The effects of age and sex on the expression of oestrogen and its receptors in rat mandibular condylar cartilages

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ABSTRACT

Objective: Oestrogen expression may indicate a difference in resistance potential to mechanical strain. The purpose of this study was to investigate the expression of oestrogen and oestrogen receptors in mandibular condylar cartilages in male and female Sprague–Dawley rats at different ages.

Materials and methods: One-hundred SD rats at the age of 2, 4, 8 weeks and 4, 12 months in both sexes, 10 in each age–sex group, were enrolled in this study. The expression of oestradiol, ERα and ERβ was detected in mandibular condylar cartilages by the method of immunohistochemistry, and enzyme-linked immunosorbent assay or western blot.

Results: Oestradiol and ERs immunoreactivity were obvious in mandibular condylar cartilages of SD rats. Oestradiol and ERα were observed in hypertrophic and mature layers, while ERβ only in hypertrophic layer. There was no sex difference of same age (except 8-week age group) in the expression of oestradiol. The expression of both ERs, however, was usually higher in male than in age-matched female rats ($P < 0.05$), except that the 8-week-old female rats showed a higher ERα expression and the 4- and 8-week-old female rats showed a higher ERβ expression than the age-matched male ones in western blot results ($P < 0.05$).

Conclusions: The results that oestradiol, ERα and ERβ are co-expressed in rat mandibular condylar cartilage, indicate that mandibular condylar cartilage is a target for oestrogen. The age and sex related differences in ERs expression may indicate a difference in potential to resist mechanical loading between genders at different ages.

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1. Introduction

Osteoarthritis (OA) is a degenerative joint disease caused by the degradation of articular cartilage. Most epidemiological studies have shown that OA occurs in a larger number of females than males, and the rates of both prevalence and incidence increase in postmenopausal women. Furthermore, the symptoms of OA are more severe in women than in men.[1–4] The marked sexual dimorphism attracted investigators to

focus on the female reproductive hormones in OA. The temporomandibular joint (TMJ) plays an important role in craniofacial growth and function, and shows a high incidence of OA.[5] In literature, the effect of oestrogen on mandibular condylar cartilage has been studied by several researchers. In TMJs from ovariectomized animals the increased condylar cartilage thickness and even degenerative changes were noticed.[6–9] Studies on the effect of different concentrations of exogenous oestrogen on cultured mandibular condylar cartilage blocks or chondrocytes also suggested that oestrogen played an important role in modulating the morphology and function of condylar cartilages.[10,11] Additional support was provided by the existence of oestrogen receptors (ERs) in mandibular condylar cartilages reported by several studies. As a nuclear hormone receptor, ER is one member of a family of activated transcription factors that can initiate or enhance the transcription of genes containing specific oestrogen response elements.[12] The two subtypes of ERs, ERα and ERβ, are distinct proteins encoded by separate genes located on different chromosomes. As early as middle of 1980s, Sherian et al.[13] and Aufdermorte et al.[14] found ERs in the mandibular condylar cartilage of female baboons, although Milam SB et al.[15] failed to detect ER in condylar cartilages of male baboons by autoradiography. With the methods of immunohistochemistry and in situ hybridization Yamada et al.[16] revealed that ERα is distributed in male rat mandibular condylar cartilage. It seemed that oestrogens have the potential to modify the development and function of mandibular condylar cartilage.

It is suggested that oestrogen can be produced not only by gonads but also by a number of extragonadal sites, including cartilage and bone, in which oestrogen can act locally in a paracrine or intracrine fashion.[17] In our previous report,[18] it was found that mandibular condylar chondrocytes expressed aromatase which is essential in oestrogen synthesis, and oestrogen may be synthesized locally through the presence of aromatase. However, in literature very few reports were found focusing on the local expression of oestrogen in mandibular condylar cartilages in vivo. Whether there is difference in the expression of oestrogen, as well as its receptors, in mandibular condylar cartilages between sexes and between those at different ages is worth investigating for a future exploration of the role of oestrogen in the onset of OA. Thus the aim of the present study was to compare the expression of oestrogen, ERα and ERβ in mandibular condylar cartilages of Sprague–Dawley (SD) rats in different age and sex groups.

