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ORIGINAL ARTICLE

Effect of estrogen replacement therapy on arthritis and bone mineral density in estrogen-replete rats with collagen-induced arthritis

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Abstract The influence of estrogen therapy on changes in arthritis and bone mineral density (BMD) was evaluated using an estrogen-replete collagen-induced arthritis (CIA) rat model. Seven-month-old female Sprague–Dawley rats ($n = 30$) were divided into the three groups; control (CONT), collagen sensitization (CIA), and CIA + 17β-estradiol administration for 7 weeks (CIA + E). BMD was measured by peripheral quantitative computed tomography in the proximal tibia every 2 weeks. Eight weeks after the initial sensitization the rats were killed and histomorphometry of tibia was performed. The hind paw thickness increased with time in CIA rats and there was a significant difference between CONT and CIA at 8 weeks after the initial sensitization. Estrogen tended to make the development of arthritis milder. In CIA, BMD at metaphyseal cancellous bone began to decrease with the onset of arthritis and became significantly lower than in CONT after 8 weeks. Compared with the CIA, the deterioration in BMD was inhibited in CIA + E. Histomorphometrical parameters of bone resorption were increased in CIA compared with CONT, and those elevations were reduced by estrogen treatment, but estrogen had no effect on bone formation parameters. In conclusion, estrogen could partially suppress arthritis and bone loss in estrogen-replete rats as well as estrogen-deplete ones.

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Introduction

Estrogen deficiency is an important contributing cause of osteoporosis, since osteoporosis is more common in women than in men primarily due to the loss of endogenous estrogen production after menopause [1, 2]. Estrogen deficiency also plays a substantial part in the progression of rheumatoid arthritis (RA)-associated osteoporosis [1, 3, 4]. Osteoporosis in patients with RA progresses with joint destruction and increases the risk of fracture, which is enhanced by estrogen deficiency [1, 3, 4].

Estrogen is involved in the immune response system and is an important modulator of RA. It is generally known that women are approximately three to four times as likely to develop RA as men [1]. It is also agreed that RA tends to improve during pregnancy but commonly flares or initially develops in the postpartum period [5]. There is evidence that protection against RA is afforded by contraceptive pills and post-menopausal replacement estrogen, although these issues remain controversial [6, 7].

In our previous study, the effects of ovariectomy and estrogen replacement therapy on arthritis severity as well as bone mineral density (BMD) were evaluated in rats with collagen-induced arthritis (CIA) [8]. We concluded that ovariectomy in CIA rats could enhance the severity of arthritis and bone loss, and that estrogen could suppress arthritis and bone loss. In that study, the CIA rats without ovariectomy were not treated with estrogen, because the model was designed to reflect the treatment of postmenopausal RA patients. Therefore, the effect of estrogen in estrogen-replete rats with CIA is uncertain. Therefore, the

aim of this study was to investigate the influence of estrogen therapy on changes in BMD and arthritis using an estrogen-replete CIA rat model.

Materials and methods

Animals

Seven-month-old female Sprague–Dawley rats (retired breeder animals with a body weight of 260–330 g; Shimizu Laboratory Supply, Kyoto, Japan) were used. These experiments were conducted at the animal study facility of Tottori University Faculty of Medicine with approval by the Animal Experiment Ethical Committee of Tottori University, and performed in Tottori University Animal Center. The animals were given tap water and solid food (calcium content 1.18/100 g, phosphorus content 1.09/100 g, vitamin D3 content 250 IU/100 g) (CE-2; Clea Japan, Tokyo, Japan) ad libitum. The animals were maintained in an animal room, which was illuminated for 12 h daily (7:00–19:00), at a room temperature of 24°C.

After 14 days of acclimation, the animals were divided into the following three groups by equalizing mean body weight: (1) injection of only the vehicle used for collagen sensitization + only the vehicle used for 17β-estradiol administration (n = 10, CONT); (2) collagen sensitization + injection of only the vehicle used for 17β-estradiol administration (n = 10, CIA); (3) collagen sensitization + 17β-estradiol administration for 7 weeks (n = 10, CIA + E).

