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Longitudinal effects of estrogen on mandibular growth and changes in cartilage during the growth period in rats

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ABSTRACT

Estrogen is a steroid hormone that induces skeletal growth and affects endochondral ossification of the long tubular bone growth plate during the growth period. However, the effects of estrogen on endochondral ossification of the mandibular condylar cartilage are unclear. In this study, ovariectomized Wistar/ST rats were used to investigate the longitudinal effects of estrogen on mandibular growth. The rats were administered different doses of estrogen. Longitudinal micro-computed tomographic scanning, histological staining and ELISA on plasma growth hormone were performed to examine the effects of estrogen on mandibular growth. The results showed that mandibular growth was suppressed throughout the growth period by estrogen in a dose-dependent manner. In addition, long-term administration of a high dose of estrogen to the rats resulted in significant increase in growth hormone throughout the growth period, significant circularization of cell nuclei in the proliferative layer, intensely staining cartilage matrix in the subchondral bone, and significant suppression of estrogen receptor (ER) alpha and beta expression in the mandibular cartilage. However, regardless of estrogen concentration, in the posterior part of the mandibular cartilage, ER expression extended to both the hypertrophic and proliferative layers. These results indicate that estrogen suppresses mandibular growth throughout the growth period. Additionally, it influences endochondral ossification via its effect on ERs.

1. Introduction

Endochondral ossification is a growth phase during which undifferentiated mesenchymal stem cells differentiate into chondrocytes, which grow from the proliferation stage to the hypertrophy and calcification stages. Then, the chondrocytes directly transform into bone cells (Jing et al., 2015). It has been reported that biomechanical factors, oxygen partial pressure, hormones, cytokines, and growth factors, among others, can influence endochondral ossification in the mandibular condylar cartilage (MCC) (Charlier et al., 1969; Dai and Rabie, 2007; Maor et al., 1999, 2002; McNamara and Carlson, 1979; Rabie et al., 2003; Ramirez-Yañez et al., 2004; Stevens and Williams, 1999), which is the main growth center of the mandible. Moreover, since osteogenesis is associated with endochondral ossification in the MCC, it causes the entire mandible to undergo anterior and downward growth (Mizoguchi et al., 2013; Tanaka, 2017).

Estrogen is a type of steroid hormone (also identified as a follicle hormone) that is produced in the ovaries and placenta during pregnancy; it is also produced in the adrenal cortex, testes, and peripheral tissues. It plays an important role in the development of secondary sexual characteristics during puberty and is responsible for regulating pregnancy and lactation. Estrogen level decreases systemically during the premenstrual period via the growth hormone (GH)/insulin-like growth factor (IGF)-I axis in the long bone growth plate, through which it can promote bone elongation. However, the rapid increase in estrogen level that occurs around the time of first ovulation triggers the closure of the epiphyseal line. Additionally, vascular endothelial growth factor level increases, which leads to closure of the epiphyseal line and the end of physical growth (Alsabban et al., 2018; Emons et al., 2010; Mitsimponas et al., 2018; Weise et al., 2001).

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Turner syndrome (TS) is a chromosomal disorder that is characterized by lack of or delayed secondary sexual characteristics and a short stature, which are mainly due to ovarian dysfunction. Estrogen replacement therapy is used to treat patients with TS to improve gonadal function during growth (Ross et al., 2011). It has been reported that patients with TS show inferior mandibular growth (Babić et al., 1997), which is suggestive of the effect of estrogen replacement on bone metabolism. However, the detailed mechanism underlying this effect is not clear. In the present study, we aimed to clarify the effects of estrogen on mandibular growth in ovariectomized rats during their growth phase. The effects of different estrogen doses and administration periods were evaluated.

2. Materials and methods

2.1. Experiment animals and treatment

All experimental animals were conducted in accordance with the Hiroshima University Regulations for Animal Experiments. The study was approved by the ethics committee of Hiroshima University (Animal Experiment Ethics Approval No. A19-142). Female Wistar/ST rats (age, 3 weeks) were used as the experimental animals. The rats were allowed to freely consume powdered feed (CE-2; Nippon Clare, Osaka, Japan) and drinking water under constant temperature and humidity conditions and a 12/12 h, light/dark cycle. The animals were preliminarily bred for one week before they were used in the study; therefore, the experiments were started when they were 4 weeks of age. The rats were divided into the following groups (n = 21 per group) and treated; sham, OVX, OVX+5E, and OVX+50E. Rats in the sham group underwent sham ovariectomy (OVX), whereas those in the OVX, OVX+5E, and OVX+50E groups were ovariectomized. Animals in the OVX+5E and OVX+50E groups were also administered 5 and 50 μg 17β-estradiol (E2; Cayman Chemical, Ann Arbor, MI, USA), respectively. The treatment day was set as day 0 for each group. Body weight was measured on days 2, 9, 16, 23, and 30 after treatment. A blood withdrawal was performed on day 2, 9, 16, 23, and 30 after treatment in each group. On day 32, the animals were euthanized and their uterine and MCC tissues were removed for analysis.

