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Sexual dimorphism of estrogen-sensitized synoviocytes contributes to gender difference in temporomandibular joint osteoarthritis

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Abstract

Objectives: Temporomandibular joint osteoarthritis (TMJOA) is approximately twice as prevalent in women than in men. Synoviocytes are believed to play a critical role in joint inflammation. However, it is unknown whether synoviocytes from different genders possess sexual dimorphisms that contribute to female-predominant TMJOA.

Materials and Methods: Freund's complete adjuvant combined with monosodium iodoacetate was used to induce TMJOA in female and male rats. Histologic and radiographic features were used to evaluate TMJOA. The expression of CD68, MCP-1, iNOS, and IL-1β was detected by immunohistochemistry and real-time PCR. Primary fibroblast-like synoviocytes (FLSs) isolated from the synovial membrane of female and male rats were used for in vitro experiments.

Results: Female rats showed aggravated TMJOA features as compared to male rats. Increased expression of iNOS and IL-1β was detected in synovial membrane from female TMJOA rats as compared to male rats. Furthermore, greater amounts of CD68-positive macrophage infiltration and increased MCP-1 expression around the synovial membrane were detected in female TMJOA rats compared to males. Primary cultured FLSs from female rats showed higher sensitivity to TNF-α treatment and recruited increased macrophage migration than male FLSs. More important, ovariectomy (OVX) by ablation in female rats repressed the sensitivity of female FLSs to TNF-α treatment due to the loss of estrogen production. Blockage of the estrogen receptor repressed estrogen-potentiated TNF-α-induced pro-inflammatory cytokine expression in OVX-FLSs. Moreover, the injection of estrogen receptor antagonists relieved the cartilage destruction and bone deterioration of TMJOA in female rats.

Conclusion: Estrogen-sensitized synoviocytes in female rats may contribute to gender differences in the incidence and progression of TMJOA.

KEYWORDS

estrogen, sexual dimorphism, synovitis, Temporomandibular joint osteoarthritis

1 | INTRODUCTION

Temporomandibular joint osteoarthritis (TMJOA), as an important subtype of temporomandibular disorders (TMDs), is characterized by synovial inflammation, cartilage degradation, subchondral bone remodeling, and chronic pain (Dworkin, & LeResche, 1992; Israel, Diamond, Saed-Nejad, & Ratcliffe, 1998; Stegenga, de Bont, & Boering, 1989; Zarb, & Carlsson, 1999). The TMJ is a unique organ in the human body, and TMJOA severely affects the quality of life of patients. TMJOA is approximately twice as prevalent (and more severe) in women than in men (Carlsson, 1999; Israel et al., 1998; Stegenga et al., 1989; Zhao, & Ma, 2006). It is not surprising that previous studies have tried to explain the sex difference in TMJOA prevalence by focusing on sex hormones, such as estrogen, which has been considered to play an important role in the symptomatology of female-predominant TMDs, synovitis, chondrocyte apoptosis, and inflammatory pain (Flake, Hermanstyne, & Gold, 2006; Guan, Kerins, Bellinger, & Kramer, 2005; Kou et al., 2011, 2014, 2015; Wang et al., 2013; Wu et al., 2010). The TMJ synovial membrane consists of a condensed one- to three-cell-thick lining layer of synoviocytes that overlies the loose connective tissue of the synovial sublining (Nozawa-Inoue et al., 2003). Synoviocytes producing inflammatory factors are believed to play a critical role in the process of joint inflammation. However, it is unknown whether the synoviocytes from different genders possess sexual dimorphism that can account for female-predominated TMJOA.

Sex differences exist in many other diseases, such as cardiovascular disease (Dunlay, & Roger, 2012), chronic musculoskeletal pain (Rollman, & Lautenbacher, 2001), and allergic disorders (Osman, Hansell, Simpson, Hollowell, & Helms, 2007). A previous study showed that rat microvascular endothelial cells exhibit gender differences in the mRNA expression of phosphodiesterase (PDE) 1 and PDE3 (Kikuiri et al., 2010). Female human umbilical vein endothelial cells (HUVECs) express a higher level of stress and immune response-related genes compared to male HUVECs (Lorenz et al., 2015). These findings indicate that cellular sexual dimorphism may contribute to the gender disparity observed in these sex-specific diseases. Dorsal root ganglion neurons derived from female rats show a markedly more hyperpolarized resting membrane potential than male neurons (Hendrich et al., 2012), which indicates a different characteristic in female and male nociceptors. Primary mast cells (MCs) are immune cells that play a critical role in many allergic and inflammatory diseases, and when they are derived from female rats and mice, they possess more intracellular mediator synthesis and granule storage than male MCs (Mackey et al., 2016). Inspired by previous studies, we assumed that female and male individuals may yield a different response in the pathological process of TMJOA. In addition, female and male TMJ-derived synoviocytes may possess different sensitivities to inflammatory stimuli, which may be one of the reasons for the epidemiological differences in TMJOA.

In this study, we showed that a similar gender disparity in TMJOA was observed in rats as in human patients. Synoviocytes from female rats exhibited higher sensitivity to inflammatory stimulus and recruited more macrophages than male counterparts. Loss of estrogen by ablation of the ovaries in female rats repressed the sensitivity of female fibroblast-like synoviocytes (FLSs) and attenuated TMJOA in female rats, and these finding may be used to prevent TMJOA in females.

2 | MATERIALS AND METHODS

2.1 | Animals

Adult female and male Sprague-Dawley (SD) rats (180–200 g for establishing the TMJOA model, 8-weeks old for FLS primary culture) purchased from Vital River Laboratory (Beijing Vital River Laboratory Animal Technology Co., Ltd., China) were used in this study. The animal experimental protocols were approved by the Peking University Animal Ethics Committee prior to the initiation of the study in the year of 2014 (approval number: LA2014221). Rats were housed on a 12-hr light/dark cycle, under controlled temperature (22 ± 1°C) and had free access to food and water.

2.2 | Induction of TMJOA and treatment of TMJOA

The rats were randomly divided into four groups with five rats in each group: male control, female control, male TMJOA, and female TMJOA groups. Complete Freund's adjuvant (CFA; Sigma) and 0.5 mg monosodium iodoacetate (MIA; Sigma) were dissolved 1:1 in 50 μl total volume and injected into the upper compartment of bilateral TMJs of rats in the TMJOA groups to create a reliable TMJOA model. The male control group and female control group received saline injections in the same volume. Two weeks later, all rats were euthanized by pentobarbital overdose. Estrogen antagonist ICI 182,780 (5 mg/ml, Sigma) and tamoxifen (0.5 μg/ml, Sigma) were used for treatment 7 days after induction of TMJOA.

2.3 | Histology and scoring of TMJOA

Tissue was harvested as described previously (Wang, Kou, Mao, Gan, & Zhou, 2012). In brief, the TMJs were removed en bloc and fixed in 4% paraformaldehyde, followed by decalcification with 10% EDTA (pH 7.4) and paraffin embedding. Paraffin sections were stained with (5 μm) hematoxylin–eosin (HE) and toluidine blue (TB) for histologic evaluation. The scoring of synovial inflammation, cartilage destruction, and subchondral bone remodeling was performed according to the histopathologic criteria in Supporting information Table S1, which were adapted and modified from previous studies (Kou et al., 2011; Wang et al., 2013; Wang, Kou, He et al., 2012). Higher scores represent more severe synovial inflammation, cartilage destruction, and subchondral bone remodeling. For any group, the total scores were averaged by the number of examined joints and are presented as the mean ± *SD*.

