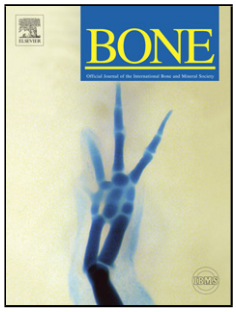

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The effect of oestrogen on mandibular condylar cartilage via hypoxia-inducible factor-2α during osteoarthritis development

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Highlights

- .Obvious OA-like lesion can be induced by unilateral anterior crossbite (UAC).
- . High level of oestrogen could aggravate the degenerative changes of condylar cartilage, while lack of oestrogen could alleviate it via oestrogen-ERβ-HIF2α pathway.
- . The effect of oestrogen on condylar cartilage is at least partially via oestrogen-ERβ-HIF2α pathway.

Abstract

Oestrogen and hypoxia inducible factor-2α (HIF2α) are key regulators in the pathogenesis of osteoarthritis (OA). However, the cellular interaction between oestrogen and HIF2αin articular cartilage during OA process remains unknown. Our previous study has revealed that high-physiological level of oestrogen aggravates the degradation of condylar cartilage in the early stage of temporomandibular joint osteoarthritis (TMJ OA). Here, we hypothesize that HIF2α involves the effect of oestrogen on mandibular condylar cartilage in the progression of TMJ OA. Our experiment *in vivo* found that the degeneration of condylar cartilage caused by unilateral anterior crossbite (UAC) model, characterized by obvious degenerative morphology, loss of cartilage extracellular matrix, up-regulation of TNF-α, HIF2α and its' down-stream OA-related cytokines (MMP-13, VEGF and Col X), could be alleviated by lack of oestrogen while aggravated by high level of oestrogen in rats. Meanwhile, our *in vitro* study found that 17β-estradiol stimulation resulted in the loss of extracellular matrix, increased expression of TNF-α, IL-1, HIF2α and its' down-stream OA-related cytokines (MMP-13, VEGF and Col X) in primary condylar chondrocytes via oestrogen receptor beta (ERβ), which could be reversed by ER antagonist, selective estrogen receptor modulators (SERMs) and HIF2α translation

inhibitor. Our results reveal that high level of oestrogen can aggravate the degenerative changes of mandibular condylar cartilage, while lack of oestrogen can alleviate it via oestrogen-ERβ-HIF2αpathway during TMJ OA progression

Key words: Oestrogen (E_2), hypoxia-inducible factor-2α (HIF2α), Temporomandibular joint (TMJ), osteoarthritis (OA)

1. Introduction

Temporomandibular disorders (TMD) is a group of wide-spread diseases embracing many orofacial signs and symptoms involving the masticatory musculature, temporomandibular joint (TMJ) and associated structures [1]. It has been estimated that over 50 million Americans suffer from TMD-related complaints [2, 3]. TMD are most commonly reported in young to middle-aged adults (20 to 50 years of age), with female-to-male ratio of patients seeking care from 3:1 to as high as 9:1[4]. Osteoarthritis (OA), a severe pathological manifestation which is featured by cartilage degradation and subchondral bone remodeling, is often present in the TMJ of severe TMD patient [5, 6]. The important characteristics of condylar cartilage degradation of OA in TMJ (TMJ OA) include loss of extracellular matrix, increasing expression of pro-inflammatory and degradative cytokines such as interleukin (IL), tumor necrosis factor-α (TNF-α), matrix metalloproteinases (MMPs), vascular endothelial growth factor (VEGF), et al, and accelerated chondrocyte hypertrophy characterized by expression of type X collagen (Col X) [7-9]. However, the molecular pathogenic mechanism underlying TMJ OA needs to be elucidated in detail.

Oestrogen has been suggested to play an important role in the development of TMD [4, 10-12]. Coincidentally, our recent study has found that high-physiological oestrogen has different effects on the prognosis of condylar cartilage and subchondral bone in the early stage of TMJ OA, which aggravates the degeneration of cartilage

while reverses the resorption of subchondral bone [9]. However, the underlying mechanism for this phenomenon remains unclear.

