Estrogen Aggravates Iodoacetate-induced Temporomandibular Joint Osteoarthritis

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X.D. Wang[1,2], X.X. Kou[1,2], Z. Meng[3], R.Y. Bi[3], Y. Liu[1], J.N. Zhang[1], Y.H. Zhou[1,2]*, and Y.H. Gan[3]*

[1]Department of Orthodontics, Peking University School and Hospital of Stomatology, Beijing, China; [2]Center for Craniofacial Stem Cell Research and Regeneration, Peking University School and Hospital of Stomatology, Beijing, China; and [3]Center for Temporomandibular Disorders and Orofacial Pain, Peking University School and Hospital of Stomatology, 22# Zhongguancun South Avenue, Haidian District, Beijing 100081, China; *corresponding authors, yanhengzhou@gmail.com and kqyehuagan@bjmu.edu.cn

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ABSTRACT

Temporomandibular joint osteoarthritis (TMJOA) is clinically characterized by female preponderance, with a female-to-male ratio of more than 2:1; however, the underlying mechanism remains obscure. We examined the effects of estrogen on TMJOA induced by monosodium iodoacetate. Female rats were randomly and equally divided into 5 groups: control, sham-ovariectomized, and ovariectomized rats treated, respectively, with 17β-estradiol (E2) at doses of 0 μg, 20 μg, and 80 μg/day until the end of the experiment. After induction of TMJOA, TMJs were evaluated by histopathology and microCT, and the expression of Fas, FasL, caspase 3, and caspase 8 was evaluated by real-time polymerase chain-reaction or immunohistochemistry. Another 5 groups of female rats were used to evaluate the effect of estrogen receptor antagonist ICI 182780 on E2 effects on TMJOA, when injected intraperitoneally into the control, sham-ovariectomized, and 80-μg-E2-treated groups. We found that E2 potentiated cartilage degradation and subchondral bone erosion in iodoacetate-induced TMJOA. E2 also potentiated mRNA expression of Fas, FasL, caspase 3, and caspase 8 in the condylar cartilage. Moreover, the estrogen receptor antagonist partially blocked E2 effects on TMJOA. These findings suggest that E2 could aggravate TMJOA, which may be an important mechanism underlying the sexual dimorphism of TMJOA.

KEY WORDS: cartilage, subchondral bone, sexual dimorphism, TMJ, estrogen receptor, apoptosis.

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INTRODUCTION

The temporomandibular joint (TMJ) is one of the most common sites affected by osteoarthritis (OA) (Kapila *et al*., 2009). Temporomandibular joint osteoarthritis (TMJOA), an important subtype of the classification of temporomandibular disorders (TMD) (Israel *et al*., 1998), is characterized by cartilage degradation, subchondral bone remodeling, and chronic pain (Stegenga, 2001). Evidence indicates that TMJOA shows a high female-to-male preponderance, nearly 2:1 (Israel *et al.*, 1998; Zhao, 2006), and primarily affects females after puberty and during the reproductive years (Warren and Fried, 2001; Nekora-Azak, 2004; Kapila *et al*., 2009), which differs from OA in the knee or other joints, especially in post-menopausal women (Roman-Blas *et al.*, 2009). However, the reasons for susceptibility to TMJOA in females remain unclear.

Sex hormones are critical factors related to the sexual dimorphism of some diseases (Callewaert *et al*., 2010). The expression of estrogen receptors α and β in TMJ suggests that TMJ is a target of estrogen (Milam *et al.*, 1987). Although the relationship between estrogen level and increasing TMJOA risk is controversial (Warren and Fried, 2001), and estrogen can protect against OA in the knee (Sowers *et al.*, 2006), there are clinical studies demonstrating that the serum levels of 17beta-estradiol (E2) are significantly higher in TMD patients (including TMJOA) than in healthy control individuals, both male and female (Landi *et al.*, 2005), and the concentration of E2 in the synovial fluid is significantly higher in TMJOA patients than in normal control individuals, and especially higher in TMJ patients with osteosclerotic changes (Cheng *et al.*, 2001). These findings imply that high estrogen levels may play a role in the physiopathology of TMJOA.

TMJ condylar cartilage is sensitive to changes in estrogen levels, and its thickness can be decreased by estrogen (Ng *et al*., 1999; Orajarvi *et al.*, 2011). However, the effects of estrogen on TMJ cartilage and subchondral bone in an OA model remain to be explored.

We previously reported a reliable TMJOA model in rats, induced by intra-articular injection of monosodium iodoacetate (MIA), an inhibitor of glyceraldehyde-3-phosphate dehydrogenase which is widely used in knee OA models; in this TMJOA model, condylar cartilage degradation and subchondral bone remodeling are clearly observed (Wang *et al.*, 2012a).