2.4 | Micro-CT examination

The TMJs in each group were dissected. Radiographs of the condyles were obtained with a high-resolution Micro-CT System (Inveon, Siemens, Germany) at 80 kV, 500 μA, and 8.89 μm effective pixel

size. The images were analyzed with software provided by the manufacturer. Sagittal images were captured with the same parameters: C_t = 2,500, W = 4,500. Transverse images were captured with the same parameters: C_t = 1,700, W = 6,000. The bone volume fraction (bone volume/total volume) was measured with the same software.

2.5 | Immunohistochemistry

The paraffin-embedded sections were blocked with 5% bovine serum albumin (BSA) and incubated with the primary antibodies against rat CD68 (1:500, MCA341GA, Serotec), interleukin (IL)-1β (1:100, ab2015, Abcam), inducible nitric oxide synthase (iNOS) (1:100, ab15323, Abcam), and monocyte chemoattractant protein-1 (MCP-1) (1:2,000, ab7202, Abcam), at 4°C overnight. After extensive washing with phosphate-buffered saline (PBS), the sections were incubated with HRP-conjugated secondary antibodies and visualized using diaminobenzidine (DAB) (Zhongshan Golden Bridge Biotechnology, Beijing, China).

2.6 | Cell culture and treatments

Fibroblast-like synoviocytes (FLSs) were isolated from the synovial membrane of TMJs from male and female rats as previously described (Nagai et al., 2006) and used for experiments in passages 2 or 3. Eight-week-old bilaterally ovariectomized rats were used for OVX-FLSs isolation. FLSs were treated with 20 ng/ml TNF-α (T 5944; Sigma) for 6 hr. Primary antibodies against rat CD68 and vimentin (1:100, bs-8533R, Bioss) were used for FLS identification (Supporting information Figure S1). Estrogen receptor antagonist ICI 182,780 (100 μM, Sigma) was added to the media 0.5 hr before treatment with 17β-estradiol (10^{-8} M, Huameihuli Biochemical).

2.7 | Enzyme-linked immunosorbent assay (ELISA)

The supernatants of cultured rat FLSs were collected, and the concentration of MCP-1 was quantified with an ELISA kit (BMS631INST, eBioscience, Vienna, Austria) according to the manufacturer's instructions.

2.8 | Western blotting

Cells were washed with PBS and lysed using a protein extraction kit (RIPA Cocktail, Thermo). Equal protein quantities were separated by SDS-PAGE and transferred onto a polyvinylidene difluoride membrane. The membranes were blocked with 5% nonfat milk and 0.1% Tween-20 for 1 hr, followed by incubation with MCP-1 and β-actin (1:2,000, sc-47,778, Santa Cruz Biotechnology) antibody overnight at 4°C. The blots were developed using a HRP-conjugated secondary antibody and enhanced chemiluminescence detection.

2.9 | Quantitative real-time PCR

Total RNA was extracted from the synovial membrane or cells with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. RNA samples were reverse-transcribed using a Reverse Transcription system (Bio-Rad). The primers used were as follows: rat β-actin sense/antisense, 5′-TGACAGGATGCAGAAGGAGA-3′/5′-TAGAGCCACCAATCCACACA-3′; rat IL-1β sense/antisense, 5′-CACCTCTCAAGCAGAGCACAG-3′/5′-GGGTTCCATGGTGAAGTCAAC-3′; rat iNOS sense/antisense, 5′-GAGTGAGGAGCAGGTTGAGG-3′/5′-CCAAGGTGTTGCCCTTTTT-3′; rat MCP-1 sense/antisense, 5′-GGACTTCAGCACCTTTGAATGTG-3′/5′-CTTGAGGTGGTTGTGGAAAAGAG-3′. The efficiency of the primers was confirmed by sequencing. Reverse transcription PCR was conducted with an cDNA synthesis kit (Invitrogen, USA) in 20 μl reaction volume containing 1.5 μg of total RNA. SYBR Green (Roche, USA) was used for real-time PCR detection using a 7,500 real-time PCR System (Applied Biosystems). The mRNA level of the target gene was acquired from the value of threshold cycle (C_t) as a relative level to that of β-actin through the formula $2^{-\Delta Ct}$ (ΔC_t = β-actin C_t−target gene C_t).

2.10 | FLSs and NR8383 transwell coculture system

A transwell system for 6-well plates was used for coculture experiments. FLSs from male and female rats were seeded into the lower chamber at 2×10^5 cells/well. After the FLSs were treated with or without 20 ng/ml TNF-α for 6 hr, NR8383 (a rat macrophage cell line) cells were loaded into each upper chamber at 1×10^6 cells/well. Cells were cocultured for 24 hr. Those cells remaining in the upper surface of the transwell chambers were removed by cotton swabs before staining with crystal violet. Cells stained by crystal violet on the bottom surface served as the migrated cells. The number of those migrated cells in three visual fields at 10× magnification in each chamber was counted (n = 3).

2.11 | Statistical analysis

Statistical analysis was performed with SPSS software, version 13.0 for Windows. All data were presented as means ± *SD*. Differences between the experimental and control groups, or female and male groups were analyzed by Student *t* tests, and comparisons between more than two groups were analyzed using one-way ANOVA with the Bonferroni adjustment. p values <0.05 were considered statistically significant.

3 | RESULTS

3.1 | Female synovial membrane produces greater amounts of pro-inflammatory factors and shows enhanced synovitis as compared to male synovial membrane during TMJOA

Synoviocyte-producing inflammatory factors are believed to play a pivotal role in joint inflammation including OA. We first tested whether the synovial inflammation in females was severe as compared to male rats using histology examination. The synovial membrane of TMJ in control female and male rats, which was