2.2. Surgical procedures

Bilateral OVX and sham surgery were performed under general anesthesia. A mixture of medetomidine (1.0 mg/mL; Kyoritsu Seiyaku, Tokyo, Japan), midazolam (5.0 mg/mL; Sandoz, Yamagata, Japan), and butorphanol (5.0 mg/mL; Meiji Seika, Tokyo, Japan) was used to induce anesthesia via intraperitoneal administration. After shaving the dorsal surgical field and disinfecting it with 10% iodine solution, an incision (1 cm) was made to elevate the ovaries outside the body. The fallopian tubes and ovaries were ligated and excised using sutures, after which the ovaries were removed. In the sham group, the ovaries were elevated and then put back in place without removal.

2.3. Estrogen replacement

E2 was used whenever estrogen had to be administered. It was dissolved in sesame oil (Nacalai Tesque, Kyoto, Japan) administered subcutaneously into the back of each rat starting on the first day after treatment and then every two days afterwards until the end of the experiment. To confirm the systemic effect of the subcutaneous injection, the uterus of each group was removed on day 32 after the start of the experiment to examine any effects on uterine tissue.

2.4. Morphometric analysis using micro-computed tomography (micro-CT)

On day 2, 9, 16, 23, and 30 after treatment in each group, the mandible of each rat was imaged using a micro-CT system (SkyScan 1173; Bruker-CT, Kontich, Belgium) under general anesthesia induced with the mixture of the anesthetic agents via intraperitoneal injection. The imaging conditions were as follows: pixel size, 16 μm; X-ray voltage, 50 kV; current, 200 μA; and Al filter, 0.5 mm. The data were reconstructed using NRecon software (SkyScan, Aartselaar, Belgium). After data reconstruction, the mandible was morphologically evaluated using Dataviewer (Bruker, Kontich). The landmarks and measurement items for mandibular assessment via morphometric analysis were set as previously described (Fujita et al., 2013; Hayashi et al., 2014) (Fig. 1). Me–Cd, Me-Go, and Me–Co were measured three-dimensionally.

2.5. Histological analysis

After deep anesthesia on day 32 after treatment in each group, perfusion fixation was performed using 0.5 M phosphate-buffered 4% paraformaldehyde. After 24 h of posterior fixation, the tissue fragments including MCC were removed and demineralized with 14% ethylenediaminetetraacetic acid tetrasodium (Wako Pure Chemical Industries, Osaka, Japan) for 6 weeks. The demineralized tissue fragments were embedded in paraffin and thinly sliced into 7 μm pieces in the sagittal direction using a rotary microtome (Microm315; Carl Zeiss, Oberkochen, Germany) to prepare serial tissue sections. The tissues were subjected to hematoxylin and eosin (HE), safranin O- fast green, and toluidine blue staining for histological analysis. The expression levels of estrogen receptor (ER) α and ERβ were also evaluated by immunohistochemical staining.

Tissues sections were prepared as indicated above and incubated for 24 h at 4 °C with primary antibodies against ERα (1:100 dilution) and ERβ (1:100 dilution) (Sigma Aldrich, St. Louis, MO, USA). Subsequently, the tissues were incubated with secondary antibodies against ERα and β (1:100 dilution) (Sigma Aldrich), streptavidin-biotin peroxidase (K0690; Universal Dako LSAB® Kit, Peroxidase), and 3,3′-diaminobenzidine (Sigma-Aldrich). The sections were counterstained with hematoxylin. Temporomandibular joint tissues and ERα and β immunolabeling were observed using a fluorescence microscope (BZ-X800; Keyence Corporation, Osaka, Japan). Tissue sections were quantified cell morphology, cell density, safranin O staining, toluidine blue staining, and ER expression according to conventional methods using Image J software.

2.6. ELISA on plasma growth hormone

On day 2, 9, 16, 23, and 30 after treatment in each group, circulating Growth hormone (GH) levels in blood plasma of each group were detected using GH ELISA kit (Invitrogen, Thermo Fisher Scientific, Massachusetts, USA) according to the manufacturer's instructions.

