphate ions. Then the first lattice line together with the Mg-CO_3 compound "huntite" may create the central dark line (CDL), which remains as the crystal nucleation site. If supply of the carbonate ions is insufficient due to exposure to harmful chemicals,[27),28)] Mg ions may retain their inhibitory effect at the central area of the crystal. However, the peripheral area escapes the influence of Mg ions and can grow continuously from the already formed crystal surface. Eventually, this process may promote the creation of the voids at the centers of enamel crystals.[27),28)] On the other hand, amorphous minerals may increase in bone because bone crystals are too thin and small. Therefore, CA appears indispensable to the initiation of crystal nucleation in calcified hard tissues.

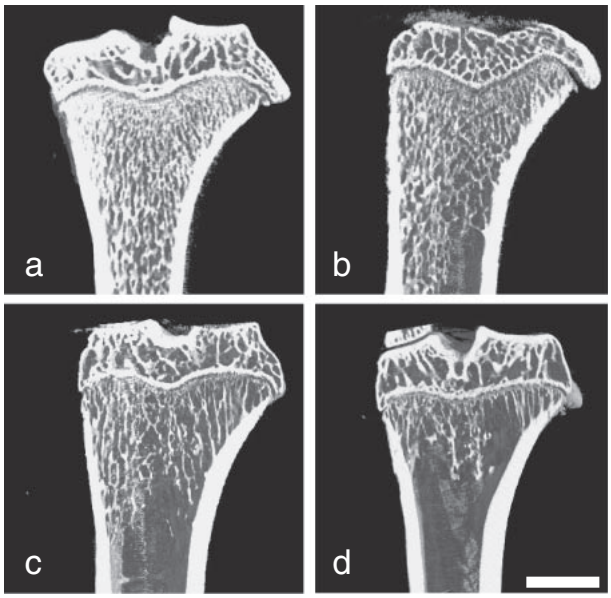

Fig. 4. Micro-computed tomography images of the rat tibias obtained from the control and other experimental rats. (a), Control; (b), Cd exposure; (c), Es deficiency; (d), Combination of Es deficiency and Cd exposure. The trabecular architecture is remarkably loosened by the combined effects of Es deficiency and Cd exposure. (a–d), same scale; bar = 0.2 cm.

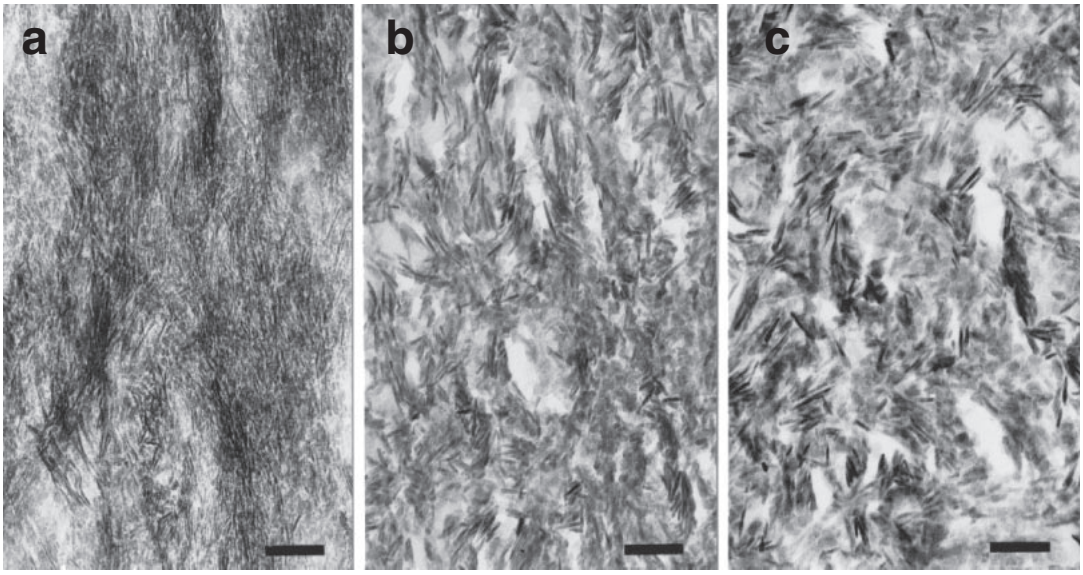

Fig. 5. Lower magnification of electron micrographs of minerals observed in the radiolucent and radiopaque areas of the calvaria influenced by the combined effects and those obtained from the control. (a), Minerals in the radiolucent area; (b), Minerals in the radiopaque area; (c), Minerals obtained from the control. Minerals in the radiolucent area seem to be meager compared to those in both the radiopaque area of the combined with Cd exposure and the control rats. Bars = 100 nm.