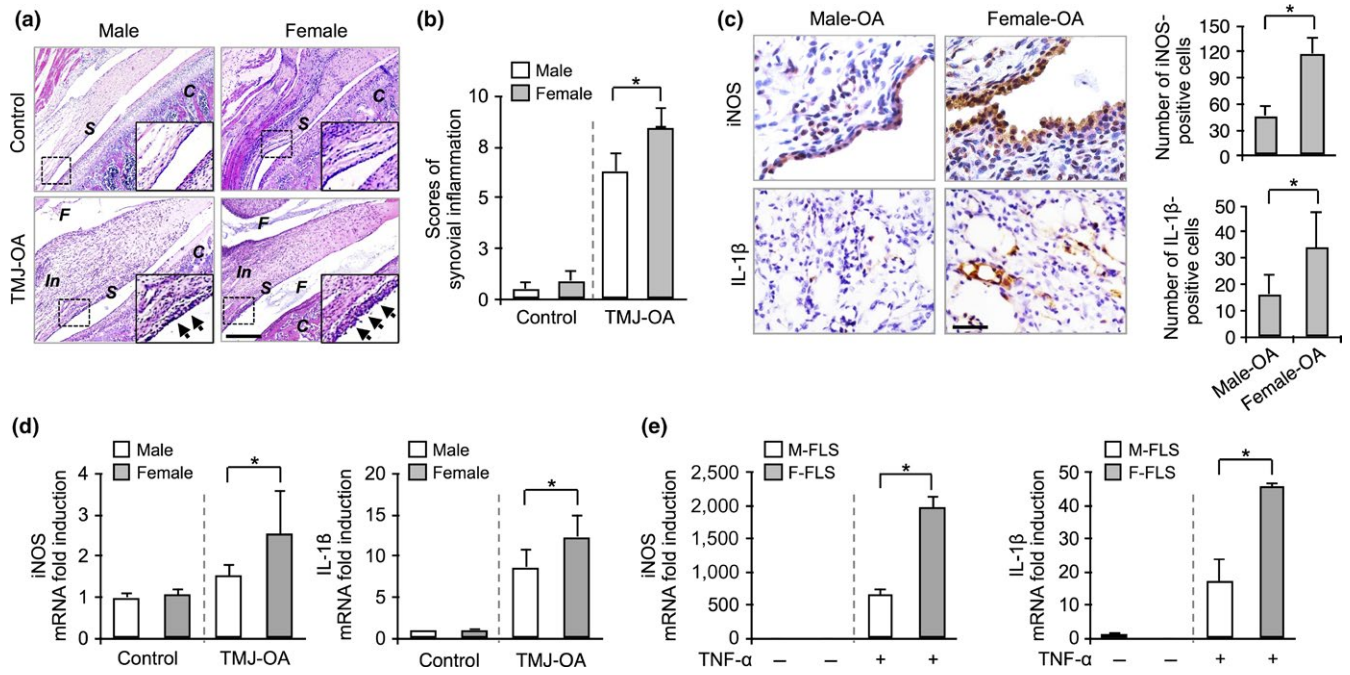

FIGURE 1 Female synovial membrane produces greater amounts of pro-inflammatory factors and shows enhanced synovitis than male synovial membrane during TMJOA. (a) Representative images of the TMJ synovial membrane in female and male rats for HE staining. Following induction of CFA + MIA for 2 weeks, both female and male TMJOA groups showed typical features of synovitis. Small and large frames indicate the original and the magnified areas. Compared with the male TMJOA group, the proliferation of the synovial membrane was more evident in the female TMJOA group (arrowhead), with a thicker synovial lining layer and an increased infiltration of mononuclear cells around the synovial membrane (bar = 200 μm). (b) Scoring of TMJOA synovitis. (c) Representative photomicrographs of iNOS and IL-1β immunostaining of sections of TMJ synovial membrane in female and male TMJOA groups. iNOS and IL-1β were strongly observed in female and male TMJOA groups, and more positive staining was detected in the female TMJOA group than in the male group (bar = 20 μm). (d) The mRNA expression of iNOS and IL-1β in female and male TMJOA synovial membrane. Increased production of iNOS and IL-1β was detected in female TMJOA synovitis compared to the male one. (e) The mRNA expression of iNOS and IL-1β in primary cultured fibroblast-like synoviocytes (FLSs) isolated from females and males. The TNF-α-induced increase in iNOS and IL-1β was greater in female FLSs than in male ones. $^*p < 0.05$, $n \geq 3$

characterized by a one- to three-cell-thick lining layer overlying the loose connective tissue of the synovial sublining, showed no difference (Figure 1a). Next, we found that the features of synovitis, including proliferation of synoviocytes, fibrin-like exudate in the articular space, and infiltration of mononuclear cells around the synovial membrane, were detected in both female and male TMJOA groups but not in the control groups (Figure 1a). More important, female rats showed more severe synovitis, especially with the hyperplastic synovial lining, than the male rats in the TMJOA groups (Figure 1a,b). Immunohistochemistry staining showed that greater amounts of iNOS and IL-1β staining were detected in the proliferated synovial lining cells and the infiltrated inflammatory cells in the female rats compared with the male rats (Figure 1c). Next, we used real-time PCR to show that there was no difference in the mRNA expression of iNOS and IL-1β in the synovial membrane from control male and female rats, but mRNA expression of iNOS and IL-1β was increased in the female synovial membrane compared with the male group after induction of TMJOA (Figure 1d). To further confirm the enhanced inflammatory features of the synoviocytes in the female rats, FLSs from the TMJ synovial membrane were isolated and cultured. Consistent with the result obtained using synovial tissue, we found that there was no difference in the mRNA expression of iNOS and IL-1β in FLSs from control male and female rats without treatment of TNF-α (Figure 1e). However, the mRNA expression of iNOS and IL-1β was significantly increased in female FLSs treated with TNF-α compared with that of the male FLSs (Figure 1e). These results suggest that female synovial cells can produce a greater amount of pro-inflammatory factors in response to inflammatory stimulus compared with male synovial cells.

3.2 | Enhanced cartilage degradation and bone deterioration in female TMJOA rats

Cartilage degradation and bone remodeling are important characteristics of OA. We next tested whether female rats also exhibit a more severe cartilage and bone phenotype using toluidine blue and H&E staining and a previously reported histopathologic scoring system (Supporting information Table S1) (Wang et al., 2013; Wang, Kou, He et al., 2012). The condylar cartilage from the control rats was a regular and smooth alignment of multilayer