Thus, we hypothesized that estrogen could aggravate the progression of TMJOA. To address this hypothesis, we evaluated the effects of varied doses of estrogen and estrogen receptor antagonist on MIA-induced TMJOA in OVX rats.

MATERIALS & METHODS

Animals

In total, 90 female Sprague-Dawley rats (180-200 g) were randomly assigned to 10 groups with 9 rats in each group. The experimental schedule is illustrated in Fig. 1A. Briefly, this study was designed as 2 experiments. Experiment I contained 5 groups—control, sham-OVX, and 3 groups of OVX rats treated, respectively, with 17β-estradiol (E2) at doses of 0 μg, 20 μg, and 80 μg/day for 3 wks; whereas experiment II contained another 5 groups—control, sham-OVX, sham+ICI (estrogen receptor antagonist ICI 182780), 80 μg-E2 (OVX and treated with 80 μg E2), and 80 μg-E2+ICI. Rats were housed under controlled temperatures with a 12-/12-hour light/dark cycle and free access to food and water. All animal procedures were approved by the Peking University Animal Ethics Committee prior to the initiation of the study (Approval number: LA2012-59). This investigation conformed with ARRIVE guidelines for pre-clinical studies.

Estrogen Replacement

Estrogen replacement was performed as described previously (Wu *et al*., 2010). Briefly, rats were bilaterally OVX or sham-OVX. One week later, the OVX rats were treated with E2 (Sigma, St. Louis, MO, USA), dissolved in ethanol and diluted to 10% in saline immediately before administration, by subcutaneous abdominal injection daily in the morning, at doses of 0 μg, 20 μg, or 80 μg *per* rat, respectively, until the end of the experiment. The control and sham-OVX groups received saline injections.

Induction of TMJOA

One week after initiation of E2 replacement, TMJOA was induced in the sham-OVX and E2 replacement groups by injection of 0.5 mg MIA (Sigma) dissolved in 50 μL saline into the upper compartment of bilateral TMJs, as described previously (Wang *et al.*, 2012a). The control group received saline injections. All rats were sacrificed by pentobarbital overdose at the end of the experiment, body weight of each rat was recorded, and the serum level of E2 was measured by radioimmunoassay with an Access Immunoassay System (Beckman Coulter, Beijing, PRC).

Histological Staining and Scoring

Tissue was harvested as described previously (Wang *et al.*, 2012b). Briefly, the left TMJ of 3 rats in each group was removed *en bloc*, fixed in 4% paraformaldehyde, and demineralized in 15% EDTA. Paraffin-embedded TMJ blocs were cut sagittally in 5-μm-thick serial sections. Sections were stained with hematoxylin and eosin (HE) for routine histopathological evaluation. Toluidine blue (TB) staining was used to evaluate proteoglycans in the cartilage matrix.

TMJOA was evaluated and scored by three senior oral pathologists who were blinded to the animal groups. The scoring of TMJOA was performed according to previous studies (Xu *et al.*, 2009) with some modifications for the extent of affected cartilage and subchondral bone deterioration (Fig. 1B). The score of each group was presented as mean ± standard deviation (SD).