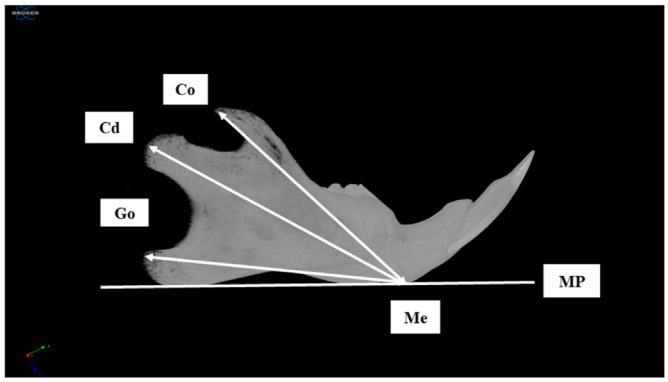

Fig. 1. Landmarks and linear measurements assessed. Me, midpoint on both sides of the lowest point of the osteophyte adjacent to the incisor in the mandibular inferior margin plane; Cd, the most posterior point of the mandibular head; Go, the most posterior point of the mandibular angular process; Co, the most posterior point of the muscular process.

2.7. Statistical analysis

The results have been presented as mean ± standard error of the mean. Statistical analyses were performed using one-way or two-way analysis of variance (ANOVA). Pairwise multiple comparisons (post-hoc Tukey–Kramer tests) were performed to identify significantly different variables when any of the ANOVA tests showed significant results. The significance level was set at $P < 0.05$ or <0.01.

3. Results

3.1. Effects of OVX and estrogen on uterine tissue

On day 32 after treatment, uterine tissues were removed to evaluate the effects of the various treatments (Fig. 2). Atrophy of the uterus was observed in the OVX group but not in the sham group, clearly showing the effect of OVX. Uterine tissues from rats in the OVX+5E and OVX+50E groups had similar morphological features without uterine atrophy compared to the sham group. However, uterine tissues in the OVX+50E group had a more hypertrophied form compared to those in the OVX+5E group.

3.2. Effects of OVX and estrogen on body weight

The effects of OVX and E2 on body weight were assessed on days 2, 9, 16, 23, and 30 after treatment. It was found that the body weight of the rats increased as the experiment progressed (Fig. 3A and B). Weight gain in the OVX group was significantly higher than in the other groups on days 9–16 (age, 5–6 weeks), but significantly lower in the OVX+5E and OVX+50E groups than in the sham and OVX groups on days 16–23 (age, 6–7 weeks). In contrast, weight gain in the OVX group was significantly higher than in the sham group. Additionally, weight gain in the OVX+5E and the OVX+50E groups was significantly lower than in the OVX group but not significantly different from that in the sham group on days 23–30 (age, 7–8 weeks). It was also noted that there was no significant difference in weight gain among the groups on days 2–9 (age, 4–5 weeks). The phenotype of the OVX+5E and the OVX+50E groups showed a smaller body size than the OVX group depending on the estrogen concentration (Fig. 3C).

3.3. Effects of OVX and estrogen on mandibular morphology

Micro-CT scans of the mandible were taken and reconstructed on days 2, 9, 16, 23, and 30 after treatment, after which mandibular length (Me–Cd, Me-Go, and Me–Co; Fig. 4A) and its amount of increase were measured over time (Fig. 4B). The results showed that mandibular length increased as the experiment progressed. All the parameters measured were significantly higher in the OVX group than the OVX+50E group on days 23 and 30. Me–Cd and Me–Co were significantly higher in the sham group than the OVX+50E group on day 30. However, there was no significant difference in any measurement item between the sham and OVX+5E groups. Furthermore, all the measurement items were significantly higher in the OVX group than the OVX+50E group on days 2–9 (age, 4–5 weeks). Me–Cd and Me–Co were also significantly higher in the OVX group than in the OVX+50E group on days 16–23 (age, 6–7 weeks) and days 23–30 (age, 7–8 weeks), respectively. There was no significant difference in mandibular growth when the rats were 5–6 weeks of age. This indicates that OVX causes significant mandibular growth at 4–5 weeks of age but does not cause a significant change in the length of the mandible. Moreover, continuous administration of E2 resulted in suppressed mandibular growth at the 6–8 weeks of age, as well as a significant difference in mandibular length.

3.4. Effects of OVX and estrogen on temporomandibular joint tissue

On day 32 after treatment, the MCC was removed for histological examination. Histological changes in the mandibular bone were evaluated by performing HE, safranin O- fast green, and toluidine blue staining (Fig. 5A, D, and F). ERα and β expression in the MCC was investigated via immunohistochemical staining (Fig. 6A and C).

The results of the HE staining indicated that tissue sections in all groups had a regular arrangement of fibrous, proliferative, prehypertrophic and hypertrophic layers (Fig. 5A). In addition, cell nuclei in the proliferative layer of tissues in the sham and OVX groups had flat morphologies, whereas those in the OVX+5E and the OVX+50E groups had circular morphologies (Fig. 5A, red box). We quantified the long against short axis of cell nuclei in the proliferative layer and found that those of the OVX+5E and the OVX+50E groups were significantly smaller than the sham and the OVX groups (Fig. 5B). Furthermore, in terms of cell densities in the proliferative layer of each groups, there were no significant differences between all the groups, however the cells in the OVX+5E and the OVX+50E groups had sparser tendency (Fig. 5C).