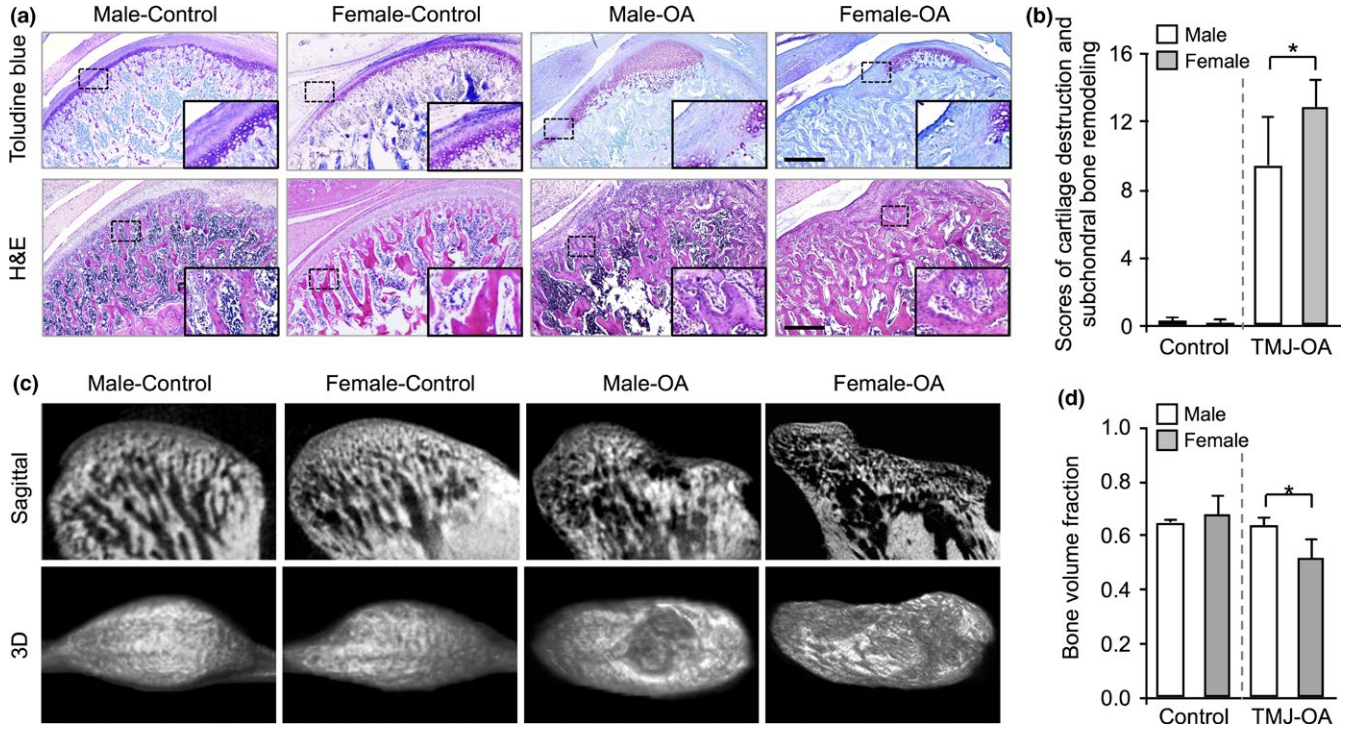

FIGURE 2 Enhanced cartilage degradation and bone deterioration in female TMJOA rats. (a) Representative images of the TMJ region in female and male rats for HE and TB staining. Both female and male TMJOA groups showed typical features of cartilage destruction and bone deterioration of TMJOA. Compared with the male TMJOA group, the extent of cartilage destruction was larger in the female TMJOA group, with a strongly irregular arrangement of chondrocytes. In the female TMJOA group, subchondral bone sclerosis was evident, with complete loss of chondrocytes (bar = 400 μm). (b) Scores of cartilage destruction and bone deterioration. (c) Representative images of the corresponding condyles by micro-CT scanning in a sagittal section view and a 3D reconstructed view. The control condyle showed intact subchondral bone with a smooth surface. The condyle in the female and male TMJOA groups showed regional loss of surface bone and multiple erosions of the subchondral bone, with a thicker trabecular bone compared with controls. The bone defects in the female TMJOA group were larger than those in the male TMJOA group. (d) Bone volume fraction in each group. $^*p < 0.05$, $n \geq 3$

chondrocytes, which showed no differences between the male and female groups (Figure 2a,b). As expected, significant destruction of cartilage and bone was detected in the TMJOA groups, as characterized by decreased and discontinued multilayer chondrocytes, and subchondral bone defect (Figure 2a). Moreover, the female rats showed more aggravated cartilage destruction and subchondral bone deterioration than male rats, which was presented as the extensive loss of chondrocytes as determined via staining and subchondral bone sclerosis (Figure 2a,b). We next performed micro-CT scanning to further confirm the bone destruction, which showed that the condyle surface of the control groups was smooth and continuous, whereas the anterior and central areas of the condyles were discontinuous in the female and male TMJOA groups (Figure 2c). Multi-erosions, characterized by translucency disrupting the bone surface of the load-bearing areas, grew deeper and more extensive with obvious defects in the female TMJOA group (Figure 2c). In addition, we found that the condyles in the female TMJOA group had thicker trabecula and more active subchondral bone remodeling, as assessed by bone volume/total volume (Figure 2d). Above all, TMJOA in female and male rats showed great sexual dimorphism.

3.3 | The female synovial membrane shows more active macrophage infiltration and produces a greater amount of MCP-1 than the male synovial membrane during TMJOA

As a large amount of infiltrated monocytes was found around the synovial membrane in TMJOA rats (Figure 1a), and macrophages play a major role in joint inflammation and inflammatory-mediated tissue damage and bone loss (De Rycke et al., 2005; Mosser, & Edwards, 2008; Tak, & Bresnihan, 2000), we speculated that infiltration of macrophages might be involved in the sexual dimorphism associated with TMJOA. Using immunohistochemistry staining of CD68, a specific marker of macrophage, we found that a greater amount of CD68-positive monocytes was observed in the synovial membrane and underlying connective tissue of the female inflamed TMJs as compared to males (Figure 3a,b), which indicated more macrophage activity in the female TMJOA group.

Next, we investigated the underlying mechanism by which female rats recruit greater amount of macrophages. MCP-1, a member of the C-C motif chemokine family and a major chemoattractant cytokine for recruiting monocytes to sites of inflammation (Charo, & Ransohoff, 2006), was detected to confirm the phenomenon of macrophage

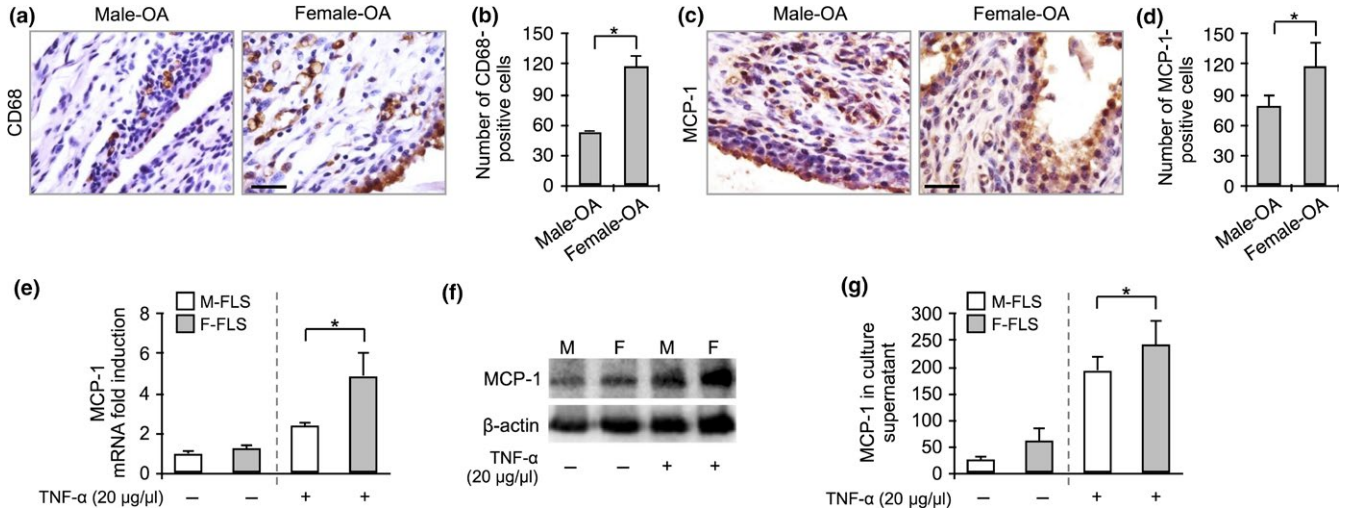

FIGURE 3 The female synovial membrane shows more active macrophage infiltration and produces a greater amount of MCP-1 than the male synovial membrane during TMJOA. (a, c) Representative photomicrographs of CD68 and MCP-1 immunostaining of sections of TMJs in the TMJOA groups. More CD68- and MCP-1-positive staining was detected in the female TMJOA group than in the male group (bar = 20 μm). (b, d) The number of CD68- and MCP-1-positive cells was larger in the female TMJOA group than that in the male group. MCP-1 secretion induced by TNF-α in primary cultured female and male fibroblast-like synoviocytes (FLSs) shown in (e) real-time PCR, (f) western blotting and (g) ELISA. MCP-1 production was significantly upregulated after TNF-α induction for 6 hr. The TNF-α-induced increase in MCP-1 was greater in the female group than that in the male group. $^*p < 0.05$, $n \geq 3$

infiltration. We found that enhanced MCP-1 staining was detected in the proliferated synoviocytes and around the synovial intimal lining of inflamed female TMJs compared with that of the male group (Figure 3c,d). This result reinforced the data supporting the concept that there are different macrophage immune responses between female and male TMJOA. To further confirm that synoviocytes contribute to MCP-1 secretion in the inflamed TMJs, primary cultured FLSs were treated with or without TNF-α in vitro. We found that no significant difference in MCP-1 expression and release was detected in FLSs from male and female rats without TNF-α treatment (Figure 3e-g). After treatment of TNF-α, the FLSs derived from female rats produced and released greater amount of MCP-1 than in males, as assessed by real-time PCR, western blot, and ELISA (Figure 3e-g). These results indicate that female and male synoviocytes may have different sensitivity to inflammatory stimulus, which may contribute to the different macrophage-related immune response in the process of TMJOA.