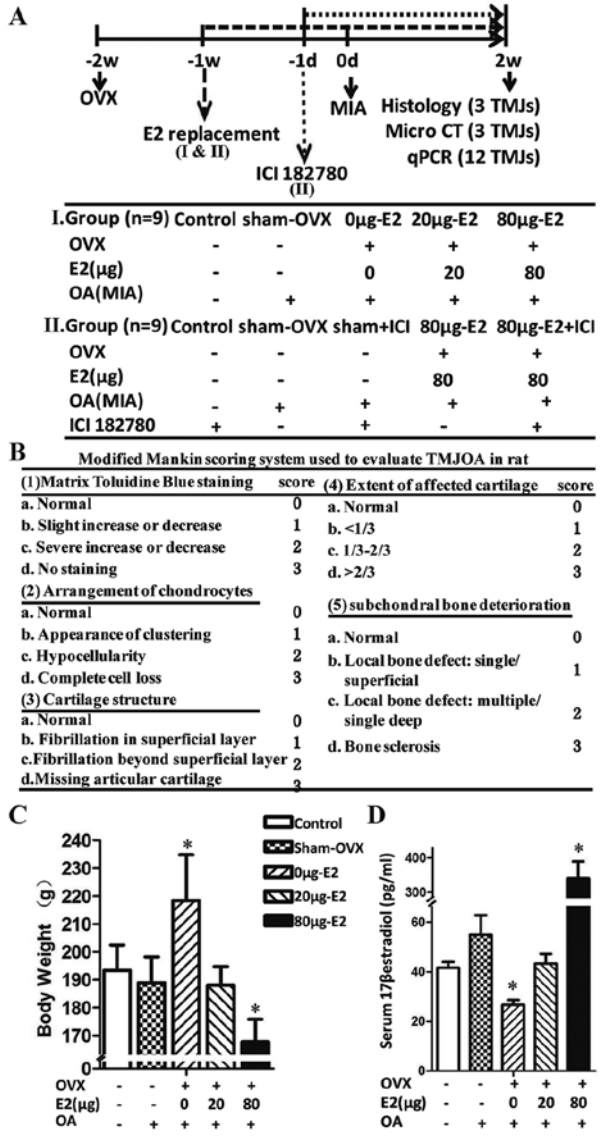

Figure 1. Study design and confirmation of estrogen replacement. **(A)** Outline of experimental design. **(B)** Modified Mankin scoring system used to evaluate TMJOA in rats. **(C)** Body weight of rats after the experiments. The body weight of OVX rats with 0-μg-E2 replacement was the highest, whereas the body weight of the 80-μg-E2 group was the lowest. Body weight of the 20-μg-E2 group was comparable with that of the sham-OVX and control groups. **(D)** Serum level of E2 in each group. Among OVX rats, the level of E2 was significantly low in the group that received 0 μg E2 and increased dose-dependently in the 20-μg and 80-μg groups. There was no difference in levels among the control group, sham-OVX group, and the 20-μg-E2 group. (C,D) All data are presented as means ± SD (n = 6; $*p < .05$ *vs.* all other groups). OVX, ovariectomized; E2, 17β-estradiol; qPCR, real-time PCR; OA, osteoarthritis; MIA, monosodium iodoacetate; ICI 182780, estrogen receptor antagonist.

MicroCT Examination

The right TMJ of 3 rats in each group was dissected. Radiographs of the condyles were obtained with a high-resolution MicroCT System (Inveon, Siemens, Germany) at 60 kV, 300 μA, and 8.5 μm effective pixel size. The images were analyzed with software