The three experimental groups described above were examined every 2 weeks until week 8 after the initial sensitization for body weight, arthritis score, hind paw thickness, and measurement of BMD using pQCT. Eight weeks after the initial sensitization, the rats were anesthetized by intraperitoneal anesthesia with 50 mg/kg body weight of ketamine hydrochloride and 10 mg/kg body weight of xylazine, killed by exsanguination, and the left tibia was resected and fixed in 70% alcohol at 4°C for analysis.

Preparation of the CIA model

Under intraperitoneal anesthesia with 50 mg/kg body weight of ketamine hydrochloride and 10 mg/kg body weight of xylazine, 1.0 ml of an emulsion containing 500 μg of bovine type II collagen in 0.3% acetic acid solution (catalog no. K-41; Cosmo-Bio, Tokyo, Japan) and 500 μg of Freund's incomplete adjuvant (catalog no. 263910; Difco, Detroit, MI, USA) were injected subcutaneously at three sites on the back of each rat. For additional sensitization, 0.5 ml of the same emulsion was injected subcutaneously at two sites on the posterior aspects of both hip joints 1 week after the initial sensitization [8–12]. In the CONT group, physiologic saline was injected subcutaneously, using the same volume and methods applied for the other two groups.

Estrogen replacement therapy

For estrogen replacement therapy, 17β-estradiol (058-04043, Wako Pure Chemical Industries, Ltd., Osaka, Japan) was dissolved in a solvent consisting of 95% corn oil (034-17015, Wako Pure Chemical Industries, Ltd., Osaka, Japan) and 5% benzyl alcohol (027-01276, Wako Pure Chemical Industries, Ltd., Osaka, Japan), and 20 μg/kg was subcutaneously injected, three times per week [8]. In the groups with vehicle, only the solvent consisting of 95% corn oil and 5% benzyl alcohol was subcutaneously injected. Administration was initiated 8 days after the initial sensitization and continued for 7 weeks.

Evaluation of arthritis

The severity of inflammation of each toe, hind paw, ankle joint, and knee joint was evaluated every 2 weeks according to the degree of severity and extent of erythema and edema of the periarticular tissue, scored 0–3, where 0 = normal, 1 = detectable, 2 = moderate, 3 = severe [8]. The total score was regarded as the arthritic score with a maximum possible score of 24 [8]. Swelling of the posterior limbs was evaluated by measuring the ankle width from the medial malleolus to the lateral malleolus using a constant-tension caliper [8]. The posterior limb swelling was expressed as the mean of the bilateral limbs.

Measurement of bone mineral density

The volumetric BMD was measured in the proximal metaphysis (2 mm distal to the growth cartilage) and diaphysis (5 mm distal from the growth cartilage) of the left tibia every 2 weeks until 8 weeks after initial sensitization using peripheral quantitative computed tomography (pQCT: Model XCT-960, Norland-Stratec, Inc., Pforzheim, Germany). Under intraperitoneal anesthesia with 50 mg/kg body weight of ketamine hydrochloride and 10 mg/kg body weight of xylazine, the scan beam was attached to the proximal metaphysis of the left tibia perpendicularly to the bone axis, and the measurement was performed at a voxel size of 0.295 mm and a slice thickness of 1 mm after identifying the metaphysis and diaphysis by scout scanning.

The parameters of measurement were determined as follows: Contmode 2, peelmode 20, cortmode 1, and threshold 0.63 for cancellous BMD (mg/cm^3) and 0.93 for cortical BMD (mg/cm^3) [8, 12, 13]. Coefficients of variation (CVs) for this measurement with repositioning were 2.8% for cancellous bone and 3.3% for cortical bone [8, 12, 13].