3.4 | Female primary cultured FLSs recruit greater amount of macrophages than male FLSs

We next used a coculture system of FLSs and a macrophage cell line to further confirm whether female FLSs are capable of recruiting greater amounts of macrophages. We found that without TNF-α treatment, no difference in NR8383 macrophage migration was detected after coculturing with male and female FLSs (Figure 4a,b). TNF-α treatment increased the number of migrated NR8383 macrophages compared with the control groups in a time-dependent manner, as assessed by flow cytometry (Supporting information Figure S2). Moreover, female FLSs recruited an increased number of NR8383 macrophage migrations after treatment with TNF-α compared with the male group (Figure 4a,b). These results indicate that female synoviocytes are more sensitive to inflammatory stimulus-induced MCP-1 secretion, which might result in the increased capacity of macrophage recruiting.

3.5 | Estrogen contributes to the sensitized inflammatory response of female FLSs

Female rats have higher levels of plasma estrogen than male rats, and previous studies showed that estrogen aggravates TMJ inflammation and TMJOA (Kou et al., 2011, 2015; Wang, Kou, He et al., 2012). We hypothesized that estrogen might contribute to the enhanced sensitivity of female FLSs to response to inflammatory stimulus. Because the plasma level of estrogen is decreased significantly in OVX rats (Kou et al., 2011; Wang, Kou, He et al., 2012), we isolated FLSs from control female rats and ovariectomized female rats to address this question. We found that there was no difference in the mRNA expression of iNOS, IL-1β, and MCP-1 in FLSs from control and OVX female rats. However, TNF-α was observed to induce increases in iNOS, IL-1β, and MCP-1 mRNA expression in female FLSs, but these were significantly inhibited in OVX-FLSs (Figure 5a). These data suggest that OVX-caused hormone change in vivo might contribute to the sensitized response of female FLSs to TNF-α in vitro.

To further confirm whether estrogen contributes to the sexual dimorphism of inflammatory response in FLSs, we used estrogen and ICI 182,780 (estrogen receptor antagonist) to treat the FLSs from OVX rats. We found that estrogen-potentiated TNF-α-induced mRNA expression of iNOS, IL-1β, and MCP-1 in FLSs from OVX rats, and ICI 182,780 treatment partially blocked the TNF-α-induced increase in the expression of these pro-inflammatory cytokines (Figure 5b).

3.6 | Blockage of estrogen by estrogen receptor (ER) antagonist injection attenuates cartilage destruction and bone deterioration in TMJOA rats

We next determined whether ER antagonist (ICI 182,780 and tamoxifen) treatment would be efficacious for TMJOA of female rats. We found that ICI 182,780 and tamoxifen injection significantly repressed the cartilage destruction and bone deterioration in the TMJOA rats, which presented as restricted loss of chondrocytes with an intact calcification chondrocyte layer and relieved subchondral bone sclerosis compared with the TMJOA rats without treatment (Figure 6a,b). In addition, micro-CT analysis also showed that ICI 182,780 and tamoxifen injection significantly attenuated the bone defect of the condyle surface and the multi-erosions of the subchondral bone in the female TMJOA group (Figure 6c,d). A histomorphometric analysis indicated that osteoclast numbers and RANKL expression around subchondral bone in the tamoxifen treated rats were significantly reduced when compared to the control groups

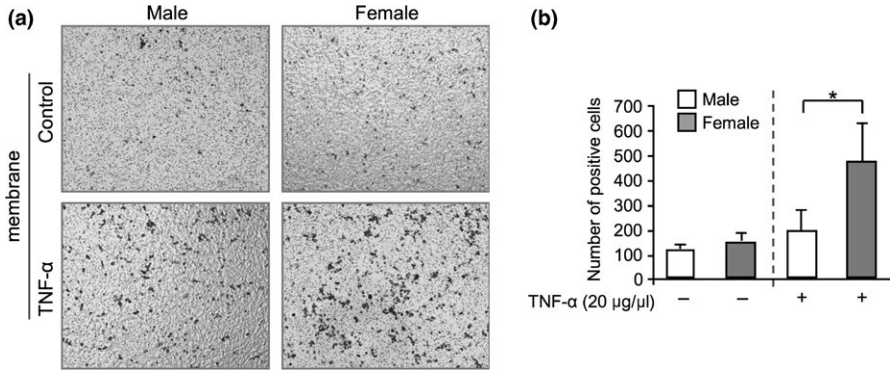

FIGURE 4 Female primary cultured fibroblast-like synoviocytes (FLSs) recruit a larger amount of macrophages than male FLSs. (a) Representative images of crystal violet staining of NR8383, a rat macrophage cell line. The number of migrated NR8383 cells increased after treatment with TNF-α compared to the control groups, whereas the migration of NR8383 in the female TNF-α group was greater than that in the male TNF-α group. (b) The amount of crystal violet-stained cells in each group. The number of migrated cells in the female TNF-α group was larger than that in the female TNF-α group. *$p < 0.05$, $n \geq 3$

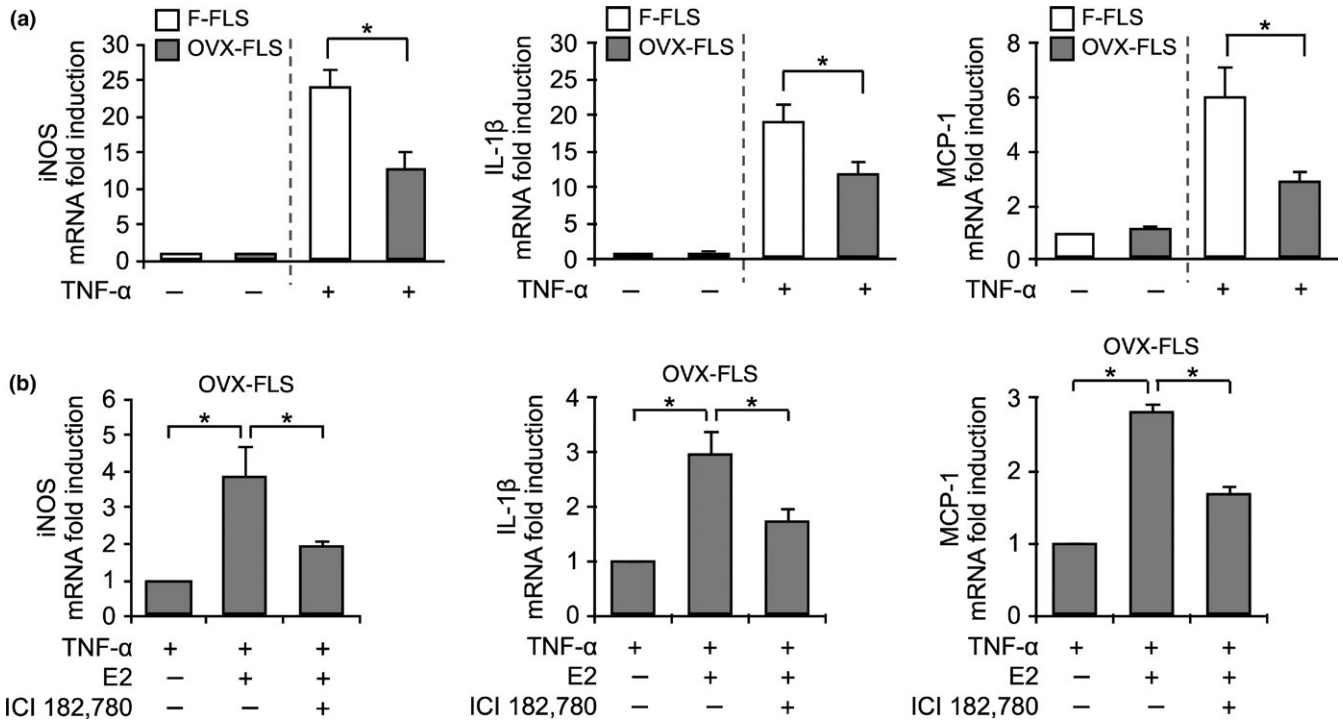

FIGURE 5 Estrogen contributes to the sensitized inflammatory response of female fibroblast-like synoviocytes (FLSs). (a) The mRNA expression of iNOS, IL-1β, and MCP-1 in female and OXV-female FLSs. The TNF-α-induced increase in iNOS, IL-1β, and MCP-1 mRNA expression in female FLSs was significantly inhibited in OVX-FLSs. (b) The mRNA expression of iNOS, IL-1β, and MCP-1 in OXV-female FLSs after induction of TNF-α, estrogen, and ICI 182,780. Estrogen-potentiated TNF-α induced mRNA expression of iNOS, IL-1β, and MCP-1 in FLSs from OVX rats, and ICI 182,780 treatment partially blocked the TNF-α-induced increase in the expression of these pro-inflammatory cytokines. *$p < 0.05$, $n \geq 3$