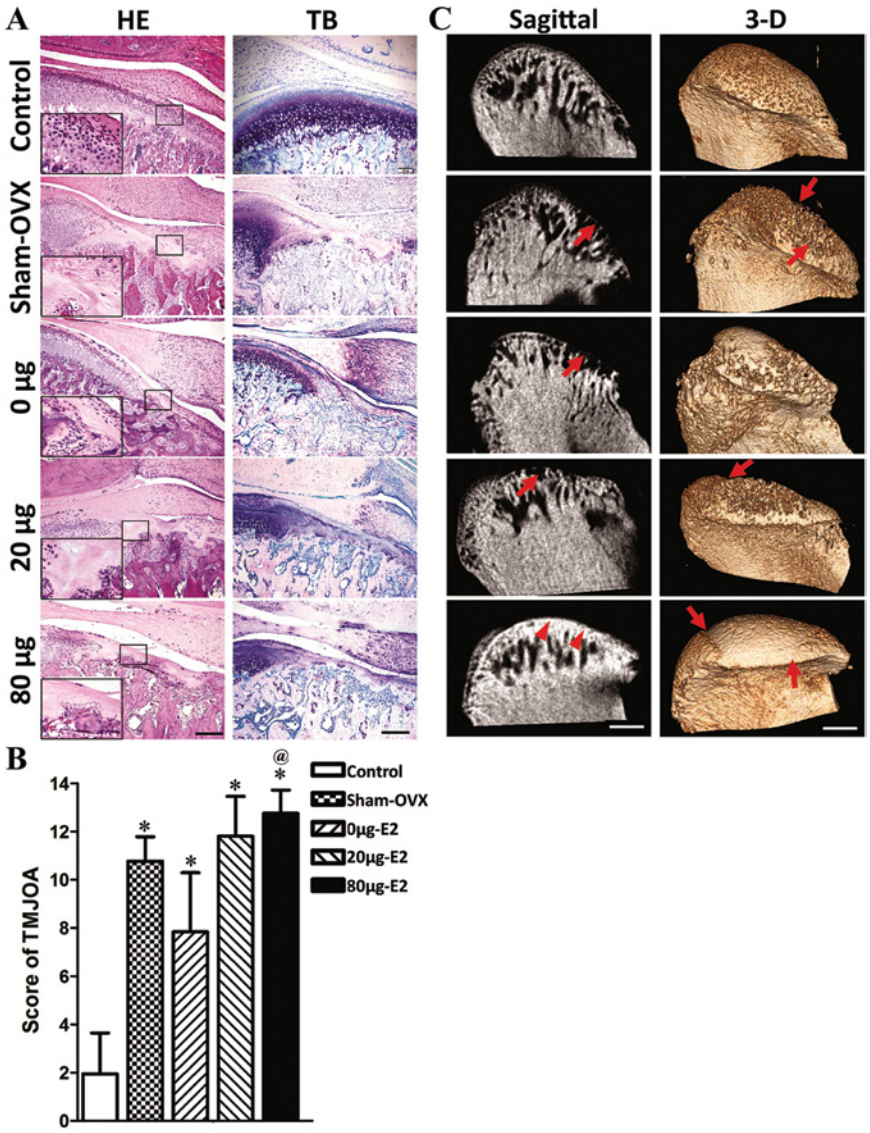

Figure 2. E2 dose-dependently potentiated MIA-induced TMJOA. **(A)** Representative images of the TMJs sectioned in the sagittal plane for HE and TB staining. Small and large black frames indicate the original and the magnified areas, respectively. Condylar cartilage in the controls was stained purple-blue by TB. Following induction of MIA for 2 wks, TMJs of the sham-OVX and the 20-μg-E2 group showed typical features of OA (magnified window: clusters and uneven matrix). In the 0-μg-E2 group, the extent of cartilage destruction was less than that in the 20-μg-E2 group, with only a slightly irregular arrangement of chondrocytes (magnified window). In the 80-μg-E2 group, the cartilage layer was thin, with complete loss of chondrocytes and TB staining, and subchondral bone sclerosis was evident (magnified window: thinned cartilage layer and cell loss) (bar = 200 μm). **(B)** Histopathological score of TMJ in each group. All data are presented as means ± SD (n = 3; *$p < .05$ *vs.* control group; @ = $p < .05$ *vs.* 0-μg-E2 group). **(C)** Representative images of the corresponding condyles by microCT 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 sham-OVX and 20-μg-E2 groups showed regional loss of surface bone and multiple erosions of the subchondral bone (arrow). The bone defects in the 0-μg-E2 group were smaller than those in the 20-μg-E2 group. In the 80-μg-E2 group, the surface bone defect was larger and the trabecular bone was thicker and more dense (arrowhead) (bar = 300 μm).

provided by the manufacturer. All sagittal images were captured with the same parameters: Ct = -550, W = 550.

Real-time Polymerase Chain-reaction (PCR) Analysis

To evaluate pro-apoptotic gene expression in the condylar cartilage and subchondral bone, we pooled bilateral condylar heads of each rat (6 rats in each group) for real-time PCR (qPCR) analysis and ground them into powder in liquid nitrogen using a cryogenic grinder (SPEX SamplePrep, Metuchen, NJ, USA) for sufficient RNA extraction. qPCR analysis was performed with the Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) as described previously (Wu *et al.*, 2010). The efficiency of primers for rat β-actin, TNFα, Fas, FasL, caspase 3, and caspase 8 was confirmed previously (Wang *et al.*, 2012a).

Immunohistochemical Staining

Immunohistochemical (IHC) staining was performed with a two-step detection kit (Zhongshan Golden Bridge Biotechnology, Beijing, PRC) as described previously (Wu *et al.*, 2010). The primary antibody was Fas (Abcam, Hangzhou, PRC; 1:100 dilution).

Estrogen Receptor Antagonist Administration

ICI 182780 (Sigma) was dissolved in 10% ethanol and used as described previously (Kou *et al.*, 2011). Briefly, ICI 182780 (500 μg in 100 μL) was injected intraperitoneally into the control, sham+ICI, and 80-μg-E2+ICI groups 24 hrs before and immediately after MIA injection and every other day until the end of the experiment, whereas the other 2 groups (sham-OVX, 80-μg-E2) were injected with vehicle. HE staining, microCT, qPCR, and IHC were performed to evaluate the changes of TMJOA.

Statistical Analysis

Statistical analyses were performed with SPSS software (ver. 11.0 for Windows). All data were presented as means ± SD and were analyzed by one-way analysis of variance (ANOVA) with a *post hoc* test. Any p value < .05 was considered to indicate statistical significance.