Bone histomorphometry

Bone labeling by intraperitoneal injection of calcein at 10 mg/kg was performed twice, 7 days and 1 day before killing (schedule of 1-5-1-1). The proximal tibia resected at killing was fixed with 70% alcohol. Villanueva bone staining was then performed [14]. The bone tissue samples were embedded in methyl methacrylate resin without decalcification. The resulting blocks of the specimens were sectioned in the frontal plane at a thickness of 5 μm with a Jung Model K microtome (Reichert Jung, Germany). For histomorphometric analysis, the following items were measured in the secondary spongiosa extending 1.3–3.9 mm distally from the proximal growth cartilage of the tibia: bone volume (BV/TV, %), osteoid volume (OV/TV, %), osteoblast surface (Ob.S/BS, %), osteoid surface (OS/BS, %), trabecular thickness (Tb.Th, μm), trabecular number (Tb.N, /mm), trabecular separation (Tb.Sp, μm), osteoclast surface (Oc.S/BS, %), eroded surface (ES/BS, %), osteoclast number (N.Oc/BS, number/mm), mineralizing surface (MS/BS, %), double-label surface (dLS/BS, %), mineral apposition rate (MAR, μm/day), adjusted apposition rate (Aj.AR, μm/day), and bone formation rate (BFR/BS, μm^3/μm^2 per day). Each parameter was expressed according to the classification of Parfitt et al. [15]. Bone histomorphometric parameters were measured by an investigator blinded to the experimental groups.

Statistical analysis

Fisher's protected least significant difference procedure was performed after a repeated measures analysis of variance (ANOVA) for the comparison among the groups of body weight, hind paw thickness, and bone histomorphometry. The BMD of the tibia measured every 2 weeks until after week 8 using pQCT was expressed as percent change relative to the value at the initial sensitization (baseline), and compared among the groups by Fisher's protected least significant difference procedure after repeated measures of ANOVA among the groups. For the comparison of the arthritis score, the Mann–Whitney test between CIA and CIA + E was performed. For body weight, hind paw thickness, arthritis score, BMD, and bone histomorphometry, only the data for rats that developed arthritis were used. Four rats that did not develop arthritis (two in the CIA and two in the CIA + E) and three animals that died under anesthesia (one in each group) were excluded from the analysis. Statistical analysis was performed using SPSS software (Dr. SPSS II for Windows Version 11.0.1J, SPSS Japan Inc., Tokyo, Japan).

Results

Body weight

Body weight tended to increase gradually with time in the CONT group, but without significant difference (Table 1). Body weight at week 6 was significantly lower in both CIA groups ($P < 0.05$), and that at week 8 was even more significantly lower in both CIA groups ($P < 0.01$) than in the CONT group. No significant difference was noted among the CIA groups at any time point.

Incidence of arthritis

Joint swelling started to occur 2 weeks after the initial sensitization, and almost all rats that developed arthritis finally had joint swelling at 4 weeks after the initial sensitization. At 2, 4, 6, and 8 weeks after the initial sensitization, the incidence of arthritis was 33, 78, 78, and 78% in CIA, and 56, 78, 78, and 78% in CIA + E rats, respectively. The hind paw thickness increased with time in CIA rats and there was a significant difference between

Table 1 Body weight

	0 weeks	2 weeks	4 weeks	6 weeks	8 weeks
CONT	316.1 ± 19.2	316.7 ± 20.0	326.7 ± 22.1	333.3 ± 28.2	339.4 ± 32.1
CIA	315.0 ± 21.5	312.2 ± 25.4	306.7 ± 34.8	301.7 ± 29.2*	291.1 ± 29.2**
CIA + E	312.2 ± 19.5	310.6 ± 20.7	308.3 ± 29.7	300.0 ± 26.1*	292.2 ± 23.5**

Data are mean ± SD

CONT control, *CIA* collagen sensitization, *CIA + E* collagen sensitization + 17β-estradiol administration