(Supporting information Figure S3), as assessed by RANKL immunohistochemical and tartrate-resistant acid phosphatase (TRAP) staining, respectively. In an interesting manner, we found that no difference of the ERα and ERβ expression was detected in female and male synoviocytes (Supporting information Figure S4), as assessed by real-time PCR and western blot.

4 | DISCUSSION

As a unique organ in the human body, the TMJ is involved in multiple actions such as chewing, speaking, and swallowing, and even expression of emotions. TMJOA can severely affect the quality of life of patients. TMJOA is known to epidemiologically exhibit a significant sex difference. In addition to the sex hormone factors, the different cellular characteristics between female and male could contribute to TMJOA gender differences. Here, we showed that experimental TMJOA in rats presented remarkable sex difference in synovial inflammation, cartilage destruction, and subchondral bone deterioration. Synoviocytes from female rats possessed higher sensitivity to inflammatory stimulus and recruited a greater amount of migrated macrophages than that of male counterparts. Loss of estrogen in female rats by OVX repressed the sensitivity of female FLSs, and injection of ER antagonist attenuated TMJOA in female rats.

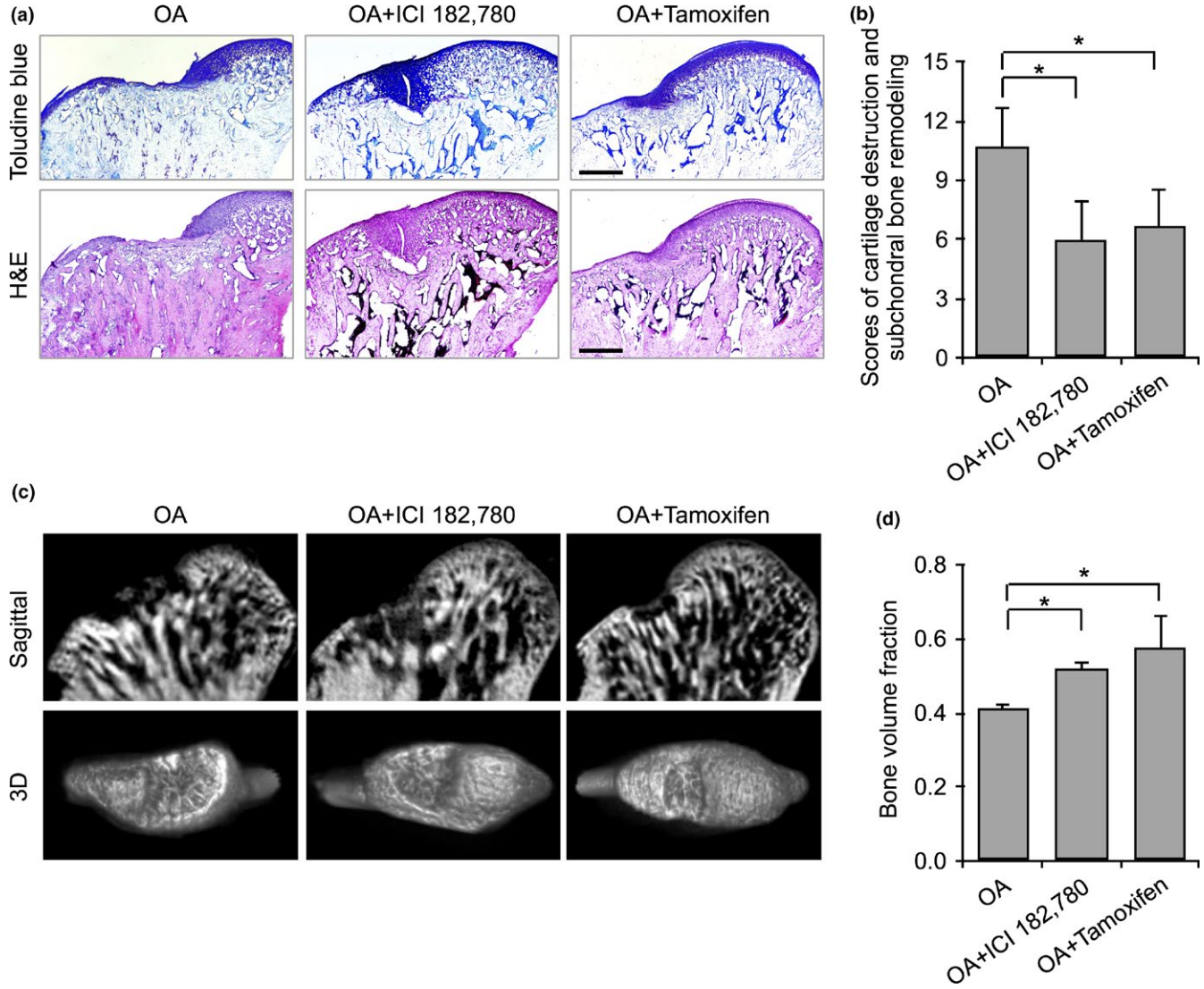

FIGURE 6 Blockage of estrogen by ER antagonist injection attenuates cartilage destruction and bone deterioration in TMJOA rats. (a) Representative images of the condyles in the TMJOA and estrogen antagonist treatment groups for HE and TB staining. ICI 182,780 and tamoxifen injection attenuated the cartilage destruction and bone deterioration in the TMJOA rats, presenting as restricted loss of chondrocytes with an intact calcification chondrocyte layer and relieved subchondral bone sclerosis compared with the TMJOA rats without treatment (bar = 400 μm). (b) Scores of the cartilage destruction and bone deterioration. (c) Representative micro-CT images of the corresponding condyles in a sagittal section view and a 3D reconstructed view. ICI 182,780 and tamoxifen injection significantly repressed the bone defect of the condyle surface and the multi-erosions of the subchondral bone in the female TMJOA group. (d) Bone volume fraction in each group. $^{*}p < 0.05$, $n \geq 3$

These findings will help us to understand the sexual dimorphism observed in TMJOA. Furthermore, our study provides new data to explain the high female to male preponderance observed in clinical practice.

More women with TMJ problems seek treatment than men, usually with craniofacial pain as their chief clinical complaint. This indicates that women suffer from a more serious pathological condition of TMD than men. For TMJOA patients, the pain is generally and mainly caused by synovial inflammation. Synovial inflammation is also related to progressive cartilage and subchondral bone loss in the frontal bevel of the condyle as the main characteristics. In our study, we found that female rats showed more severe synovial inflammation, cartilage destruction, and subchondral bone deterioration in the TMJOA model than male rats. This result basically reflects the similar gender disparity of TMJOA that is observed in human patients.