The result of the safranin O- fast green staining indicated that proteoglycans were well detected from the proliferative layer to the hypertrophic layer in all the groups and were stained strongly especially in the hypertrophic layer. However, the proliferative layer was less stained and the fibrous layer was not detected (Fig. 5D). Specifically, the OVX+50E group was widely stained red by safranin O than the OVX+5E group (Fig. 5E). In the subchondral bone, cartilage matrix was less stained in the OVX group than in the OVX+50E group (Fig. 5D, yellow box).

The result of the toluidine blue staining indicated that cartilage matrix was detected from the proliferative layer to the hypertrophic layer in

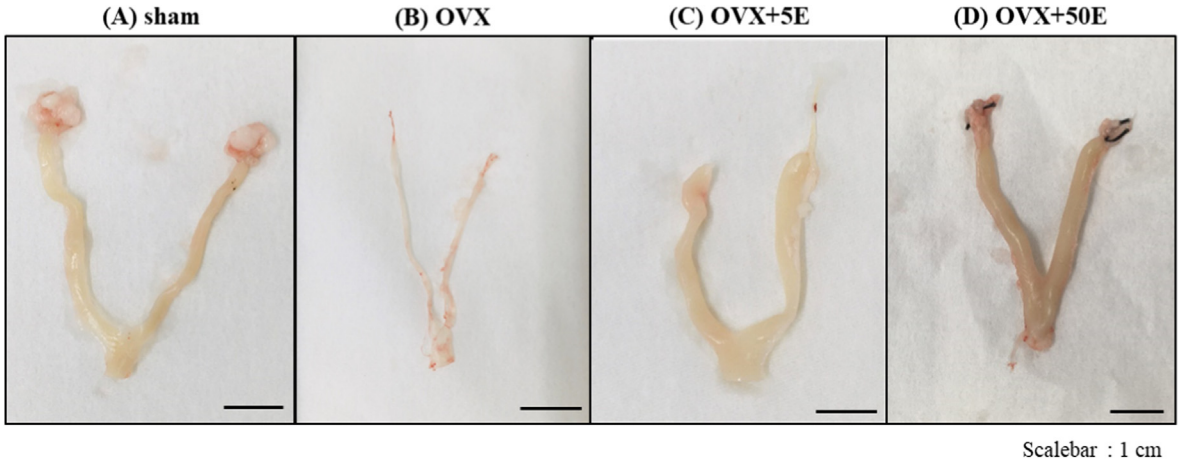

Fig. 2. Effects of ovariectomy (OVX) and estrogen on uterine tissue on day 32. (A) sham, (B) OVX, (C) OVX+5E, (D) OVX+50E. Scale bars are 1 cm.

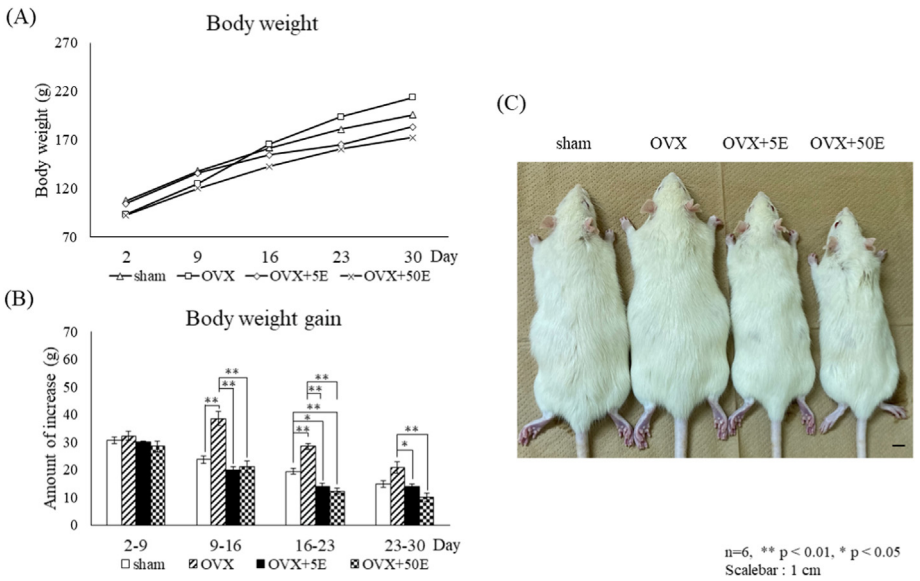

Fig. 3. Effects of ovariectomy (OVX) and estrogen on the body weight of the rats. (A) Changes in body weight on days 2, 9, 16, 23 and 30. (B) Body weight gain over days 2–9, 9–16, 16–23 and 23–30. (C) The phenotypes of each group on day 30. Scale bars are 1 cm.