* $P < 0.05$ (vs. CONT), ** $P < 0.01$(vs. CONT)

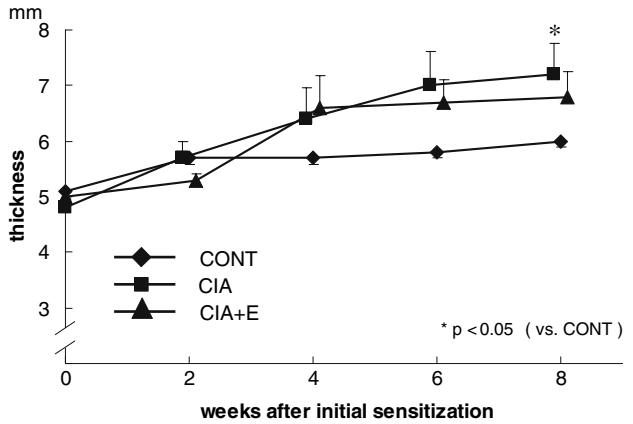

Fig. 1 Hind paw thickness in rats with collagen-induced arthritis (*CIA*) treated with vehicle (*CIA*), or with administration of 17β-estradiol for 7 weeks (CIA + E), as well as in controls (CONT) without CIA. Values are mean ± SEM. *$P < 0.05$ versus CONT. There was a significant difference between CIA and CONT at 8 weeks after the initial sensitization ($P < 0.05$)

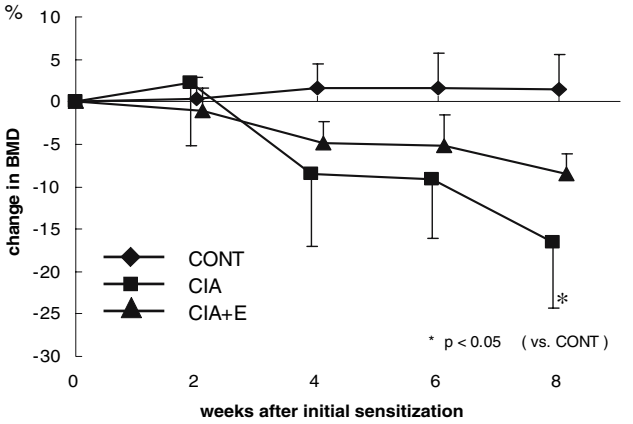

Fig. 2 Percent change in cancellous BMD of the tibial metaphysis. Values are the mean ± SEM.*$P < 0.05$ versus CONT. See Fig. 1 for definitions of the treatment groups. In CIA, BMD of cancellous BMD began to decrease with the onset of arthritis and became significantly lower than in CONT after 8 weeks ($P < 0.05$)

CIA and CONT at 8 weeks after the initial sensitization ($P < 0.05$) (Fig. 1). There were no significant differences between CIA and CIA-E or CONT and CIA-E.

The median arthritis scores were 4.7, 17.9, 17.4, and 17.9 in CIA at 2, 4, 6, and 8 weeks after the initial sensitization, respectively. Those in CIA-E were 6.3, 16.0, 17.7, and 17.4, respectively. There was no significant difference between CIA and CIA-E.

Bone mineral density

Tibial metaphysis

In CIA, BMD of cancellous bone began to decrease with the onset of arthritis and became significantly lower than in CONT after 8 weeks ($P < 0.05$) (Fig. 2). In CIA-E, the magnitude of the decrease was smaller than that in CIA, and no significant difference compared with that in CONT was observed during the observation period. There was no significant difference between CIA and CIA-E.

BMD of cortical bone was not reduced in any of the groups throughout the observation period.

Tibial diaphysis

In both CIA groups, BMD of cancellous bone tended to decrease with time after the onset of arthritis; however, there was no significant difference from that in CONT during the observation period (Fig. 3). BMD of cortical bone was not reduced in any of the groups throughout the observation period.

