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Sex Differences in Chondrocyte Maturation in the Mandibular Condyle from a Decreased Occlusal Loading Model

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Abstract

Temporomandibular joint disorders (TMDs) predominantly afflict women of childbearing age. Defects in mechanical loading-induced temporomandibular joint (TMJ) remodeling are believed to

be a major etiological factor in the development of TMD. The goal of this study was to determine if there are sex differences in CD-1 and C57BL/6 mice exposed to a decreased occlusal loading TMJ remodeling model. Male and female CD-1 and C57BL/6 mice, 21 days old, were each divided into two groups. They were fed either a normal pellet diet (normal loading) or a soft diet and had their incisors trimmed out of occlusion (decreased occlusal loading) for 4 weeks. The mandibular condylar cartilage was evaluated by histology, and the subchondral bone was evaluated by micro-CT analysis. Gene expression from both was evaluated by real-time PCR analysis. In both strains and sexes of mice, decreased occlusal loading caused similar effects in the subchondral bone, decreases in bone volume and total volume compared with their normal loading controls. However, in both strains, decreased occlusal loading caused a significant decrease in the expression of collagen type II (*Col2*) and Sox9 only in female mice, but not in male mice, compared with their normal loading controls. Decreased occlusal loading causes decreased bone volume in both sexes and a decrease in early chondrocyte maturation exclusively in female mice.

Keywords

Temporomandibular joint; Mouse model; Mechanical loading; Fibrocartilage; Subchondral bone

The temporomandibular joint (TMJ) is composed of the mandibular condyle, a disc, and the articulating surfaces of the temporal bone. The articulating surfaces of the mandibular condyle are covered with fibrocartilage. Temporomandibular joint disorders (TMDs) are a collective term embracing several clinical problems that involve the masticatory musculature and/or the TMJ [1]. Approximately 10% of TMD patients have irreversible damage to the joint [2]. TMD affects 6–12% of the adult population [3, 4], with 80% being females of childbearing age [5]. Because of the high prevalence of TMDs in women of reproductive age, it has been postulated that sex hormones may make an individual susceptible to TMDs [6–11]. However, the cellular and molecular mechanisms of sex hormone actions on the TMJ are not fully known.

Changing the mechanical environment of the TMJ causes the mandibular condylar cartilage to remodel. The mandibular condylar cartilage remodels in response to mechanical strains by regulating chondrogenesis and endochondral ossification, to achieve a better balance between mechanical stress and the load-bearing capacity of the joint [12]. Two recent review articles have postulated that defective mechanical loading-induced TMJ remodeling is a major etiological factor in the development of TMJ degenerative diseases [2, 13]. There are a variety of mechanical loading-induced TMJ remodeling animal models [14]. We chose a mouse model because of the power of mouse genetics and for proof of concept. However, experiments on higher animal models are needed (i.e., pigs, primates) to validate the results. To examine the effects of TMJ mechanical loading-induced remodeling in mice, investigators [15, 16] have trimmed the incisors out of occlusion (believed to decrease the occlusal force and the amount of protrusion [17, 18]) and replaced the standard hard-pellet diet with a soft diet (believed to decrease the amount of occlusal molar force [17, 18]).

There is strong evidence that the mandibular condylar cartilage is derived from periosteum [19, 20]. In long bones, mechanical loading-induced periosteal bone formation is greater in males than in females (see [21] for review). In our previous study, we characterized the effects of decreased occlusal loading on the mandibular condyle in female CD-1 mice [16]. Therefore, the goal of this study was to examine if there were sex differences in response to decreased occlusal loading in the mandibular condyle in two different strains of mice. We hypothesized that, similar to long bones, decreased occlusal loading-induced periosteal condylar cartilage maturation would be further inhibited in female compared with male mice. Greater understanding of the sex differences in chondrocyte maturation due to

decreased occlusal loading is important in deciphering the sex predilection for degenerative diseases of the TMJ.

Materials and Methods

Mice

All experiments were performed under an institutionally approved protocol for the use of animals in research (University of Connecticut Health Center 2008-472). One hundred eighteen 21-day-old (eruption of molars and occlusion are complete at this age [22]) CD-1 (35 mice each gender) and C57BL/6 (24 mice each gender) mice (Charles River, Wilmington, MA) were used for this study.

Male and female CD-1 and C57BL/6 mice were each divided into two groups—(1) normal loading for 4 weeks and (2) decreased occlusal loading—and fed a soft-dough diet (Transgenic Dough Diet, with the same nutritional composition as the normal-pellet diet; BioServ, Frenchtown, NJ) with mandibular incisor trimming of approximately 1 mm of tooth structure every other day by an orthodontic light wire clipper for 4 weeks (the unimpeded eruption rate of the mouse mandibular incisor is approximately 0.4 mm a day [23], so incisor trimming keeps the anterior open bite between 0.5 and 1.0 mm). After the 4 weeks of the experiment, mice were killed.