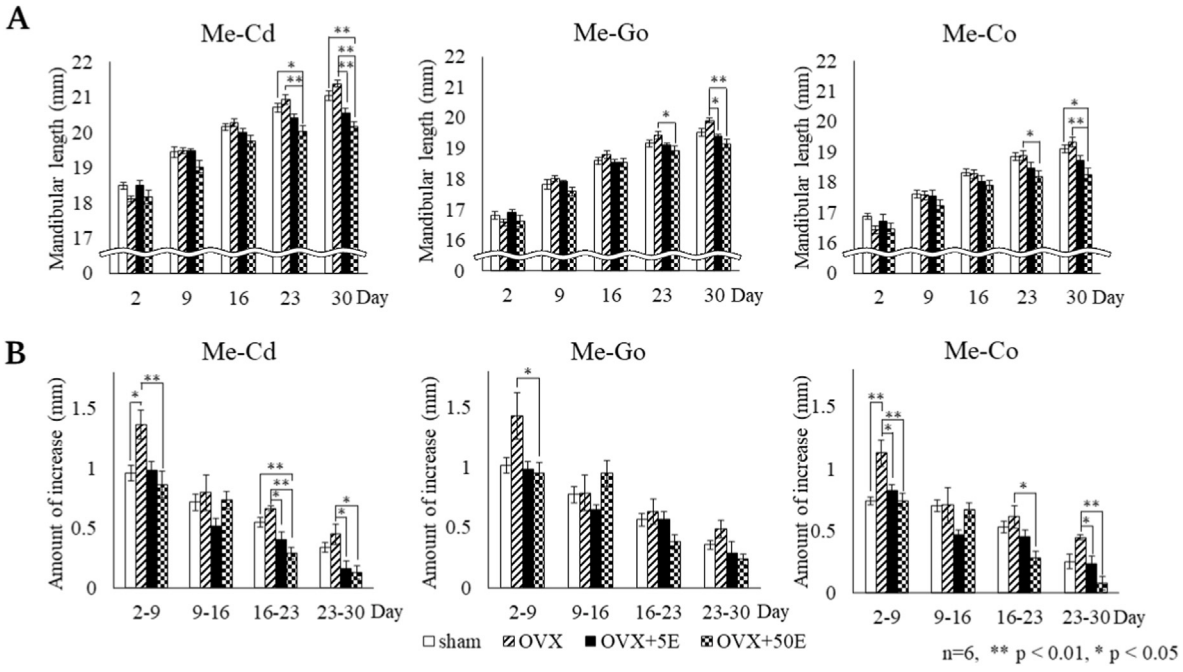

Fig. 4. Effects of ovariectomy (OVX) and estrogen on mandibular morphology. Me–Cd, Me-Go, and Me–Co were measured three-dimensionally using Dataviewer software. (A) Changes in mandibular length from day 2–30. (B) Amount of increase in mandibular length over day 2–9, 9–16, 16–23 and 23–30. Me, midpoint on both sides of the lowest point of the osteophyte adjacent to the incisor in the mandibular inferior margin plane; Cd, the most posterior point of the mandibular head; Go, the most posterior point of the mandibular angular process; Co, the most posterior point of the muscular process.

all the groups and was especially stained strongly in the hypertrophic layer. In the subchondral bone, as well as safranin O- fast green staining, cartilage matrix was less stained in the OVX group than in the OVX+50E group. (Fig. 5F, green box).

The result of immunohistochemical analysis indicated ERα and β expression was observed in all the groups (Fig. 6A and C). ERα expression in the OVX+50E group was lower than that in the OVX and the OVX+5E groups (Fig. 6A yellow arrow heads and B). However, ERβ expression in the OVX+50E group was lower than that in all other groups (Fig. 6C yellow arrow heads and D). It was observed that, in all the groups, ERα and β were expressed in the prehypertrophic layer from the anterior to the posterior area of the MCC, as well as in the proliferative and pre-hypertrophic layers in the most posterior part of the MCC (Fig. 6, red arrow heads).