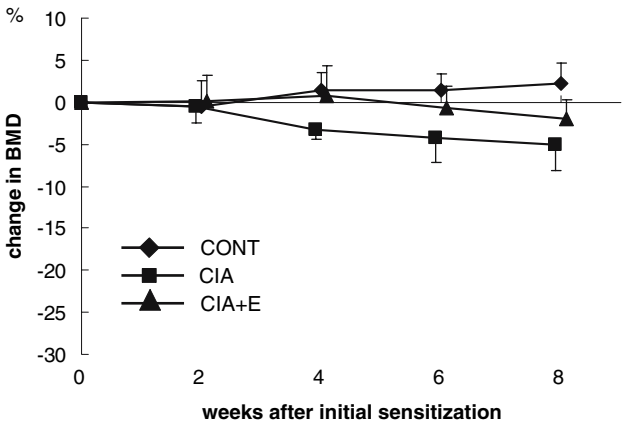

Fig. 3 Percent change in cancellous BMD of the tibial diaphysis. Values are the mean ± SEM. See Fig. 1 for definitions of the treatment groups

Histomorphometry

BV/TV and Tb.Th were lower, while Tb.Sp, ES/BS, Oc.S/BS, N.Oc/BS, and O.Th were higher in CIA compared with CONT (Table 2). There was no significant difference between CONT and CIA + E, except regarding ES/BS. No significant difference was noted in OV/BV, Tb.N, OS/BS, or Ob.S/BS among the groups. dLS was noted in 7, 6, and 4 animals in CONT, CIA, and CIA + E, respectively. MAR, Aj.AR, and BFR/BS showed no difference among the groups.

Discussion

The type II CIA rat is a model of RA that has been widely used for research on the pathology and treatment of RA

Table 2 Bone histomorphometry

	CONT	CIA	CIA + E
BV/TV (%)	30.0 ± 4.3	21.7 ± 7.2*	25.2 ± 2.9
OV/BV (%)	0.1 ± 0.2	0.5 ± 0.7	0.3 ± 0.4
TbTh (μm)	81.7 ± 14.3	63.9 ± 17.1*	67.6 ± 6.9
Tb.N (N/mm)	3.7 ± 0.5	3.4 ± 0.5	3.7 ± 0.2
Tb.Sp (μm)	193.0 ± 36.4	238.1 ± 52.1*	201.2 ± 13.7
OS/BS (%)	2.0 ± 2.8	4.7 ± 5.2	3.9 ± 5.0
Ob.S/BS (%)	1.4 ± 2.1	3.5 ± 4.8	3.1 ± 4.1
ES/BS (%)	25.4 ± 7.3	36.7 ± 9.1*	35.2 ± 8.3*
Oc.S/BS (%)	4.8 ± 2.1	8.8 ± 2.5*	7.1 ± 2.5
N.Oc/BS (N/mm)	1.5 ± 0.6	2.1 ± 0.3*	1.6 ± 0.5
O.Th (μm)	1.7 ± 0.7	2.3 ± 0.3*	2.0 ± 0.3
MS/BS (%)	2.6 ± 2.3	4.8 ± 4.9	3.3 ± 4.6
MAR (μm/day)	0.9 ± 0.2	0.9 ± 0.6	1.4 ± 0.2
Aj.AR (μm/day)	1.2 ± 0.9	0.9 ± 0.8	1.4 ± 0.4
BFR/BS ($\mu m^3/\mu m^2$/day)	0.009 ± 0.008	0.022 ± 0.029	0.017 ± 0.023

Data are mean ± SD

CONT control, *CIA* collagen sensitization, *CIA + E* collagen sensitization + 17β-estradiol administration

* $P < 0.05$ (vs. CONT)

since it was reported by Trentham et al. [16] in 1977. The rat has since been used as the model for studying pathology and therapy with regard to RA. We previously demonstrated that the BMD of cancellous bone close to the joint begins to decrease early after the onset of arthritis, as shown by a longitudinal observation of mature CIA rats, and therefore it was concluded that mature CIA rats are a useful model for periarticular osteoporosis in the early stage of RA [9–12].