Histology and Immunohistochemistry

Whole mouse heads were sectioned in half and prepared either for paraffin-embedded sections or for frozen embedded sections. For paraffin sections, the TMJs were fixed in 10% formalin for 1 week at room temperature. Samples were washed with tap water for 5 min and decalcified in 14% EDTA (Sigma-Aldrich, St. Louis, MO; pH 7.1) for 1 week. Subsequently, samples were processed for standard paraffin embedding and cut in sagittal serial sections. For frozen sections, dissected TMJs were placed in a biopsy cassette (Thermo Shandon, Pittsburgh, PA) and fixed for 2 days in freshly prepared 4% paraformaldehyde dissolved in PBS and adjusted to pH 7.4 with NH_4OH . The TMJ was decalcified in 14% EDTA for 3 days, soaked in 30% sucrose in PBS for 1 day, and embedded in $10 \times 10 \times 5$ mm disposable Vinyl specimen molds (Electron Microscopy Sciences, Hatfield, PA) filled with frozen embedding medium Shandon Cryomatrix (Thermo Fisher Scientific, Waltham, MA). Serial sections of the whole joint were obtained by cryosectioning on a CM1900 Cryostat (Leica, Nussloch, Germany) equipped with the CryoJane Frozen Sectioning Kit (Instrumedics, Richmond, IL). The CryoJane system is designed to capture a frozen cryostat section on special cold adhesive tape [24] to assist transferring the section to a cold glass microscope slide coated with an ultraviolet light-curable, pressure-sensitive adhesive (CryoJane Adhesive Coated Slides, Instrumedics). The section is permanently bonded to the adhesive on the slide with a flash of ultraviolet light.

TMJ sections were used for immunohistochemistry or H&E staining [25].

Mandibular Condylar Cartilage Thickness

H&E-stained sagittal sections corresponding to the midcoronal portion of the mandibular condylar head of each animal were selected and captured using a digital camera. For each mouse, the condyle cartilage thickness was measured in a blinded, nonbiased manner using the OsteoMeasure computerized image analysis system (OsteoMetrics, Atlanta, GA) interfaced with an Optiphot microscope (Nikon, Melville, NY). Briefly, a $200 \times 200 \text{ mm}^2$ field was placed at the central portion of the mandibular condylar cartilage and the outline of the condylar cartilage was demarcated by the same investigator, who did not know from which mice the sections originated. The OsteoMeasure program then calculated the average

cartilage thickness for the field. Measurements were performed on three sections from each mouse and three mice from each of the groups.

BrdU Labeling

Three hours prior to death, mice were injected intraperitoneally with 0.1 mg bromodeoxyuridine (BrdU) per gram body weight. BrdU immunohistochemical analysis was completed using a staining kit following the manufacturer's instructions (Invitrogen, Carlsbad, CA). Negative controls consisted of omitting the primary antibody step and incubating with blocking solution. Images of proliferating cells in the mandibular condylar cartilage were taken using the Axiocam MRC (Carl Zeiss MicroImaging, Thornwood, NY) digital camera. To quantify BrdU staining, a rectangular box of fixed area was superimposed on 20× images of each section and a labeling index (number of BrdU-positive cells/total number of cells) calculated. Three to five sections, corresponding to the same anatomical area (mid-sagittal), were counted for each animal; and the average of these sections was the BrdU labeling index given to that mouse.

Micro-CT

Bone morphometry of the mandibular condyle from five to eight mice per group was quantified using cone beam micro-focus X-ray computed tomography (μCT40; Scanco Medical, Bassersdorf, Switzerland). Serial tomographic images were acquired at 55 kV and 145 μA, collecting 2,000 projections per rotation at a 300 ms integration time. Three-dimensional 16-bit gray-scale images were reconstructed using standard convolution back-projection algorithms with Shepp and Logan filtering. Segmentation of bone from marrow and soft tissue was performed in conjunction with a constrained gaussian filter to reduce noise. Volumetric regions were selected to include the mandibular condyle (see Fig. 4a). The thickness and spacing of the trabecular bone were measured.