RESULTS

E2 Replacement Inhibited Weight Gain and Increased Serum Levels of E2 in a Dose-dependent Manner

E2 replacement therapy inhibited the gain of body weight of OVX rats in a dose-dependent manner, compared with that in the control and sham-OVX rats ($p < .05$; Fig. 1C). The body weight of the 0-μg-E2 group was the highest ($p < .05$) among the groups; the weight of the 20-μg-E2 group was similar to that of the sham-OVX and control groups ($p > .05$), and the weight of the 80-μg-E2 group was the lowest ($p < .05$). The changes of weight were consistent with previous results that administration of E2 prevents OVX rats from gaining body weight because of affecting energy metabolism (Pedersen *et al.*, 2001). There was no change in food intake within a limited period of time among the groups (data not shown). The serum levels of E2 in the 0-μg-E2 and 80-μg-E2 groups were the lowest and highest, respectively, and that in the 20-μg-E2 group was comparable with the physiological level of the control and sham-OVX groups (Fig. 1D), which confirmed the effectiveness of ovariectomy and E2 replacement.

E2 Potentiated MIA-induced TMJOA in a Dose-dependent Manner

Histopathological analysis showed that, following induction of MIA for 2 wks, TMJs of the sham-OVX group showed typical features of OA, including regional loss of chondrocytes, proliferation of peripheral chondrocytes, subchondral bone erosion, and uneven TB staining in the cartilage matrix, compared with the control group (Fig. 2A). The destruction of cartilage and subchondral bone was the mildest in the 0-μg-E2 group among the groups, with only a slightly irregular arrangement of chondrocytes and decreased staining of matrix by TB. The destruction in the 20-μg-E2 group was comparable with that in the sham-OVX group. However, severe thinning of the cartilage with complete chondrocyte loss and corresponding loss of TB staining in the anterior slope of the condyle were seen in the 80-μg-E2 group, and subchondral bone sclerosis was evident (Fig. 2A). The scores of TMJOA were significantly higher in the MIA-induced groups compared with the control group ($p < .05$). Moreover, the scores of the OVX groups treated with E2 showed a trend of increase with the increase of E2, with the score of the 80-μg-E2 group significantly higher than that of the 0-μg-E2 group ($p < .05$). However, there were no statistical differences between the scores of the 0-μg-E2 and the 80-μg-E2 groups, and between the scores of the 20-μg-E2 and the 80-μg-E2 groups ($p > .05$, n = 3; Fig. 2B), likely due to limitations resulting from the small sample size.