2. Materials and methods

2.1. Animals and tissue preparation

The study was approved by the Animal Research Committee of the Fourth Military Medical University. One hundred SD rats, at the age of 2, 4, 8 weeks and 4, 12 months in both sexes, 10 in each age and sex group, were provided by the animal centre of the Fourth Military Medical University.

Under deep anaesthesia with intraperitoneal injection of pentobarbital sodium (50 mg/kg body weight), 50 rats, 5 in each age and sex group for immunohistochemistry, were perfused with 200 mL normal saline through ascending aorta, followed by 400 mL paraformaldehyde (4% in phosphate buffer saline, pH 7.4). The TMJs were dissected and post-fixed overnight at 4 °C with the same fixative, and then decalcified for 1 week in Kristensen's fluid (sodium formate 52.2 g, formic acid 174.2 mL, 1000 mL distilled water). TMJ samples were then dehydrated in graded ethanol before being embedded in paraffin. The paraffin-embedded TMJs were cut into 5 μm middle-sagittal sections which were parallel to the lateral surface of the condyle neck of the mandible ramus. Then all sections were mounted on clean poly-L-lysine precoated glass slides.

2.2. Immunohistochemical staining

Three commercially available primary antibodies were used in immunohistochemical staining. They were anti-human oestradiol rabbit polyclonal IgG (AB924, Chemicon Bio. Inc., USA), anti-human ERα rabbit polyclonal IgG (SC542, Santa Cruz Bio. Inc., USA) and anti-human ERβ rabbit polyclonal IgG (SC8974, Santa Cruz Bio. Inc., USA).

Three sections from the middle part of each joint were selected for this study. Immunohistochemical staining was carried out with a three-step avidin–biotin complex method as described previously.[18] Briefly, after deparaffinization and rehydration, the sections were treated with 3% hydrogen peroxide at room temperature for 10 min, to eliminate endogenous peroxidase activity. Then the antigenic sites were exposed by digestion with Antigen Retrieval Solution (Wuhan Boster Biological Technology Ltd., China) for 10 min, and nonspecific binding sites were blocked by incubating the sections for 30 min at 37 °C in normal serum. The sections were incubated overnight at 4 °C with (1) anti-human oestradiol rabbit polyclonal IgG (4 μg/mL), (2) anti-human ERα rabbit polyclonal IgG (4 μg/mL), (3) anti-human ERβ rabbit polyclonal IgG (3 μg/mL). The bound primary antibody was then localized by biotin-labelled IgG (BeiJing ZhongShan Golden Bridge Biotechnology Co., Ltd., China) at 37 °C for 30 min and then an avidin-peroxidase complex at 37 °C for 30 min. The antibody staining was performed using peroxidase/diaminobenzidine (DAB) yellow kit (Wuhan Boster Biological Technology Ltd., China). The sections were lightly counterstained with hematoxylin, and then dehydrated in ethanol series, cleared in xylene and coverslipped. For control experiments, the sections were incubated by omitting the primary antibodies.

On each time of staining 1 of the sections in each age and sex group was selected, and totally 10 sections were stained together so that the staining results were comparable between groups.

2.3. Enzyme-linked immunosorbent assay (ELISA) for determination of oestradiol

Under deep anaesthesia with intraperitoneal injection of pentobarbital sodium (50 mg/kg body weight), the other 50 rats, also 5 in each age and sex group for ELISA and western blot were sacrificed. Cartilage samples were pulverized in liquid nitrogen, and then extracted with RIPA Lysis Buffer (Beyotime Institute of Biotechnology, China) for an hour at 4 °C. Insoluble material was removed by centrifugation at 10,000 rpm for 10 min. Protein in the supernatant was

estimated by BCA method according to the instructions of manufacturer (Pierce Biotechnology, Inc.).

Thirty micrograms total protein from each group was assayed by ELISA. Contents of oestradiol were quantified by goat anti-rat oestradiol ELISA kits (QRCT-301330012133EIA\UTL, Adlitteram Diagnostic Laboratories, Inc., San Diego, USA) according to the manufacturer's instructions. The minimum

detectable concentration of oestradiol in this assay was less than 1.0 pg/mL.