RNA Extraction and PCR Amplification

The condylar head, which contains both condylar cartilage and subchondral bone, was isolated and dissected from the mice. Total RNA was extracted with TRIzol reagent (Invitrogen) following the manufacturer's protocol. Total RNA was converted to cDNA using the ABI High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) following the manufacturer's protocol. Real-time PCR was performed for expression of different genes in separate wells (singleplex assay) of a 96-well plate in a reaction volume of 20 μl. *Gapdh* was used as endogenous control. Three replicates of each sample were amplified using assay-on-demand gene expression for the particular gene of interest, using predesigned unlabeled gene-specific PCR primers and TaqMan MGB FAM dye-labeled probe. The PCR mixture (including 2 × TaqMan Universal PCR Master Mix, 20×assays-on-demand gene expression assay mix, 50 ng of cDNA) was run in the Applied Biosystems ABI Prism 7300 sequence detection system (Life Technologies, Carlsbad, CA) utilizing universal thermal cycling parameters. For the genes for which the efficiencies of target and endogenous control amplification were approximately equal, relative expression in a test sample compared with a reference calibrator sample ($\Delta\Delta C_t$ method) was used for data analysis. For the genes that were not amplified with the same efficiency as the endogenous control, the relative standard curve method, in which target quantity was determined from the standard curve and divided by the target quantity of the calibrator, was used. Gene expression was performed for parathyroid hormone-related peptide (*Pthrp*), SRY box-containing gene 9 (*Sox9*), collagen type II (*Col2*), and collagen type X (*Col10*).

Statistical Analysis

The statistical significance of differences among means was determined by analysis of variance (ANOVA) with post hoc comparison of more than two means by the Bonferroni method using GraphPad Prism (GraphPad, La Jolla, Ca).

Results

Decreased occlusal loading did not cause any significant differences in the weight of the mice for the duration of the experiment in either strain or sex compared with the normal loading group (data not shown).

In both CD-1 and C57BL/6 mice, after 4 weeks of decreased occlusal loading, there was a significant decrease in the thickness of the mandibular condylar cartilage in both males (Fig. 1c, d) and females (Fig. 1g, h) compared with their normal loading controls.

Chondrocyte Maturation Markers in Response to Decreased Occlusal Loading in Females

Gene expression of chondrocyte maturation markers from the mandibular condylar head was analyzed in CD-1 and C57BL/6 mice exposed to decrease occlusal loading for 4 weeks. In both strains, decreased occlusal loading for 4 weeks significantly decreased *Sox9* and *Col2* mRNA expression in female (Fig. 2a, c), but not in male (Fig. 2b, d), mice compared with their normal loading controls. In addition, decreased occlusal loading for 4 weeks caused a significant decrease in the expression of *Col10* expression in CD-1 male mice, in CD-1 female mice, and in C57BL/6 female mice compared with their normal loading controls (Fig. 2a–c).

Cell Proliferation in Response to Decreased Occlusal Loading in Female C57BL/6 Mice

To further characterize the sex differences in the mandibular condylar cartilage to our decreased occlusal loading model, proliferation was examined by BrdU labeling. Decreased occlusal loading for 4 weeks caused a significant decrease in the BrdU cell ratio (number of BrdU-positive cells/total number of cells) in the mandibular condylar cartilage in female C57BL/6 mice compared with their normal loading controls (Fig. 3b).

Subchondral Bone in Response to Decreased Occlusal Loading

In both strains, micro-CT analysis revealed that decreased occlusal loading for 4 weeks caused similar changes in male and female mice consisting of significant decreases in total volume (Fig. 4a) and bone volume (Fig. 4b) compared with their normal loading controls. Furthermore, decreased occlusal loading caused a significant decrease in trabecular thickness in male CD-1 mice compared with their normal loading controls (Fig. 4f). In C57BL/6 decreased occlusal loading caused a significant decrease in trabecular number in female mice (Fig. 4d) and an increase in trabecular spacing in both sexes (Fig. 4e).

Discussion

In this study, we found that there were sex differences in the mandibular condylar cartilage in response to decreased occlusal loading. In the mandibular condylar cartilage, we found that decreased occlusal loading for 4 weeks caused a significant decrease in gene expression of early (*Sox9*, *Col2*) chondrocyte maturation markers compared with the normal loading group in female, but not in male, mice. The reasons behind the sex differences are unknown; however, one possible explanation is that increased levels of estrogen in female mice cause inhibition of mandibular condylar cartilage maturation and, therefore, affect the response of the condylar cartilage to mechanical loading. In support, a number of studies have shown that estrogen inhibits the growth of the mandibular condyle. For example, addition of

estrogen to rat mandibular condylar organ cultures causes a decrease in the condylar cartilage thickness and proliferation [26], and ovariectomy causes an increase in the thickness of the mandibular condylar cartilage [27–30] that is reversed by the administration of exogenous estrogen [29].