In our previous study, it was revealed that the decrease in BMD was much more prominent in ovariectomized rats with CIA than in estrogen-replete ones, and that estrogen replacement significantly inhibited the decrease in BMD in ovariectomized rats with CIA [8]. Both the arthritis score and hind paw thickness were significantly lower in ovariectomized CIA rats with estrogen replacement than in those without estrogen [8]. In the current study, we evaluated the effect of estrogen in estrogen-replete rats with CIA. In this model, estrogen tended to make the development of arthritis milder compared with that in the group not treated with estrogen. Compared with the CIA group, the deterioration in BMD was inhibited by estrogen at metaphyseal cancellous bone, as shown by histomorphometrical analysis. These findings indicate that the development and severity of arthritis in CIA rats are partially ameliorated and bone loss is suppressed by estrogen even in the estrogen-replete status. However, the effect of estrogen on arthritis in estrogen-replete rats may be smaller than that in estrogen-deplete ones, since there was no statistical significance in hind paw thickness between the two groups.

Multifactorial mechanisms have been considered to explain the anti-inflammatory effect of estrogen. Estrogen plays a role in the blockade of cytokines such as IL-6, IL-1, and TNF-α [17–19], which are increased in synovial fluid in RA joints and elevated in ovariectomized mice [20], and also plays roles in the inhibition of macrophage infiltration in damaged tissues, and in the decrease of inflammatory mediators. Therefore, the marked decrease of BMD in the ovariectomized rats with CIA is considered to result from the synergistic increase of cytokine activity due to the disappearance of inhibition by estrogen.

RA symptoms are reduced by pregnancy and administration of contraceptive agents in pre-menopausal women [21, 22]. This effect of estrogen is not related to a deficiency or reduction in estrogen in RA, because there are no significant differences in sex hormone values between pre-menopausal women with RA and controls [23, 24]. Therefore, the mechanism of its effect is unclear, and few studies have been performed to clarify the effect of estrogen on arthritis in hormone-replete animals. Jansson et al. [25] demonstrated that estrogen decreases the rate of arthritis in ovariectomized as well as intact CIA mice. They also showed that estrogen treatment lowers the incidence of arthritis in non-castrated and castrated males, showing that the anti-arthritic effect of estrogen is not dependent on gender. An anti-inflammatory effect of administration of estrogen or selective estrogen receptor modulator on carrageenan paw edema was observed in carrageenan-induced acute inflammation in normal male rats [26]. Our finding that estrogen decreased the severity of arthritis in normal CIA rats agrees with these observations. Histomorphometrical analysis revealed that parameters of bone resorption, such as Oc.S and N.Oc, were increased in CIA rats compared with CONT, and those elevations were reduced by estrogen treatment. Estrogen had no effect on bone formation parameters. Estrogen mainly plays a role in the inhibition of osteoclastic bone resorption in postmenopausal women or estrogen-depleted animals [27]. This study demonstrated that estrogen inhibited osteoclastic bone resorption even in estrogen-replete animals with CIA.

There are several limitations in this study. First, rats were used in the study. The rat cortical bone has no Haversian system, so cortical bone remodeling is absent, in contrast to human cortical bone. However, in cancellous bone, bone remodeling does occur, so the effects of estrogen could be evaluated. Second, there was no CIA with ovariectomy group. Because the models in this study were designed to complement our previous study, in which we observed a positive effect of estrogen in estrogen-depleted CIA rats, the ovariectomized rats were not treated with estrogen.

In conclusion, this study demonstrated that estrogen could suppress arthritis and bone loss in estrogen-replete rats. These findings, using an animal model, indicate the potential usefulness of estrogen as an effective treatment for pre-menopausal RA patients as well as post-menopausal ones.

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