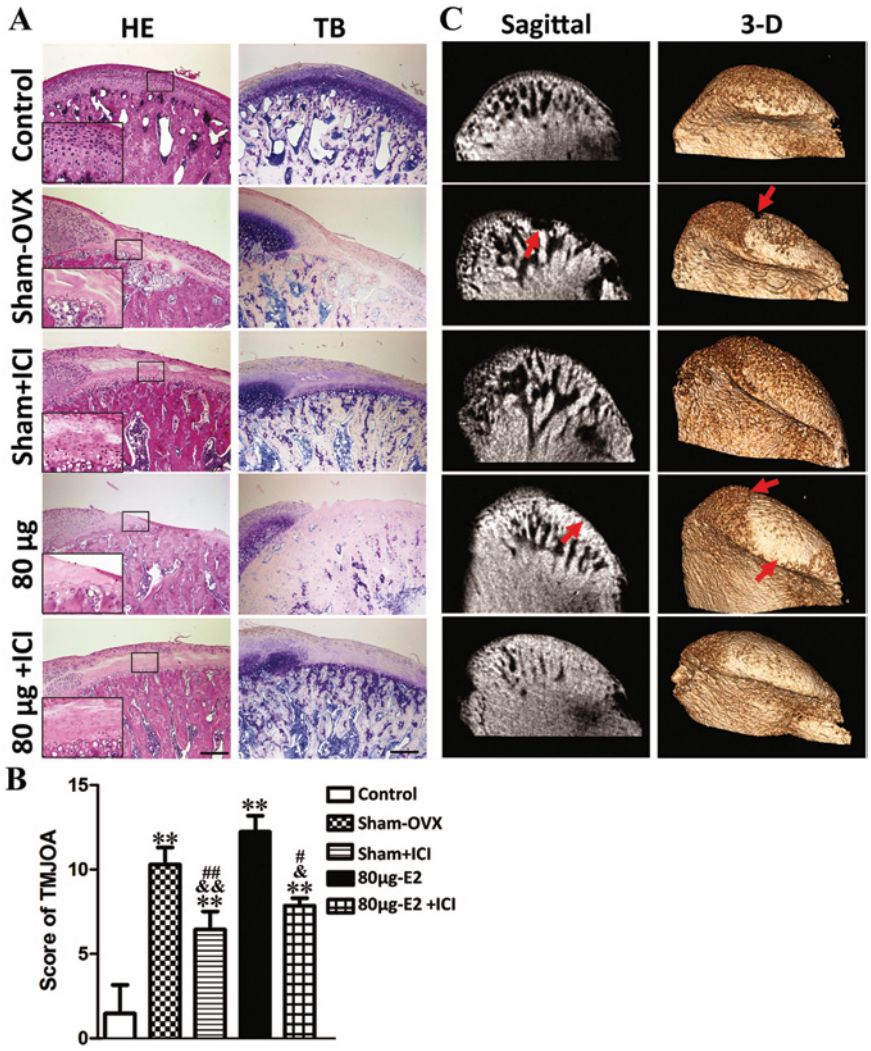

Figure 3. Blockage of E2 effects on TMJOA by estrogen receptor antagonist ICI 182780. **(A)** Representative images of the TMJ in HE and TB staining. Small and large black frames indicate the original and the magnified areas, respectively. The cartilage destruction seen in the sham-OVX (magnified window: chondrocyte loss and subchondral bone erosion) and the 80-μg-E2 groups (magnified window: cell loss in the whole cartilage) was partially reversed in the sham+ICI and 80-μg-E2+ICI groups, respectively, with the deep-layer chondrocytes apparently unaffected and the subchondral bone also intact (magnified window: unaffected calcified layer of chondrocytes) (bar = 200 μm). **(B)** Histopathological score of TMJ in each group. The histopathological scores of TMJOA in the sham-OVX and 80-μg-E2 groups were partially reversed in the sham+ICI and 80-μg-E2+ICI groups, respectively. All data are presented as means ± SD (n = 3; ** $p < .01$ *vs.* control group; & = $p < .05$, && = $p < .01$ *vs.* sham-OVX group; # = $p < .05$, ## = $p < .01$ *vs.* 80-μg-E2 group). **(C)** Representative radiographic images of the condyles by microCT scanning in a sagittal section view and a 3D reconstructed view. The bone erosion (arrow) in the sham-OVX group and surface bone loss in the 80-μg-E2 group were reversed by ICI injection (bar = 300 μm).