Synoviocytes from female rats are more sensitive to inflammatory stimulus and produce greater amounts of pro-inflammatory factors. Local immune cells such as macrophages may contribute to the production of pro-inflammatory cytokines, including iNOS and IL-1β, in TMJ tissue (Bogdan, 2001; Tsutsui, Cai, & Hayashi, 2015). In our study, we observed that increased productions of iNOS and IL-1β were detected in the synoviocytes from female TMJOA rats compared to the same condition in male rats, same phenomenon was demonstrated in primary cultured FLSs. Although different gender-isolated FLSs responded differently to pro-inflammatory cytokines, female FLSs showed higher sensitivity to inflammatory stimulus. The gender disparity demonstrated in female and male FLSs may be related to the high serum level of E2 in female individuals but not in males. The specific microenvironment, in which estrogen is in high concentration, may affect the biological behavior of female synoviocytes.

Female rats showed more active macrophage infiltration than male rats in the synovial membrane of the inflamed TMJ. Macrophages, as one of the important immune cells in innate immunity, mediate inflammatory responses and contribute to tissue damage and bone loss (Dale, Boxer, & Liles, 2008; De Rycke et al., 2005; Mosser, & Edwards, 2008; Tak, & Bresnihan, 2000). In this study, we observed more extensive CD68-positive macrophage infiltration around the synovial membrane in female rats, accompanied by increased expression of MCP-1 in the synoviocytes. It is well known that MCP-1 activates the mononuclear phagocyte system under chronic inflammation and recruits monocytes to sites of inflammation (Charo, & Ransohoff, 2006). MCP-1 is detected in fluids and inflammatory synovial tissues of TMJOA patients (Ogura et al., 2010). The inflammation-mediated increase in MCP-1 secretion in TMD indicated that the mononuclear phagocyte system is involved in the pathological process of TMJOA. Our in vitro findings further confirmed that FLSs from female rats showed higher sensitivity to TNF-α treatment, which resulted in increased secretion of MCP-1, and recruited increased macrophage migration than male FLSs. These results indicate that the sexual difference of female and male synoviocytes may contribute to the enhanced macrophage infiltration in females and thus might lead to the severe cartilage destruction and bone loss observed in female TMJOA rats.

Our study demonstrated that TMJOA exhibits significant sexual dimorphism in vivo and in vitro. Previous studies reported that the TMJ is a target tissue of estrogen (Puri, Hutchins, Bellinger, & Kramer, 2009). In this study, we found that loss of estrogen in female rats repressed the sensitivity of female synoviocytes and attenuated TMJOA in female rats. The estrogen receptor subtypes estrogen receptor alpha (ERα) and beta (ERβ) were differentially expressed in condylar chondrocytes (Wang, Hayami, & Kapila, 2009). We found that although ER antagonist treatment can abolish estrogen-potentiated TNF-α-induced pro-inflammatory cytokine expression, there was no difference in the ERα and ERβ expression in female and male synoviocytes. These findings indicate that estrogen but not the ER may contribute to the sexual dimorphism of synoviocytes mediated by gender differences in TMJOA. Epigenetic processes may influence the behavior of cells during performing function without changing underlying DNA sequence. According to previous research, estrogen may enhance cellular memory formation by epigenetic processes, specifically histone alterations and DNA methylation (Frick, Zhao, & Fan, 2011). In this study, we found that OVX of female rats significantly abolished the sensitized inflammatory response of female FLSs, which indicate that estrogens play a critical role in enhancing sensitivity of female FLSs to response to inflammatory stimulus. It is reasonable to speculate that the estrogen-enhanced sensitization to inflammatory stimulus in FLSs might share the similar epigenetics processes as in cellular memory formation. However, more investigations and experiments should certainly be conducted to dig deeper in the underlying mechanism.

In conclusion, synoviocytes from females and males possess different biological characteristics that contribute to female-predominant TMJOA synovitis. Estrogen sensitization is involved in the sexual dimorphism of synoviocytes. Loss of estrogen repressed the sensitivity of female FLSs and attenuated TMJOA in female rats. Our findings provide new data that will assist with elucidating gender disparity in TMJOA and will help to explore a new approach for the treatment of TMJOA.

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CONFLICT OF INTERESTS

The authors declare no potential conflict of interests with respect to the authorship and/or publication of this article.

AUTHOR CONTRIBUTIONS

Xue Xin-Tong did most of the researches and analyzed the data. Zhang Ting helped with some of the in vitro researches. Cui Sheng-Jie, He Dan-Qing, Wang Xue-Dong, Yang Rui-Li, Liu Da-Wei and Liu Yan provided help on data analysis. Gan Ye-Hua helped to design the study. Kou Xiao-Xing designed the study and helped to analyze the data. Zhou Yan-Heng helped to design the study.

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REFERENCES

Bogdan, C. (2001). Nitric oxide and the immune response. *Nature Immunology*, *2*, 907–916. https://doi.org/10.1038/ni1001-907

Carlsson, G. E. (1999). Epidemiology and treatment need for temporomandibular disorders. *Journal of Orofacial Pain*, *13*, 232–237.

Charo, I. F., & Ransohoff, R. M. (2006). The many roles of chemokines and chemokine receptors in inflammation. *New England Journal of Medicine*, *354*, 610–621. https://doi.org/10.1056/NEJMra052723

Dale, D. C., Boxer, L., & Liles, W. C. (2008). The phagocytes: Neutrophils and monocytes. *Blood*, *112*, 935–945. https://doi.org/10.1182/blood-2007-12-077917

De Rycke, L., Baeten, D., Foell, D., Kruithof, E., Veys, E. M., Roth, J., & De Keyser, F. (2005). Differential expression and response to anti-TNFalpha treatment of infiltrating versus resident tissue macrophage subsets in autoimmune arthritis. *The Journal of Pathology*, *206*, 17–27. https://doi.org/10.1002/(ISSN)1096-9896

Dunlay, S. M., & Roger, V. L. (2012). Gender differences in the pathophysiology, clinical presentation, and outcomes of ischemic heart failure. *Current Heart Failure Reports*, *9*, 267–276. https://doi.org/10.1007/s11897-012-0107-7

Dworkin, S. F., & LeResche, L. (1992). Research diagnostic criteria for temporomandibular disorders: Review, criteria, examinations and specifications, critique. *Journal of Craniomandibular Disorders : Facial & Oral Pain*, *6*, 301–355.

Flake, N. M., Hermanstyne, T. O., & Gold, M. S. (2006). Testosterone and estrogen have opposing actions on inflammation-induced plasma extravasation in the rat temporomandibular joint. *American Journal of Physiology: Regulatory, Integrative and Comparative Physiology*, *291*, R343–R348.