2.4. Western blotting and detection

Forty micrograms total protein from each group was separated by SDS-PAGE and transferred to polyvinylidene difluoride
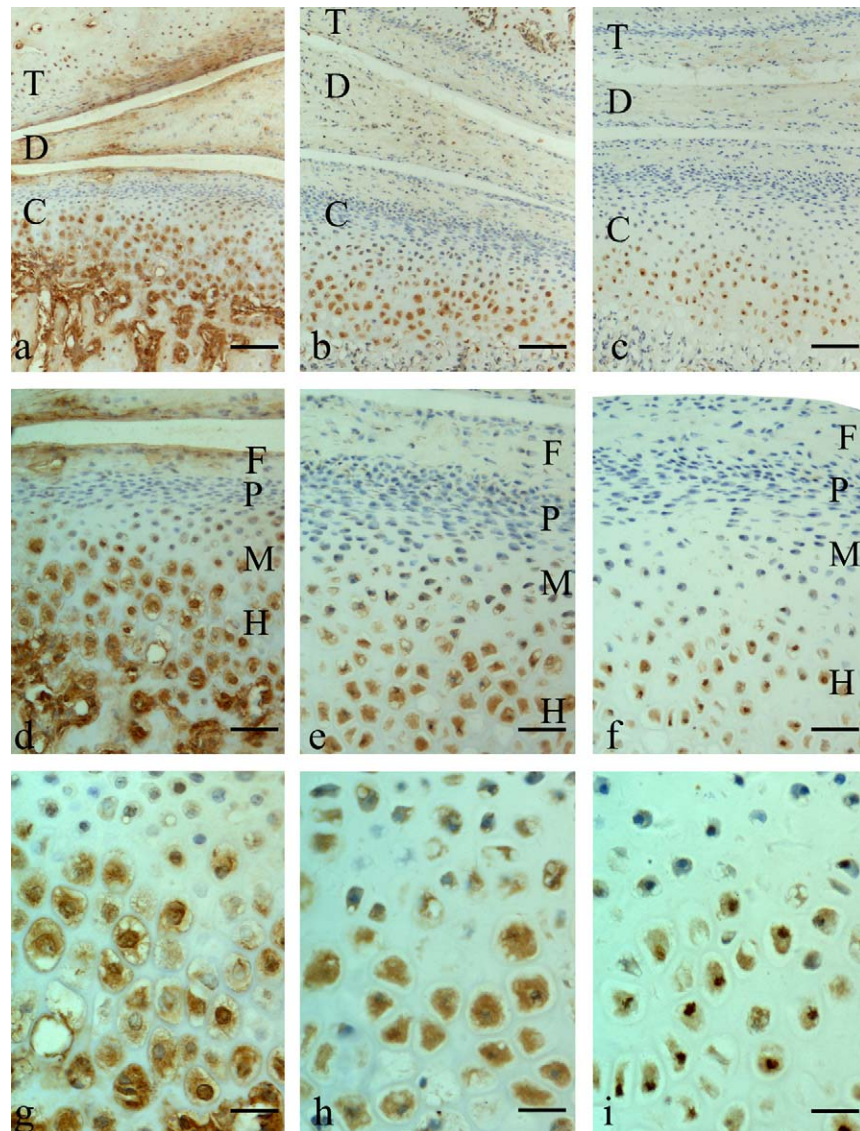

Fig. 1 – The expression of oestradiol, ERα and ERβ in TMJ. (a) The overall view of immunoreactivity for oestradiol. More intense oestradiol immunoreactivity was observed in condylar cartilage and subchondral bone tissue. (b) The overall view of immunoreactivity for ERα. More intense ERα immunoreactivity was observed in condylar cartilage. (c) The overall view of immunoreactivity for ERβ. More intense ERβ immunoreactivity was observed in condylar cartilage. (d) Immunoreactivity for oestradiol in condylar cartilage. Intense immunoreactivity was localized to the hypertrophic layer and mature layer of cartilage. (e) Immunoreactivity for ERα in condylar cartilage. Intense immunoreactivity was localized to the hypertrophic layer and mature layer of cartilage. (f) Immunoreactivity for ERβ in condylar cartilage. Different from ERα, intense immunoreactivity was localized to the hypertrophic layer of cartilage. (g) Immunoreactivity for oestradiol in hypertrophic layer. Strong immunoreactivity signals were found in cytoplasms of chondrocytes. (h) Immunoreactivity for ERα in hypertrophic layer. Strong immunoreactivity signals distributed equally in both cytoplasms and nuclei of chondrocytes. (i) Immunoreactivity for ERβ in hypertrophic layer. Strong ERβ immunoreactivity signals were primarily localized to nuclei of chondrocytes, which was also different from that of ERα. T = temporal bone, D = articular disc, C = mandibular condylar cartilage, F = fibrous layer, P = proliferative layer, M = mature layer, H = hypertrophic layer. Scale bar is 100 μm in a, b, c, 50 μm in d, e, f, and 25 μm in g, h, i.