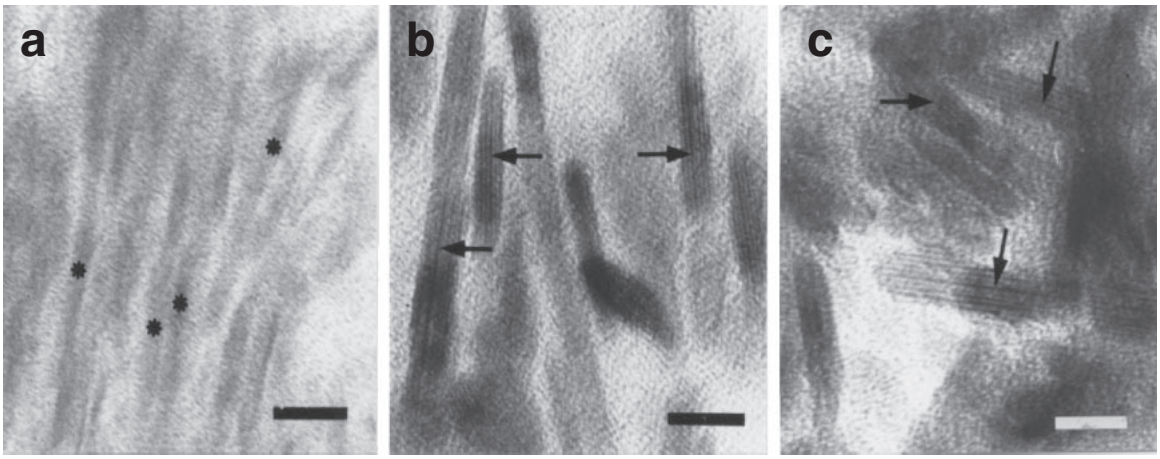

Fig. 6. Higher magnification of electron micrographs of minerals corresponding to Fig. 5. Minerals observed in the radiolucent area (a) are amorphous minerals and not crystallized, while crystals showing the central dark lines (CDLs) are observed in the radiopaque area (b) of the calvaria in the Es deficiency combined with Cd exposure rat. In the control rat calvaria CDLs are clearly observed in apatite crystals (c). Asterisks indicate amorphous minerals. Arrows indicate the central dark lines. Bars = 10 nm.

Although Es deficiency is considered the main risk factor for osteoporosis in women by affecting osteoblast differentiation,[39] its effect on the process of crystal formation has not yet been elucidated. In this study, we focused only on the relationship between CA and Es deficiency, although some other high-molecular-weight proteins, whose precise roles for crystallization remain unclear, are also adversely affected by Es deficiency. The present study revealed that Es deficiency could suppress the synthesis of CA by enamel-forming cells, thereby leading to crystal structure defects, such as an increase in the amount of amorphous minerals in calcified hard tissues, as observed in the cases of exposure to ions of harmful chemicals such as fluoride and Cd ions.[27),28] This suggests that Es may regulate directly the synthesis of CA during the process of crystal formation in calcified hard tissues.

With regard to the combination effects with Cd exposure, which also known as an environmental risk factor for osteoporosis,[4)–6] soft X-ray radiographs clearly demonstrated a labyrinthine pattern in calvaria (Fig. 3). Further, electron microscopy revealed that the radiolucent regions in the labyrinthine pattern were mainly composed of numerous amorphous minerals. Viewing from μCT analysis of the tibia in ovariectomized animal, the declining trabecular architecture showed the osteoporotic change. This osteoporotic change was further accelerated by the combination with Cd exposure (Fig. 4). These imply that rather than causing excessive bone resorption, osteoclasts may function normally during the process of bone remodeling. This is consistent with the fact that increase in the osteoclast number in bones could not be confirmed under any experimental condition.[2] Based on these findings, we provide a plausible explanation for the itai-itai disease as follows: The long-term Cd exposure increases the amorphous minerals in the bone due to the failure of crystal nucleation, resulting in osteomalacia. After the menopause, Es deficiency may adversely affect the osteoblast differentiation and thereby result in an imbalance in bone remodeling. The combination effects may further aggravate the bone fragility. Consequently, the high rate of occurrence of itai-itai disease in postmenopausal women may be associated with the deterioration of bone formation, not excessive bone resorption.

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