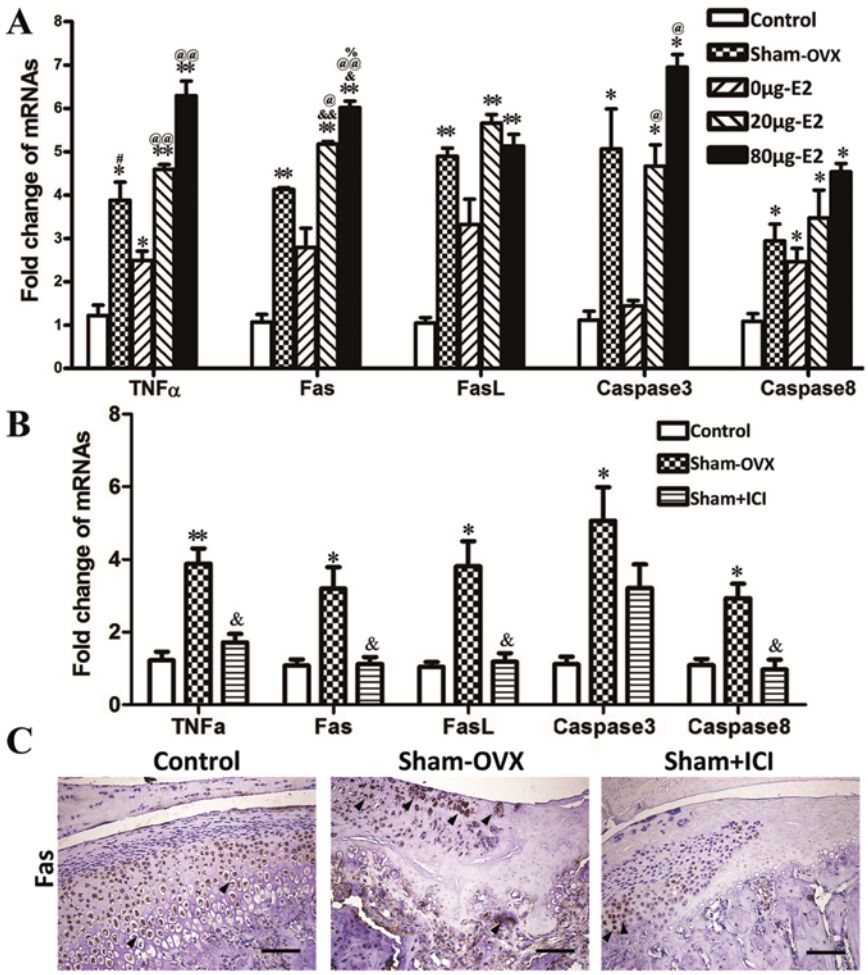

Figure 4. Pro-apoptotic genes in condylar cartilage potentiated by E2 and partially reversed by estrogen receptor antagonist ICI 182780. **(A)** Changes in mRNA expression of pro-apoptotic genes in the condyle after induction of TMJOA by MIA injection for 2 wks were evaluated by qPCR. mRNA expressions of TNFα, Fas, and caspase 3 were up-regulated by E2 in a dose-dependent manner. mRNA expressions of FasL and caspase 8 in the sham-OVX, 20-μg-E2, and 80-μg-E2 groups were higher than in the control group, but there was no significant difference between the 0-μg-E2 and 80-μg-E2 groups. **(B)** Up-regulated expression of pro-apoptotic genes in the sham-OVX group was reversed by ICI 182780 (n = 6; *$p < .01$, **$p < .01$ *vs.* control group; & = $p < .05$ *vs.* sham-OVX group; @ = $p < .05$, @@ = $p < .01$ *vs.* 0-μg-E2 group; % = $p < .05$, %% = $p < .01$ *vs.* 20-μg-E2 group; # = $p < .05$, ## = $p < .01$ *vs.* 80-μg-E2 group). **(C)** Representative images of IHC staining of Fas in the anterior slopes of condyles of TMJs. Expression of Fas (arrowhead) was mainly in the hypertrophic chondrocytes in the control group. In the sham-OVX group, staining of Fas was stronger and diffused in the superficial, proliferative, and hypertrophic layers adjacent to the lesion, whereas in the sham+ICI group, staining of Fas was restricted and reduced in the hypertrophic layer (bar = 100 μm).

In the sagittal and 3D images, the subchondral bone surface of the anterior and central areas of the condyle of the sham-OVX group was discontinuous, accompanied with regional bone defects, compared with the control condyle (Fig. 2C). However, in the 0-μg-E2 group, the subchondral bone surface was less disrupted and the bone defects were smaller compared with those in the sham-OVX group. The bone lesions of the 20-μg-E2 group were similar to those of the sham-OVX group, whereas in the 80-μg-E2 group, there was larger scale bone loss on the surface of the condyle, and the trabecular bone was thicker and more dense, presenting characteristics of sclerosis.

Estrogen Receptor Antagonist Partially Blocked Effects of E2 on TMJOA

Histopathological analysis showed that the destruction of cartilage was restricted to only the upper 3 layers, whereas the deep hypertrophic and calcification layers and the subchondral bone remained intact in the sham+ICI group, compared with the sham-OVX group (Fig. 3A). Moreover, the cartilage thinning and subchondral bone sclerosis were relieved significantly in the 80-μg-E2+ICI group, compared with the 80-μg-E2 group (Fig. 3A). The scores of TMJOA were significantly lower in the sham+ICI group and the 80-μg-E2+ICI group than in the sham-OVX group and the 80-μg-E2 group ($p < .05$; Fig. 3B). Correspondingly, the radiographic images also showed that bone loss was partially blocked by ICI 182780 injection (Fig. 3C).

Estrogen Receptor Antagonist Partially Blocked E2 Potentiated Pro-apoptotic Genes

As shown in Fig. 4A, qPCR analysis showed that E2 potentiated mRNA expression of TNFα, Fas, and caspase 3 in a dose-dependent manner. Expression of mRNA was the lowest in the 0-μg-E2 group and highest in the 80-μg-E2 group among the E2 replacement groups ($p < .05$). Expression of FasL and caspase 8 was higher in the sham-OVX and E2 replacement groups than in the control group ($p < .05$), but with no difference among the 0-μg-E2, 20-μg-E2, and 80-μg-E2 groups ($p > .05$). After the blocking of estrogen receptors with ICI 182780, the induction of these pro-apoptotic genes was reversed in the sham+ICI group compared with the sham-OVX group ($p < .05$; Fig. 4B).

Moreover, IHC showed that stronger staining of Fas was observed, diffusely in the proliferative and hypertrophic layers, adjacent to the lesion in the sham-OVX group, compared with the control and sham+ICI groups (Fig. 4C).

DISCUSSION

We found that E2 aggravated MIA-induced TMJOA in OVX rats. This was supported by multiple lines of evidence. First, E2 histopathologically and radiographically potentiated the cartilage

degradation and subchondral bone erosion and sclerosis of MIA-induced TMJOA. Second, E2 potentiated the expression of the pro-apoptotic genes in the death receptor family in the condylar cartilage. Third, estrogen receptor antagonist ICI 182780 partially blocked the effects of E2 on TMJOA and on the expression of the pro-apoptotic genes. To the best of our knowledge, this is the first report demonstrating that E2 aggravates TMJOA in an animal model.