membranes (Amersham Biosciences). Membranes were then incubated with primary antibodies that recognize human ERα (1:200, Santa Cruz Bio. Inc., USA), ERβ (1:200, Santa Cruz Bio. Inc., USA) or β-actin (1:1000, Santa Cruz Bio. Inc., USA) overnight at 4 °C. Then membranes were incubated with horseradish peroxidase-conjugated secondary antibody (1:2000, ZhongShan Goldenbridge Biotechnology Co., Ltd., China) for 2 h at room temperature. Finally, membranes were visualized using an ECL kit (Pierce Biotechnology, Inc.).

2.5. Quantitative analysis

For immunohistochemistry, all staining sections were observed under a Leica DM2500 microscope (Leica, Wetzlar, Germany). Image acquisitions (×100) were performed by a Leica DFC490 system (Leica, Wetzlar, Germany) and the part that including all layers of the cartilage, the fibrous, proliferative, mature, hypertrophic and calcified layers, was captured at the centre of anterior, middle and posterior third of the condylar cartilage. Then the images were analysed with Leica Qwin Plus software (Leica Microsystems Imaging Solutions Ltd, Cambridge, United Kingdom) as described previously.[18] Briefly, all picture files of the sections that stained at the same time were opened with the software of Photoshop 7.0 (Adobe Systems Inc., USA). Three continuous regions, 64 pixels × 64 pixels, in the central part of the anterior, middle and posterior third of condylar cartilage, were selected in the mature and hypertrophic layers in which oestradiol and ERs showed intense immunoreactivity (Fig. 1a–c). The positive chondrocytes in each region were counted at the same threshold of staining by one of the authors (ZF Ma) who knew nothing about the grouping of animals. In this way the counting of the positive cells was well controlled. The numbers of the positive chondrocytes were used for statistical analysis.

2.6. Statistical analysis

The SPSS 11.0 package (SPSS Inc., Chicago, IL, USA) was used to analyse and describe the data. Student's t-test method was used to compare the differences between groups. An alpha level of 0.05 was used for all statistical tests.

3. Results

3.1. Immunolocalization of oestradiol and ERs

No immunoreactivity was observed in the controls in which the primary antibodies were omitted. Immunocytochemistry for oestradiol and ERs demonstrated intense immunoreaction in rat mandibular condylar cartilage (Fig. 1a–c). Oestradiol and ERα were expressed in the hypertrophic and mature layers, abundant in the hypertrophic layer, while ERβ only in hypertrophic layer (Fig. 1d–f). Oestradiol was primarily localized to cytoplasms of chondrocytes (Fig. 1g), ERβ primarily to nuclei (Fig. 1i) but ERα to both cytoplasms and nuclei (Fig. 1h).

3.2. The quantitative analysis of immunohistochemistry

The expression of the oestradiol showed no significant difference between male and female rats in all 5 age groups
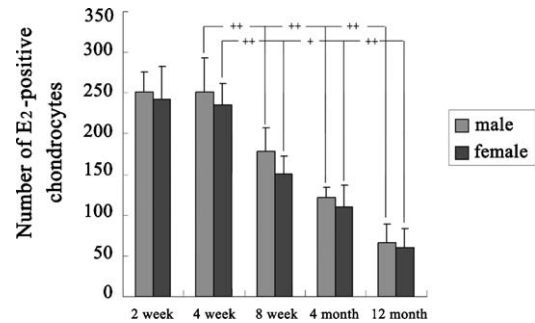

Fig. 2 – Density of oestradiol-positive chondrocytes in mandibular condylar cartilage. Error bars represent standard error (n = 5). Levels of significance were determined using Student's t-test. ⁺P < 0.05 and ⁺⁺P < 0.01 indicate significant difference between different age groups of same sex.