As an avascular tissue, articular cartilage is maintained in a hypoxia environment throughout whole life [13]. In recent years, hypoxia inducible factor-2α (HIF2α) has been proved to play an important role in the progression of OA. HIF2α expresses highly in osteoarthritic cartilage versus non-diseased cartilage of mice and human [14, 15]. And HIF2α could directly enhance the promoter activities of Col X, MMP-13 and VEGF through specific binding their hypoxia-response element (HRE) in the promoters to accelerate chondrocyte hypertrophy, matrix degradation and vascular invasion of cartilage to exacerbate OA progression [16]. HIF2α also directly induces the higher expression of catabolic factors including matrix metalloproteinases (MMPs), aggrecanase-1 (ADAMTS4), nitric oxide synthase-2 (NOS2) and prostaglandin-endoperoxide synthase-2 (PTGS2), thus leading to the destruction of articular cartilage [17]. On the contrary, HIF2α-deficient mice show resistance to OA development [16]. Moreover, in the study of TMJ OA, the overload-stress induced mortality of condylar chondrocytes is also related to the elevated expression of HIF2α [18]. So, it is suggested that HIF2α mainly involve the destructive progression of cartilage in OA development.

In recent years, a great number of studies have demonstrated the interaction between oestrogen and hypoxia in different pathological processes of different tissues, such as bone, ovary and brain [19, 20]. However, in literature, few studies have focused on whether HIF2α involves the effect of oestrogen on articular cartilage during OA process. The hypothesis of the present study is that HIF2α involves the effect of oestrogen on mandibular condylar cartilage in the progression of TMJ OA.

2. Materials and Methods

2.1. Experimental animals

All operations involving animals were conducted according to the Institutional Animal Care Guidelines and were approved by the Administration Committee of Experimental Animals at Fourth Military Medical University (FMMU). One hundred and twenty 6-week-old female SD rats (weight 140-160 g) were provided by the Laboratory Animal Center of FMMU. All animals were randomly assigned to 2 groups according to the experimental time points (8 and 12 weeks), and each group was divided into 5 subgroups: the control (C), unilateral anterior crossbite (UAC), sham-ovariectomy with UAC (Sham+UAC), ovariectomy with UAC (OVX+UAC), and ovariectomy with excessive oestrogen injection at 0.5mg/kg/day plus UAC (E+UAC). With12 rats in each subgroup, all rats were housed in a pathogen-free room and fed sterilised food and redistilled water during the study.

In the UAC, Sham+UAC, OVX+UAC and E+UAC subgroup, the UAC model was established in the rats as previously described [21]. Briefly, two small metal tubes were bonded to the left maxillary incisor and left mandibular incisor to create a UAC relation of the left side incisors under deep anesthesia. The rats in the C subgroup were subjected to the same procedure but without maintaining the tubes on the incisors. Each operation was completed within 3 mins, and all efforts were made to minimise the suffering of the rats. One week before the UAC operation, the rats in the OVX+UAC and E+UAC subgroup were bilaterally ovariectomized, while the rats in the Sham+UAC subgroup only received OVX procedure without excising the bilateral ovaries. Just after OVX operation, the rats in the E+UAC subgroup were injected with 17β-estradiol (E_2, ab120657, Abcam, Cambridge, MA, UK) subcutaneously daily in the morning, at a dose of 0.5mg/kg/day, until the end of the experiment as previously described [9]. The other groups received saline injections. The experiment schedule and design are shown in Fig. 1A. The tissues preparation, histochemical and immunohistochemical staining, RNA extraction and real-time PCR were described in details in Supplemental Material and Methods.