3.5. *Effect of OVX and estrogen on growth hormone*

After blood sampling, plasma was separated and the absorbance at 450 nm was measured to calculate GH levels (Fig. 7). GH in the OVX+50E group was significantly higher than that in the OVX group on all days, and also significantly higher than the sham group on days 9, 16, 23, and 30. GH in the OVX+5E group was significantly higher than the

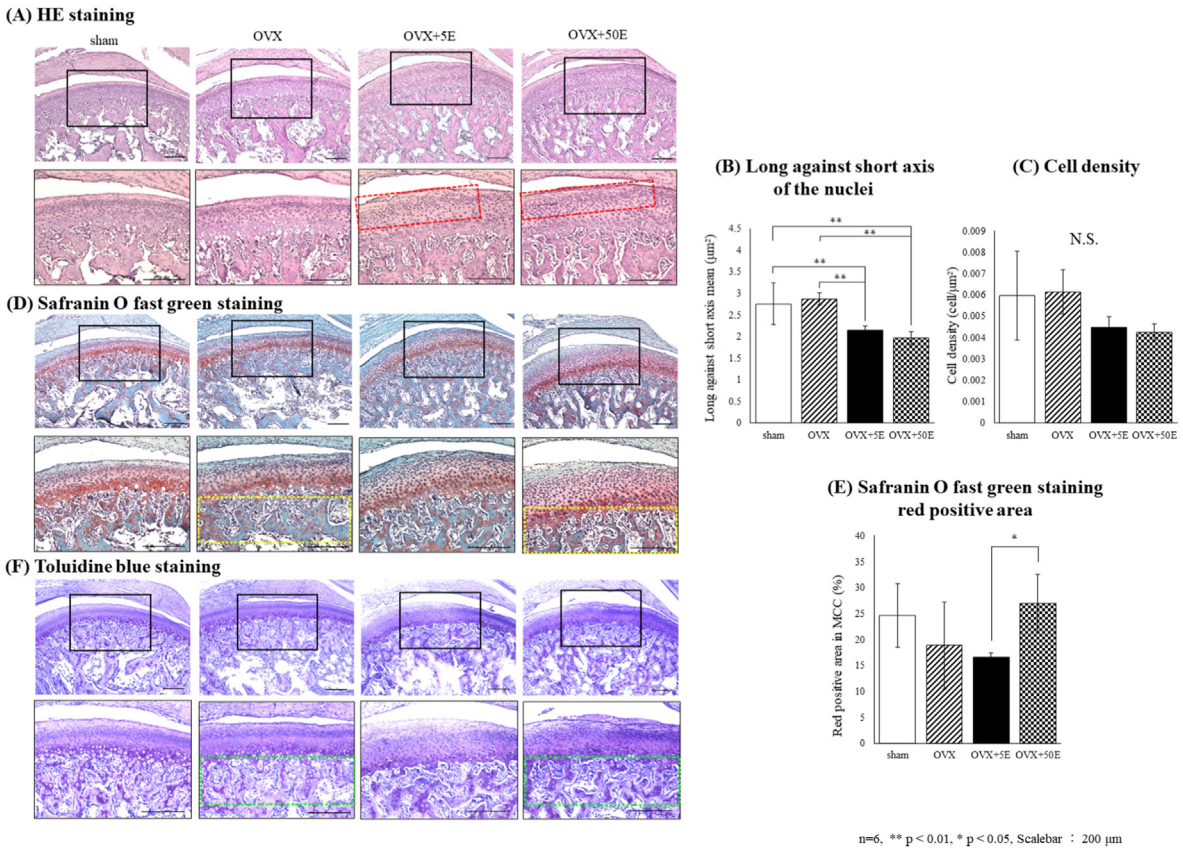

Fig. 5. Effects of ovariectomy (OVX) and estrogen on temporomandibular joint tissue on day 32. (A) hematoxylin and eosin staining (HE), (B) long against short axis of the nuclei in the proliferative layer, (C) cell density in the proliferative layer, (D) safranin O- fast green staining, (E) red positive area in safranin O- fast green staining in the MCC, (F) toluidine blue staining. The cell nuclei in the proliferating layer in the OVX+5E and the OVX+50E groups approximated a circular morphology compared to the sham and OVX groups (red box of A). In the subchondral bone, cartilage matrix was less stained in the OVX group than in the OVX+50E group (yellow box of D and green box of F). Scale bars are 200 μm.

sham group on days 16, 23, and 30, and was also significantly higher than the OVX group on days 16 and 30.

4. Discussion

In this study, we found that mandibular growth was suppressed by estrogen. Histologically, estrogen has an effect on the proliferative layer of the MCC as well as the production of cartilage matrix. Moreover, ERs are highly expressed in the posterior part of the MCC. It has been shown in previous studies that skeletal growth is influenced by low estrogen levels during the early growth period (Belgorosky et al., 2009; Küchler et al., 2021; Kalu, 1991; Morishima et al., 1995; Okuda et al., 1996; Omori et al., 2020; Smith et al., 1994; Ye et al., 2018). However, the longitudinal effects of different dose of estrogen on mandibular growth during the growth period are largely unclear.