(P > 0 .05). When compared between the different age groups, as shown in Fig. 2, it was found that there was no significant difference in the expression of oestradiol between the 2- and 4-week old groups (P > 0.05). The number of the oestradiol-positive chondrocytes in both sexes declined gradually from 4 weeks to 12 months of age (P < 0.05).

The expression of ERα in male rats was significantly higher than that of female rats in 2-, 4- and 8-week old and 12-month old groups (P < 0.05), but not in the 4-month old groups (P > 0.05). In male rats the expression of ERα was the highest in 2-week old group (P < 0.01), then declined and remained at a stable level from 4-week to 12-month old groups (P > 0.05). However, in female rats it was stable from 2 weeks to 8 weeks of age (P > 0.05), but decreased at the age of 4 months and 12 months (P < 0.05) (Fig. 3).

The expression of ERβ in male rats was significantly higher than that of female rats in 2-, 4- week and 4-, 12-month old groups (P < 0.05), but not in the 8-week old groups. In male groups, the expression of ERβ in 4-week old group was lower than that in 2-week old group (P < 0.05), without significant differences from 8 weeks to 12 months of age (P > 0.05). In female rats, it was stable from 2 to 4 weeks of age, then
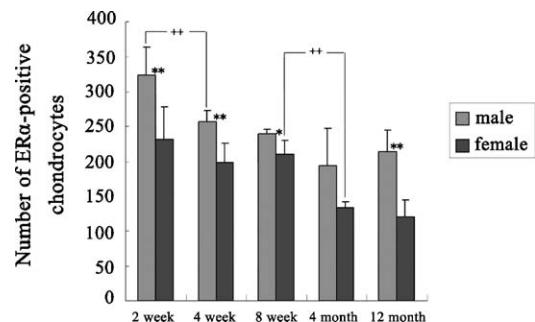

Fig. 3 – Density of ERα-positive chondrocytes in mandibular condylar cartilage. Error bars represent standard error (n = 5). Levels of significance were determined using Student's t-test. *P < 0.05 and **P < 0.01 indicate significant difference between different sexes of same age. ⁺⁺P < 0.01 indicates significant difference between different age groups of same sex.
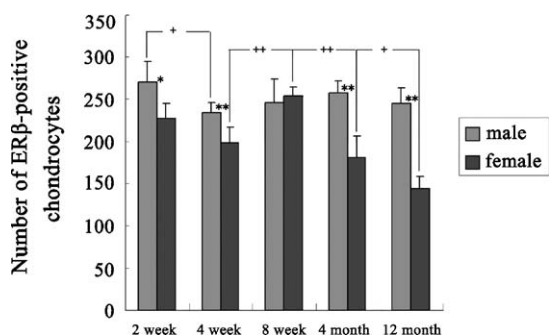
Fig. 4 – Density of ERβ-positive chondrocytes in mandibular condylar cartilage. Error bars represent standard error (n = 5). Levels of significance were determined using Student's t-test. *P < 0.05 and **P < 0.01 indicate significant difference between different sexes of same age. +P < 0.05 and ++P < 0.01 indicate significant difference between different age groups of same sex.
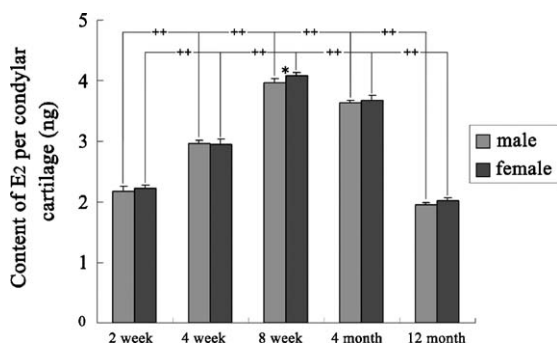

Fig. 5 – Content of oestradiol per condylar cartilage by ELISA (nanogram). Error bars represent standard error (n = 4). *P < 0.05 indicates significant difference between different sexes of same age. ++P < 0.01 indicates significant difference between different age groups of same sex.

reached the highest level at 8 weeks of age ($P < 0.05$), and declined from 4 months towards 12 months of age ($P < 0.05$) (Fig. 4).