2.2. Grouping, culture and treatment of primary rat chondrocytes

As for the *in vitro* experiments, the procedures of primary rat chondrocytes isolation and culture were described in Supplemental Material and Methods. The primary chondrocytes were grown to 60-70% confluence and serum-starved overnight in phenol red-free DMEM (Hyclone, Logan, UT, USA) supplemented with penicillin and streptomycin. Then, as for determining the exact oestrogen receptor (ER) signalling, and the relationship between oestrogen and HIF2α in TMJ OA, the chondrocytes were treated with following different signaling modulators in different groups: 1μM 17β-estradiol (E_2, ab120657, Abcam, Cambridge, MA, UK) in E_2 group, 1μM E_2 plus 1μM pan-ER antagonist ICI182780 (S1191, selleck, Huston, TX, USA) in E_2+ICI group, 1μM E_2 plus 5μM HIF2α translation inhibitor Methyl-3-(2-(cyano(methylsulfonyl)methylene)hydrazino)thiophene-2-carboxylate (400087, Merck, Darmstadt, Germany) in E_2+Meth group, 1μM E_2 plus 1μM selective estrogen receptor modulators (SERMs) tamoxifen (S1283, selleck, Huston, TX, USA) in E_2+Tamo group, 1μM E_2 plus 1μM SERMs endoxifen (S7839, selleck, Huston, TX, USA) in E_2+Endo group, 20 nM ER alpha (ERα)-specific agonist Propyl pyrazole triol (1426/10, Tocris Biosciences, Minneapolis, MN, USA) in PPT group, 1μM E_2 plus 20 nM ERα-specific antagonist MPP dihydrochloride (1991/10, Tocris Biosciences, Minneapolis, MN, USA) in E_2+MPP group, 20 nM ER beta (ERβ)-specific agonist Erteberel (GC11822, Glpbio, Montclair, CA, USA) in Erte group, 1μM E_2 plus 20 nM ERβ-specific antagonist PHTPP (2662/10, Tocris Biosciences, Minneapolis, MN, USA) in E_2+PHTPP group. After 12h stimulation, the chondrocytes were collected for RNA and protein extraction. For better simulating the natural developing environment of primary chondrocytes, at the stage of stimulation by different agents, the oxygen partial pressure of incubator (Thermo Fisher Scientific, Waltham, MA, USA) for chondrocytes culture was adjusted to 5%, which is similar with inner oxygen pressure of condylar cartilage. The experiment *in vitro* was repeated 3 times (n=3). The protein and RNA extraction, immunoblotting and real-time PCR were described in Supplemental Material and Methods.

2.3. Statistical Analysis

Statistical analysis was conducted using the SPSS 21.0 package software (SPSS Inc., Chicago, IL, USA). All data acquisition and analysis were accomplished blindly. For comparisons of the means of measurement among the four or more groups of *in vivo and in vitro* experiment, one-way ANOVA test was applied, and Tukey's multiple comparisons test was used to compare every 2 groups. The results were expressed as the mean with 95% confidence intervals (95% CI), and *P* values of less than 0.05 were defined as being statistically significant.

3. Results

3.1. Body and uterus weight

The weight of uterus was shown in Fig. 1B. There were no significant differences between the C, UAC and Sham+UAC subgroup at both time points. However, compared with the Sham+UAC subgroup, the average uterus weight of rats was significantly lower in the OVX+UAC subgroup while significantly higher in the E+UAC subgroup at both 8 weeks (both $P<0.01$) and 12 weeks (both $P<0.01$).

As for the average body weight of rats shown in Fig. 1B, no significant differences were found between the C, UAC and Sham+UAC subgroups either. However, at 8 and 12 weeks, compared with the Sham+UAC subgroup, the average body weight of rats was significantly higher in the OVX+UAC subgroup (Both $P<0.01$) while significantly lower in the E+UAC subgroup ($P<0.05$ and $P<0.01$ respectively).

The change of uterus and body weight indicated that OVX procedure and E_2 supplement after OVX procedure were effective to decrease and increase the serum oestrogen level of rats respectively.

3.2. OVX alleviated while E_2 supplement aggravated the OA-like change of cartilage induced by UAC

In the mandibular condylar cartilage of rats in the C subgroup at 8 and 12 weeks, the surface of cartilage was smooth and complete. Four typical cellular layers (i.e., the fibrous layer, proliferative layer, pre-hypertrophic layer and hypertrophic layer) were arranged regularly, with good continuity in each layer (Supplemental Fig. 1 and Fig 2A). However, at 8 and 12 weeks, the mandibular condylar cartilage of rats in the UAC, Sham+UAC, OVX+UAC and E+UAC subgroups showed OA-like lesions at different degree, which were mainly characterized by obscure cellular layer boundary and irregular chondrocytes arrangement (Supplemental Fig. 1B).