Frick, K. M., Zhao, Z., & Fan, L. (2011). The epigenetics of estrogen: Epigenetic regulation of hormone-induced memory enhancement. *Epigenetics*, *6*, 675–680. https://doi.org/10.4161/epi.6.6.16177

Guan, G., Kerins, C. C., Bellinger, L. L., & Kramer, P. R. (2005). Estrogenic effect on swelling and monocytic receptor expression in an arthritic temporomandibular joint model. *Journal of Steroid Biochemistry and Molecular Biology*, *97*, 241–250. https://doi.org/10.1016/j.jsbmb.2005.05.013

Hendrich, J., Alvarez, P., Joseph, E. K., Ferrari, L. F., Chen, X., & Levine, J. D. (2012). In vivo and in vitro comparison of female and male nociceptors. *The Journal of Pain: Official Journal of The American Pain Society*, *13*, 1224–1231. https://doi.org/10.1016/j.jpain.2012.09.009

Israel, H. A., Diamond, B., Saed-Nejad, F., & Ratcliffe, A. (1998). Osteoarthritis and synovitis as major pathoses of the temporomandibular joint: Comparison of clinical diagnosis with arthroscopic morphology. *Journal of Oral and Maxillofacial Surgery*, *56*, 1023–1027; discussion 1028. https://doi.org/10.1016/S0278-2391(98)90246-4

Kikuiri, T., Kim, I., Yamaza, T., Akiyama, K., Zhang, Q., Li, Y., … Shi, S. (2010). Cell-based immunotherapy with mesenchymal stem cells cures bisphosphonate-related osteonecrosis of the jaw-like disease in mice. *Journal of Bone and Mineral Research*, *25*, 1668–1679. https://doi.org/10.1002/jbmr.37

Kou, X. X., Li, C. S., He, D. Q., Wang, X. D., Hao, T., Meng, Z., … Gan, Y. H. (2015). Estradiol promotes M1-like macrophage activation through cadherin-11 to aggravate temporomandibular joint inflammation in rats. *The Journal of Immunology*, *194*, 2810–2818. https://doi.org/10.4049/jimmunol.1303188

Kou, X. X., Wang, X. D., Li, C. S., Bi, R. Y., Meng, Z., Li, B., … Gan, Y. H. (2014). Estradiol-potentiated cadherin-11 in synovial membrane involves in temporomandibular joint inflammation in rats. *Journal of Steroid Biochemistry and Molecular Biology*, *143*, 444–450. https://doi.org/10.1016/j.jsbmb.2014.07.002

Kou, X. X., Wu, Y. W., Ding, Y., Hao, T., Bi, R. Y., Gan, Y. H., & Ma, X. (2011). 17beta-estradiol aggravates temporomandibular joint inflammation through the NF-kappaB pathway in ovariectomized rats. *Arthritis and Rheumatism*, *63*, 1888–1897. https://doi.org/10.1002/art.30334

Lorenz, M., Koschate, J., Kaufmann, K., Kreye, C., Mertens, M., Kuebler, W. M., … Stangl, V. (2015). Does cellular sex matter? Dimorphic transcriptional differences between female and male endothelial cells. *Atherosclerosis*, *240*, 61–72. https://doi.org/10.1016/j.atherosclerosis.2015.02.018

Mackey, E., Ayyadurai, S., Pohl, C. S., D' Costa, S., Li, Y., & Moeser, A. J. (2016). Sexual dimorphism in the mast cell transcriptome and the pathophysiological responses to immunological and psychological stress. *Biology of Sex Differences*, *7*, 60. https://doi.org/10.1186/s13293-016-0113-7

Mosser, D. M., & Edwards, J. P. (2008). Exploring the full spectrum of macrophage activation. *Nature Reviews Immunology*, *8*, 958–969. https://doi.org/10.1038/nri2448

Nagai, H., Miyamoto, Y., Nakata, A., Hatakeyama, S., Iwanami, Y., & Fukuda, M. (2006). Isolation and characterization of synovial cells from the human temporomandibular joint. *Journal of Oral Pathology and Medicine*, *35*, 104–110. https://doi.org/10.1111/j.1600-0714.2006.00369.x

Nozawa-Inoue, K., Amizuka, N., Ikeda, N., Suzuki, A., Kawano, Y., & Maeda, T. (2003). Synovial membrane in the temporomandibular joint–its morphology, function and development. *Archives of Histology and Cytology*, *66*, 289–306. https://doi.org/10.1679/aohc.66.289

Ogura, N., Satoh, K., Akutsu, M., Tobe, M., Kuyama, K., Kuboyama, N., … Kondoh, T. (2010). MCP-1 production in temporomandibular joint inflammation. *Journal of Dental Research*, *89*, 1117–1122. https://doi.org/10.1177/0022034510376041

Osman, M., Hansell, A. L., Simpson, C. R., Hollowell, J., & Helms, P. J. (2007). Gender-specific presentations for asthma, allergic rhinitis and eczema in primary care. *Primary Care Respiratory Journal*, *16*, 28–35. https://doi.org/10.3132/pcrj.2007.00006

Puri, J., Hutchins, B., Bellinger, L. L., & Kramer, P. R. (2009). Estrogen and inflammation modulate estrogen receptor alpha expression in specific tissues of the temporomandibular joint. *Reproductive Biology and Endocrinology*, *7*, 155. https://doi.org/10.1186/1477-7827-7-155

Rollman, G. B., & Lautenbacher, S. (2001). Sex differences in musculoskeletal pain. *Clinical Journal of Pain*, *17*, 20–24. https://doi.org/10.1097/00002508-200103000-00004

Stegenga, B., de Bont, L. G., & Boering, G. (1989). Osteoarthrosis as the cause of craniomandibular pain and dysfunction: A unifying concept. *Journal of Oral and Maxillofacial Surgery*, *47*, 249–256. https://doi.org/10.1016/0278-2391(89)90227-9

Tak, P. P., & Bresnihan, B. (2000). The pathogenesis and prevention of joint damage in rheumatoid arthritis: Advances from synovial biopsy and tissue analysis. *Arthritis and Rheumatism*, *43*, 2619–2633. https://doi.org/10.1002/1529-0131(200012)43:12<2619::AID-ANR1>3.0.CO;2-V

Tsutsui, H., Cai, X., & Hayashi, S. (2015). Interleukin-1 family cytokines in liver diseases. *Mediators of Inflammation*, *2015*, 630265.

Wang, W., Hayami, T., & Kapila, S. (2009). Female hormone receptors are differentially expressed in mouse fibrocartilages. *Osteoarthritis Cartilage*, *17*, 646–654. https://doi.org/10.1016/j.joca.2008.09.015

Wang, X. D., Kou, X. X., He, D. Q., Zeng, M. M., Meng, Z., Bi, R. Y., … Zhou, Y. H. (2012). Progression of cartilage degradation, bone resorption and pain in rat temporomandibular joint osteoarthritis induced by injection of iodoacetate. *PLoS One*, *7*, e45036. https://doi.org/10.1371/journal.pone.0045036

Wang, X. D., Kou, X. X., Mao, J. J., Gan, Y. H., & Zhou, Y. H. (2012). Sustained inflammation induces degeneration of the temporomandibular joint. *Journal of Dental Research*, *91*, 499–505. https://doi.org/10.1177/0022034512441946

Wang, X. D., Kou, X. X., Meng, Z., Bi, R. Y., Liu, Y., Zhang, J. N., … Gan, Y. H. (2013). Estrogen aggravates iodoacetate-induced temporomandibular joint osteoarthritis. *Journal of Dental Research*, *92*, 918–924. https://doi.org/10.1177/0022034513501323

Wu, Y. W., Bi, Y. P., Kou, X. X., Xu, W., Ma, L. Q., Wang, K. W., … Ma, X. C. (2010). 17-Beta-estradiol enhanced allodynia of inflammatory temporomandibular joint through upregulation of hippocampal TRPV1 in ovariectomized rats. *Journal of Neuroscience*, *30*, 8710–8719. https://doi.org/10.1523/JNEUROSCI.6323-09.2010

Zarb, G. A., & Carlsson, G. E. (1999). Temporomandibular disorders: Osteoarthritis. *Journal of Orofacial Pain*, *13*, 295–306.

Zhao, Y. P., & Ma, X. C. (2006). Temporomandibular disorders related pain interaction with age, sex and imaging changes of osteoarthrosis. *Zhonghua Kou Qiang Yi Xue Za Zhi*, *41*, 757–758.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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