3.3. ELISA

As shown in Fig. 5, the content of oestradiol per rat condylar cartilage ranged from 1.95 to 4.08 ng. There was no significant difference between male and female rats except 8-week old groups. The 8-week old female rats showed a higher oestradiol content than the age-matched male ones. In both male and female rats, the content of oestradiol increased gradually from 2 weeks to 8 weeks of age, and reached the highest level at 8 weeks of age, and was high at 4 months of age, but significantly low at 12 months of age ($P < 0.05$).

3.4. Western blot analysis

To provide further evidence that rat condylar cartilages express both ERα and ERβ proteins, western blot analysis was performed (Fig. 6). The immunoreactive 66 kDa ERα and
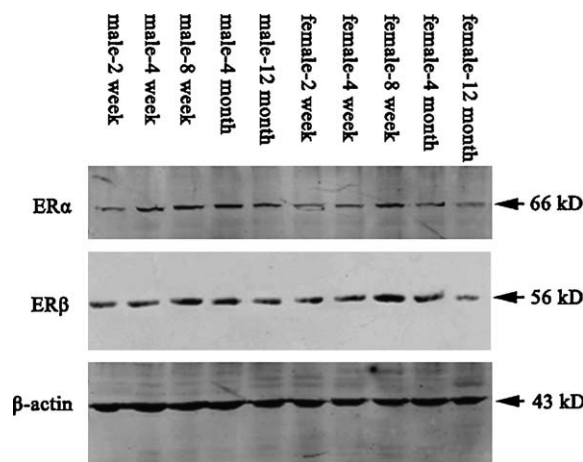

Fig. 6 – Western blot analysis for ERα and ERβ in the whole rat condylar cartilage. The immunoreactive 66 kDa ERα and 56 kDa ERβ protein bands were detected.

56 kDa ERβ protein bands were detected in all examined groups.

As shown in Fig. 7, the expression of ERα in male rats was significantly higher than that of female rats in 4-week old, 4 and 12-month old groups, while in 8-week old groups that in male was lower than female ones, with no significance in 2-week old group. In both male and female rats, the expression of ERα increased gradually from 2 weeks to 8 weeks of age, and reached the highest level at 8 weeks (female rats) or 4 months (male rats) of age ($P < 0.05$). After that, the expression of ERα in both sexes decreased gradually with age.

As shown in Fig. 8, the expression of ERβ in male rats was significantly higher than that of female rats in 4 and 12-month old groups ($P < 0.05$), while in 4 and 8-week old groups that in male was lower than female ones ($P < 0.05$), with no significance in 2-week old group. In both male and female rats, the expression of ERβ increased gradually from 2 weeks to 8 weeks of age, and reached the highest level at 8 weeks of age
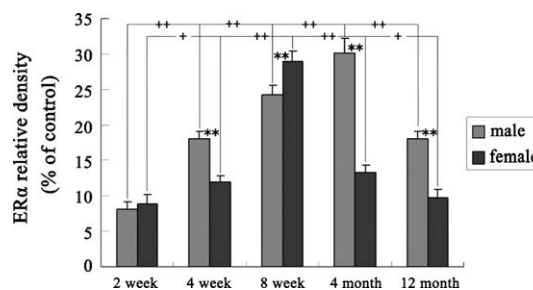

Fig. 7 – The expression levels for ERα as determined by western blot analysis in mandibular condylar cartilage. The content of ERα was determined by a densitometric analysis with that of control as 100%. The values represented the mean ± S.D. of three independent experiments. **P < 0.01 indicates significant difference between different sexes of same age. +P < 0.05 and ++P < 0.01 indicate significant difference between different age groups of same sex.
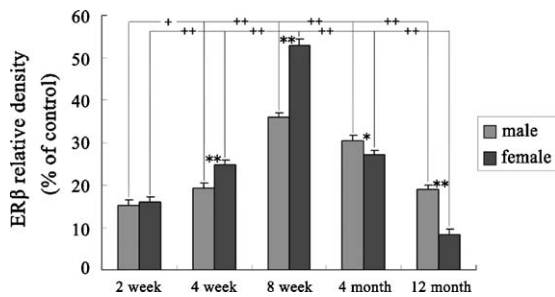
Fig. 8 – The expression levels for ERβ as determined by western blot analysis in mandibular condylar cartilage. The content of ERβ was determined by a densitometric analysis with that of control as 100%. The values represented the mean ± S.D. of three independent experiments. ⃰P < 0.05 and ⃰⃰P < 0.01 indicate significant difference between different sexes of same age. ⁺P < 0.05 and ⁺⁺P < 0.01 indicate significant difference between different age groups of same sex.