As for the thickness of the entire cartilage layers shown in Fig. 2B, both the centre and posterior third condylar cartilage were significantly thinner in the UAC subgroup compared with the C subgroup at 8 weeks (both $P<0.01$) and 12 weeks (both $P<0.01$), while no significant difference was found between the UAC and Sham+UAC subgroup at both time points. Furthermore, compared with the Sham+UAC subgroup, both the centre and posterior third cartilage at 8 weeks were significantly thicker in the OVX+UAC subgroup ($P<0.05$ and $P<0.01$ respectively), while at both 8 and 12 weeks that were significantly thinner in the E+UAC subgroup (centre third: both $P<0.05$; posterior third: $P<0.05$ and $P<0.01$ respectively). Moreover, for detecting which layers of condylar cartilage are thinner in rats, we calculated the thickness of fibrous layer plus proliferative layer (defined as superficial layer) and pre-hypertrophic layer plus hypertrophic layer (defined as deep layer). It was found that the decrease of condylar cartilage in the centre and posterior third condylar cartilage was more obvious in the deep layers than that in superficial layer (supplemental Fig 1C).

3.3. OVX alleviated while E_2 supplement aggravated the loss of extracellular matrix induced by UAC

In the C subgroup at 8 and 12 weeks, as revealed by Safranin O staining, the proteoglycan was abundant and distributed regularly in the pre-hypertrophic and hypertrophic layers, with a clear boundary between the proliferative layer and subchondral bone (Fig. 2A). However, the percentage of proteoglycan of condylar cartilage in the UAC subgroup was significantly lower at 8 and 12 weeks compared with the C subgroup (Fig. 2C, both $P<0.01$), while no significant difference was found between the UAC and Sham+UAC subgroup. Meanwhile, compared with the Sham+UAC subgroup, the percentage of proteoglycan was significantly increased by OVX procedure while decreased by E_2 supplement at 8 weeks (Fig. 2C, both $P<0.05$) and 12 weeks (Fig. 2C, $P<0.01$ and $P<0.05$ respectively).

Type II collagen (Col II) is another important component of the extracellular matrix. As shown in Fig 2A, Col II was also predominately distributed in the pre-hypertrophic and hypertrophic layers. Similar to the distribution of proteoglycan, the UAC procedure significantly decreased the percentage of Col II-positive area at 8 and 12 weeks compared with the C subgroup (Fig. 2C, both $P<0.01$), while no significant difference was found between the UAC and Sham+UAC subgroup. However, at 8 and 12 weeks, compared with the Sham+UAC subgroup, the percentage of Col II-positive area was significantly lower in the E+UAC subgroup (Fig. 2C, $P<0.01$ and $P<0.05$ respectively), while that of Col II-positive area in the OVX+UAC subgroup showed uptrend but without significant difference.

3.4. OVX decreased while E_2 supplement increased the expression of UAC-induced pro-inflammatory cytokines

The expression of potent pro-inflammatory cytokine, TNF-α, which was primarily distributed in the hypertrophic layer, was shown in Fig. 3A. At 8 and 12 weeks, compared with the C subgroup, both the percentages of TNFα-positive chondrocytes and the TNFα mRNA level of condylar cartilage were significantly higher in the UAC subgroup (Fig. 3B, both $P<0.01$; Fig. 3C, both $P<0.01$). And no

significant difference of TNF-α expression in the condylar cartilage was found between the UAC and Sham+UAC subgroup at both time points. However, at 8 and 12 weeks, compared with the Sham+UAC subgroup, the percentages of TNFα-positive chondrocytes and the TNFα mRNA level of condylar cartilage were significantly lower in the OVX+UAC subgroup (Fig. 3B, $P<0.01$ and $P<0.05$ respectively; Fig. 3C, $P<0.05$ and $P<0.01$ respectively) while significantly higher in the E+UAC subgroup (Fig. 3B, both $P<0.01$; Fig. 3C, both $P<0.05$).

3.5. OVX decreased while E_2 supplement increased the expression of HIF2α and its' downstream OA-related cytokines induced by UAC

In the literature, HIF2α mainly induced the destructive effect on cartilage in the development of osteoarthritis. As shown in Fig. 4A, HIF2α was mainly distributed in the hypertrophic layer of condylar cartilage. At 8 and 12 weeks, compared with the C subgroup, both the percentages of HIF2α-positive chondrocytes and the HIF2α mRNA level of condylar cartilage were significantly higher in the UAC subgroup (Fig. 4B and 4C, all $P<0.01$). But no significant difference was found between the UAC and Sham+UAC subgroup at both level. However, at 8 and 12 weeks, the percentages of HIF2α-positive chondrocytes and HIF2α mRNA level of condylar cartilage were significantly lower in the OVX+UAC subgroup (Fig. 4B, $P<0.05$ and $P<0.01$ respectively; Fig. 4C, both $P<0.01$) while significantly higher in the E+UAC subgroup (Fig. 4B, $P<0.01$ and $P<0.05$ respectively; Fig. 4C, both $P<0.01$) compared with the Sham+UAC subgroup.