In C57BL/6 mice, decreased occlusal loading caused a significant decrease in proliferation in only the female mice compared with normal loading controls, while in CD-1 mice, decreased occlusal loading caused no effect on proliferation in both sexes. The mandibular condylar cartilage can be organized into four zones: articular, polymorphic, flattened, and hypertrophic. Cells in the polymorphic zone do not express *Col2*, are actively proliferating, and exit the proliferative pool to mature into *Col2*-expressing chondrocytes in the flattened zone [31]. Therefore, decreased occlusal loading-induced inhibition of chondrocyte maturation may be due to inhibition of cells exiting the proliferative pool in female CD-1 mice, while in female C57BL/6 mice it may be due to decrease in total cell numbers of the polymorphic zone.

Unlike in the mandibular cartilage, we found that decreased occlusal loading in both male and female mice caused similar effects in the subchondral bone. In both strains and sexes we found that decreased occlusal loading for 4 weeks caused a significant loss of bone volume and total volume. In CD-1 mice the decrease in bone volume caused by decreased occlusal loading seemed to be dependent on decreased trabecular thickness in both males and females ($P = 0.13$; and in our previous study, where we had a larger sample size, $n = 10$ [16], it was significant), whereas in C57BL/6 mice the decrease appeared to be due to a decrease in trabecular number and increased spacing. Taken together, the results suggest that there were strain differences in the response to decreased occlusal loading. Differences in the micro-CT parameters of the trabecular bone in response to PGE₂ have also been reported for C57BL/6 compared with CD-1 mice [32].

In bone, estrogen has been shown to cause inhibition of periosteal bone formation [33–35] and mechanical loading–induced periosteal bone formation [36–39] and no effect on endosteal bone formation. Since the mandibular condylar cartilage is believed to be derived from periosteum [19], this might explain why we found greater inhibition of mandibular condylar cartilage maturation in female mice and similar subchondral bone volume inhibition in male and female mice in response to decreased occlusal loading.

In conclusion, in this study we found that males and female mice respond to decreased occlusal loading in a sex-specific manner. In the mandibular condylar cartilage, decreased occlusal loading causes decreased early chondrocyte maturation exclusively in female, but not in male, CD-1 or C57BL/6 mice. Future studies are planned to delineate the role of estrogen and its receptors in mediating these responses. Greater understanding of sex differences in the adaptive capacity of the TMJ to mechanical loading are crucial to clarify the etiology of the disease process and for the development of regenerative therapies.

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Fig. 1.

H&E staining of sagittal sections of the TMJ from 49-day-old male (**a, c, e, g**) and female (**b, d, f, h**) CD-1 and C57BL/6 mice exposed to normal (**a, b, e, f**) or decreased occlusal loading (incisor trimming and soft-diet administration) for 4 weeks (**c, d, g, h**). Condylar cartilage thickness measurements of CD-1 male and female mice (**i**) and C57BL/6 male and female mice (**j**). Bar 100 μm. *Significant difference between normal and decreased occlusal loading groups ($P < 0.05$)

Fig. 2.

Female (a, c) and male (b, d) CD-1 and C57BL/6 mice, 21 days old, were exposed to normal and decreased occlusal loading (incisor trimming and soft diet administration) for 4 weeks. Real-time PCR analysis was performed for *Pthrp*, *Sox9*, *Col2*, *Ihh*, and *Col10* gene expression from the mandibular condylar head. Points are the mean and SEM for $n = 6$.

*Significant difference between normal and decreased occlusal loading groups ($P < 0.05$)

Fig. 3.

Proliferation in 21-day-old male and female CD-1 and C57BL/6 mice exposed to normal or decreased occlusal loading (incisor trimming and soft-diet administration) for 4 weeks. For each mouse, three to five sections were analyzed. Mice were injected 3 hours prior to death with BrdU. The total number of BrdU-labeled cells was divided by the total number of cells to calculate the ratio of BrdU label for each mouse. Points are the means and SEM for $n = 6$ mice per group. *Significant differences between normal and decreased occlusal loading groups ($P < 0.05$)

Fig. 4.

Micro-CT analysis of subchondral bone from male and female 21-day-old CD-1 and C57BL/6 mice exposed to 4 weeks of normal loading or decreased occlusal loading. Analysis of total volume (**a**), bone volume (**b**), bone volume fraction (BVF) (**c**), trabecular number (**d**), trabecular spacing (**e**), and trabecular thickness (**f**). Points are the mean and SEM for $n = 6$. *Significant difference between normal and decreased occlusal loading groups ($P < 0.05$)