E2 aggravated the progression of TMJOA. The present study revealed the detrimental effects of estrogen on the cartilage degradation and subchondral bone erosion and sclerosis of MIA-induced TMJOA in rats. This effect could be blocked by administration of the estrogen receptor antagonist. Our results appeared to be opposite the generally chondro-protective effects of estrogen on knee OA (Oestergaard *et al*., 2006). However, the expressions of estrogen receptors α and β are significantly higher in the TMJ than in the knee, and it is therefore speculated that the differential expression of estrogen receptors may result in the varied downstream effects in response to hormones within these joints (Wang *et al.*, 2009). Our results appeared to support this speculation. Therefore, the difference of E2 effects on TMJ in our study and on the knee in a previous report could be due to the differential expression of estrogen receptors in the 2 joints. Further studies are needed to confirm whether effects of E2 on MIA-induced TMJOA and knee OA are different. Moreover, the detrimental effects of estrogen on the different profiles of TMJ have been reported previously. For example, estrogen induces matrix metalloproteinases in the TMJ disc but not in the knee meniscus, which suggests that estrogen may selectively contribute to TMJ diseases (Kapila *et al*., 2009). Estrogen also impaired TMJ cartilage remodeling *in vitro* (Ng *et al*., 1999) and *ex vivo* (Talwar *et al.*, 2006), and a high level of estrogen is related to subchondral bone sclerosis in the TMJ (Cheng *et al.*, 2001). Therefore, the results of our and previous studies may somehow explain the reason why TMJOA occurs more in female patients during the reproductive years, while knee OA is more prevalent in post-menopausal women.

Estrogen-receptor-dependent pathways are important in the regulation of TMJOA. We showed that injection of estrogen receptor antagonist ICI 182780 resulted in a significant reduction in cartilage and subchondral bone destruction in TMJOA and also a significant decrease in the expression of Fas in the cartilage, suggesting that the estrogen receptors and their downstream pathways were critically involved in TMJOA. Our results are consistent with those from our previous report that estrogen aggravates CFA-induced TMJ inflammation *via* the estrogen receptor (Kou *et al*., 2011) and are also consistent with the results that estrogen inhibits chondrocyte proliferation through the estrogen receptor (Ikeda *et al.*, 2012). Moreover, deficiency of estrogen receptor β increases mandibular condylar cartilage thickness in mice (Kamiya *et al.*, 2013). Our results demonstrate that E2 aggravated the progression of MIA-induced TMJOA depending on the pathways of estrogen receptors.

Estrogen aggravated TMJOA through enhancing chondrocyte apoptosis, which contributes to the disruption of cartilage integrity in OA (Aigner and Kim, 2002). Genes of the death receptor family, including Fas and FasL, are associated with chondrocyte apoptosis (Chagin *et al*., 2007; Hussain *et al*., 2012) and are up-regulated in MIA-induced TMJOA, as we reported previously (Wang *et al*., 2012a). We found that E2 dose-dependently induced chondrocyte loss and mRNA expression of Fas and caspase 3 in the cartilage of MIA-induced TMJOA, and these effects were significantly reversed by the estrogen receptor antagonist, suggesting that high levels of E2 could induce chondrocyte apoptosis and ultimately contribute to the disruption of cartilage in TMJOA. Our results are consistent with previous findings that estrogen induces apoptosis of growth plate chondrocytes (Zhong *et al.*, 2011) but are inconsistent with the anti-apoptotic effect of estrogen in knee chondrocytes (Hattori *et al.*, 2012). Again, this difference could be due to differential expression of estrogen receptors in the chondrocytes of the 2 joints, or to unknown reasons. Nevertheless, our results suggest that the induction of chondrocyte apoptosis by estrogen contributes to TMJOA.

The present study is limited by a single model of MIA-induced TMJOA. The sample size seemed small in histopathological evaluation of the TMJOA scores, since there was no statistical significance between scores of the 0-μg-E2 group and those of the sham-OVX group. A larger sample size may be needed. In addition, the potential roles of other female hormones, such as progesterone and relaxin, in TMJOA have yet to be examined.

In conclusion, we showed that estrogen aggravates MIA-induced TMJOA through inducing chondrocyte apoptosis. These findings will enhance our understanding of sexual dimorphism of TMJOA or TMD and contribute to the exploration of new intervention methods.

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