($P < 0.05$). After that, the expression of ERβ in both sexes decreased gradually with age.

4. Discussion

With the use of immunohistochemistry, ELISA and western blot in the present study, the local expressions of oestradiol, ERα and ERβ were detected in rat mandibular condylar cartilages. Some difference was found in age or sex distribution between the results of immunohistochemistry and western blot or ELISA. That is assumed mainly due to different detection method. The immunohistochemistry method provides information for the density of oestradiol or ERs positive chondrocytes. But the western blot and ELISA methods provide information for the amount of protein content of oestradiol or ERs in whole cartilage. The different sizes of condylar cartilages, the different densities of chondrocytes, and variation in the amount of protein in different cells, i.e. those in proliferative or hypertrophic layers, explains, at least partially, the different results with different methods. In the present study, oestradiol, ERα and ERβ were found co-exist in rat condylar cartilage. This result indicates that the condylar cartilage not only synthesize oestrogen, but also is a target for oestrogen in TMJ.[14–16,19] The differences in location and expression levels of ERα and ERβ suggested a difference in the function or mechanism of the two subtypes of ERs.[20–22] Moreover, the peaked expressions of oestradiol in ELISA and ERs in western blot at the age of 8 weeks indicate an increased demand of oestrogen in the condylar cartilage during the second growth of the rat.

Circulating levels of oestrogen are different in male and female animals.[17,23] The present study showed no significant sex difference (except 8-week age group) in the oestradiol level, which indicated that the level of local oestrogen in condylar cartilage is less associated, if there is, with the serum oestrogen. In 2002, Sylvia VL et al.[24] found that rat costochondral chondrocytes produced oestradiol locally. This was confirmed later by Takeuchi S et al.[25] In addition, our previous study also found that oestrogen can be synthesized locally in

mandibular condylar chondrocytes by aromatase.[18] The locally synthesized oestrogen in cartilage is reported to have a function of stimulating chondrocytes proliferation and protects them from spontaneous apoptosis.[26] Thus in the TMJ cartilage the locally synthesized oestrogen should not be neglected when discussing the development and function of condylar cartilage.

Although the oestradiol expression has no significant sex difference between male and female rats, the expression of ERα and ERβ in condylar cartilage showed a significant sex difference, supporting the view of the difference of condylar cartilage in response to mechanical stimulus between sexes. In a research on osteoblast-like cells, Lee et al.[27] found that ERα−/− mice produced three times less new cortical bone in response to the same mechanical stimulus as their ERα+/+ littermates. Similar result was reported by Jessop HL et al.[28] Moreover, primary cultures of osteoblast-like cells derived from ERα−/− mice failed to increase in number in response to mechanical stimulation, and rescue of this required transfection with fully functional ERα.[27] It is suggested that ERα played a vital role in the mechanisms by which strain regulates bones' ability to withstand loading without damage.[29] Conversely, osteoblast-like cells derived from ERβ−/− mice showed an enhanced proliferative response to strain compared with ERβ+/+ mice, which suggests that ERβ modulates the effects of ERα in the strain-related response.[28,29] Obviously, there is a close relationship between ERs level and adaptive responsiveness of bone cells. In the present study the expression of ERs in male rats was usually higher than that of age-matched female ones, which is consistent with the previous report that the expression of ER was higher in men than in women.[22] These results support the clinical impression that condylar cartilage of men has more potential to resist mechanical loading than women. However, in the results of western blot, the expression of ERα in 8-week old and ERβ in 4- and 8-week old rats was significantly higher in female than male. The age-related difference in resistance potential to mechanical loading is also attractive. The protective roles of ERs in this process need further investigations.

In summary, the present study has demonstrated that the mandibular condylar cartilage is a target for oestrogen because of the co-expression of oestradiol and ERs. The age-related higher expression of ERs in male or female rats indicates a difference in potential to resist mechanical loading of the mandibular condyle between genders and with age.

Acknowledgments

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