To confirm the systemic effects of OVX and subcutaneously administered estrogen, the uterus of each rat was removed and subjected to morphological analysis. The uteruses of rats in the OVX group were significantly atrophied, which was in agreement with the findings of a previous study (Wang et al., 2013). In contrast, the uteruses of OVX rats that were administered estrogen were not atrophied. Additionally, they had a similar appearance as those of rats in the sham group (Fig. 2).

The ovaries are the main source of estrogen; therefore, a rat model of low estrogen was established by removing ovaries. This model has been widely used for several years to study the effects of estrogen (Tanaka et al., 2000). The ovaries produce other hormones such as progesterone in addition to estrogen. However, it has been previously established that progesterone does not have an effect on bone metabolism in OVX rats (Yamamoto et al., 1998). It has been shown that the growth phase of rats begins around 35 days of age, whereas the adult phase begins at 63 days of age (Montano et al., 1995; Ojeda et al., 1976). Therefore, in this study the longitudinal were performed when the rats were 4–8 weeks of age, as they were in the growth phase during that period.

E2 administration results in an increases in serum E2 level and the suppression of weight gain in a dose-dependent manner (Wang et al., 2013). It has been reported that serum E2 level in OVX rats can be restored to normal supplementation with E2 at a dose of 20 μg (Wang et al., 2013). The same result was obtained in the present study performed with 5 and 50 μg E2 (Fig. 3).

It has been suggested that low estrogen levels exert a systemically effect via the GH/IGF-I axis in the long bone growth plate and promote bone elongation (Frank, 2003; Juul, 2001), whereas high estrogen levels regulate growth plate fusion (Emons et al., 2011; Locatelli and Bianchi, 2014; Weise et al., 2001). In this study, it was found that estrogen administration increased plasma GH levels throughout the growth period (Fig. 7). Our morphological evaluation via micro-CT scanning showed that estrogen-induced inhibition of mandibular growth occurred throughout the growth period and finally affected mandibular length (Fig. 4). This suggests that estrogen, which has the ability to increase GH levels, has a suppressive effect on mandibular growth throughout the growth period regardless of its level. Moreover, it has been reported that the mechanical stress caused by masticatory muscles affects the growth of the coronoid process (Anthwal et al., 2015). It was noted in this study that Me–Co and Me–Cd measurements produced similar results (Fig. 4B), which suggests that estrogen may have an effect on the inhibition of coronoid process growth, or that the coronoid process was affected by condyle growth.

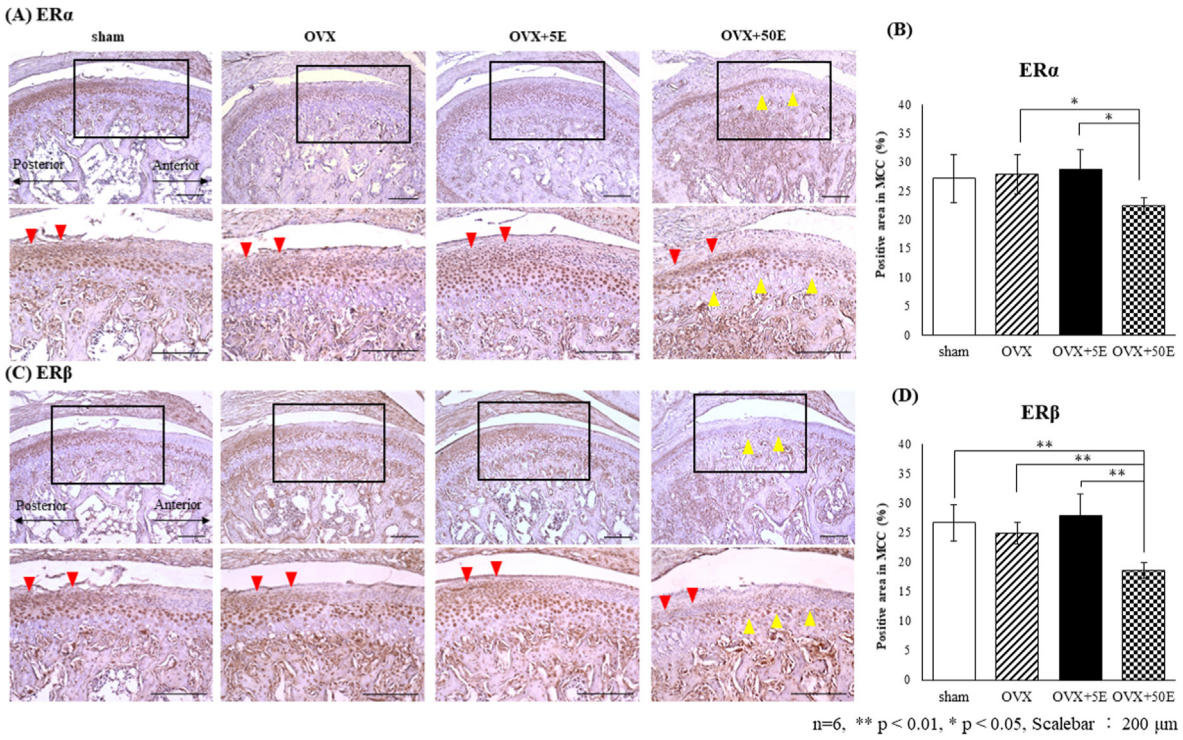

Fig. 6. Effects of ovariectomy (OVX) and estrogen on estrogen receptor (ER) α and β of temporomandibular joint tissue on day 32. (A) Immunohistochemical staining of ERα, (B) ERα positive area in MCC, (C) Immunohistochemical staining of ERβ, (D) ERβ positive area in MCC. ERα and β expressions were lower in the OVX+50E group than in the other groups (yellow arrow heads). ERα and β were expressed in the proliferative and prehypertrophic layers of the posterior part of the mandibular condylar cartilage (red arrow heads). Scale bars are 200 μm.

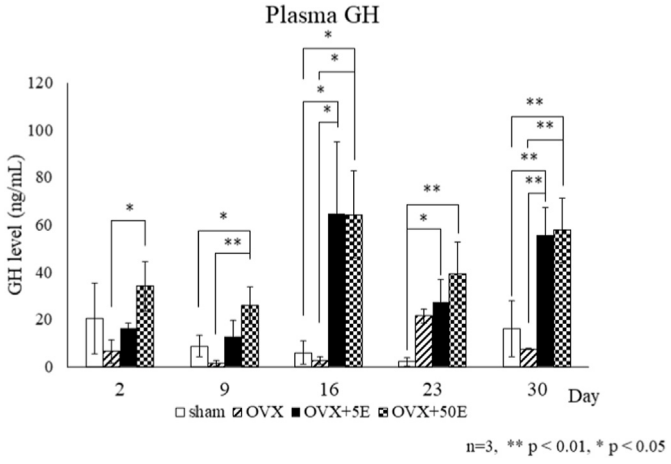

Fig. 7. Effects of ovariectomy (OVX) and estrogen on growth hormone (GH) on days 2, 9, 16, 23, and 30. Blood samplings were performed on each day and plasma was separated. ELISA was performed to measure absorbance at 450 nm.

MCC is the most prominent growth center of the mandible (Mizoguchi et al., 2013; Tanaka, 2017). Chondrocyte maturation associated with endochondral ossification in the MCC shows a transitional phase from proliferation and migration of progenitor cells to maturation and hypertrophy. In this study, showed that the MCC showed similar endochondral ossification in the HE staining (Fig. 5A). These findings are consistent with previous results of experiments performed in animals as well as in organ and cell cultures (Robinson et al., 2018; Talwar et al., 2006). Additionally, the cell nuclei in the proliferative layer of the estrogen-treated group approximated a circular morphology, compared to the elliptical morphology of the sham and the OVX groups. Therefore, it is suggested that estrogen affects cell nuclei morphology in the proliferating layers of MCCs (Fig. 5B).

In the present study, safranin O- fast green staining and toluidine blue staining revealed that the OVX+50E group was widely stained red by safranin O in the MCC than the OVX+5E group (Fig. 5E). In addition, it was revealed that the subchondral bone in the OVX group had less calcified cartilage than the OVX+50E group (Fig. 5 yellow box of D and green box of F). Therefore, it was suggested that estrogen administration increased the production of cartilage matrix or may affect chondrocyte differentiation. Furthermore, estrogen may increase cartilage matrix production during subchondral bone formation, and thereby promote the formation of scaffolds used by bone replacement.

Estrogen forms complexes with ERα and β in the cytoplasm; the complexes then enter the nucleus, where they exert various biological effects (Gruber et al., 2002). Previous studies have shown that ERβ deficiency results in an increase in MCC growth in female mice (Chen et al., 2014; Kamiya et al., 2013). In this study, we found that long-term estrogen administration suppressed ER expression (Fig. 6A and C yellow arrow heads, B and D). E2-induced suppression for ER expression may be negative feedback to maintain MCC homeostasis. We also found that ERs were widely expressed from the anterior area to the posterior area of the prehypertrophic layer in all the groups, whereas they were also expressed in the proliferative layer in the posterior part of the MCC (Fig. 6A and C, red arrow heads). This suggests that estrogen specifically acted on the posterior part of the condyle during its growth and affected the upward and backward elongation of the condyle.

5. Conclusion

The results of this study show that estrogen possibly has an inhibitory effect on mandibular growth during the growth period. Consequently, it can affect mandibular growth regulation in the upward and backward directions via its effect on ERs.

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Declarations of competing interest

The authors declare that they have no competing interests.

Data availability

No data was used for the research described in the article.

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