Chondrocyte hypertrophy, cartilage matrix degradation and vascular invasion in the osteoarthritic cartilage are characterized by expression of Col X, MMP-13 and VEGF respectively, and all these three OA-related cytokines are directly regulated by HIF2α [16]. As shown in Fig 4A and 5A, it was obvious that all of them were mainly distributed in the hypertrophic layer of condylar cartilage. Similar to the expression change of HIF2α, at 8 and 12 weeks, the expression of VEGF, MMP-13 and Col X in

condylar cartilage were significantly higher in the UAC subgroup than that in the C subgroup, at both the percentage of immunohistochemical-positive chondrocytes (VEGF: Fig. 4B, both $P<0.01$; MMP-13: Fig. 5B, both $P<0.01$; Col X: Fig. 5B, both $P<0.01$) and mRNA level (VEGF: Fig. 4C, both $P<0.01$; MMP-13: Fig. 5C, both $P<0.01$; Col X: Fig. 5C, both $P<0.01$). No significant difference was found between the UAC and Sham+UAC subgroups in the expression of VEGF, MMP-13 and Col X in condylar cartilage. However, at 8 and 12 weeks, compared with the Sham+UAC subgroup, the expression of VEGF, MMP-13 and Col X in the OVX+UAC subgroup were significantly lower at the percentage of immunohistochemical-positive chondrocytes (VEGF: Fig. 4B, both $P<0.01$; MMP-13: Fig. 5B, $P<0.05$ and $P<0.01$ respectively; Col X: Fig. 5B, both $P<0.05$) and mRNA level (VEGF: Fig. 4C, both $P<0.05$; MMP-13: Fig. 5C, both $P<0.05$; Col X: Fig. 5C, $P<0.05$ and $P<0.01$ respectively), while the expression of VEGF, MMP-13 and Col X in the E+UAC subgroup were significantly higher at the percentage of immunohistochemical-positive chondrocytes (VEGF: Fig. 4B, both $P<0.01$; MMP-13: Fig. 5B, both $P<0.01$; Col X: Fig. 5B, both $P<0.01$) and mRNA level (VEGF: Fig. 4C, both $P<0.01$; MMP-13: Fig. 5C, both $P<0.05$; Col X: Fig. 5C, both $P<0.01$).

3.6. E_2 led to degenerative changes in condylar primary chondrocytes which could be reversed by both pan-ER antagonist and HIF2α translation inhibitor

For further exploring the relationship between oestrogen and HIF2α in the cartilage of TMJ OA, the *in vitro* experiments were conducted. As shown in Fig. 6, compared with the C group, E_2 stimulation significantly decreased the protein and mRNA level of aggrecan of primary chondrocytes (Fig. 6B and 6C, both $P<0.01$). However, compared with the E_2 group, the protein and mRNA level of aggrecan significantly increased in the E_2+ICI group (Fig. 6B and 6C, both $P<0.05$) and the E_2+Meth group (Fig. 6B, $P<0.01$; Fig. 6C, $P<0.05$). As for another important component of the extracellular matrix, the mRNA level of Col II also significantly

decreased in the E_2 group compared with the C group (Fig. 6C, $P<0.01$), while that of Col II increased significantly in the E_2+ICI and E_2+Meth group compared with E_2 group (Fig. 6C, both $P<0.01$).

TNF-α and IL-1 are potent pro-inflammatory cytokines in the development of OA. As shown in Fig. 6C, compared with the C group, the mRNA level of TNF-α and IL-1 were significantly higher in the E_2 group (Fig. 6C, $P<0.01$; $P<0.01$). However, both the ER antagonist and HIF2α translation inhibitor significantly reversed the E_2-induced increasing trend of TNF-α and IL-1 (Fig. 6C, all $P<0.01$) mRNA level.

3.7. Both pan-ER antagonist and HIF2α translation inhibitor reversed the increased expression of HIF2α and its' downstream OA-related factors induced by E_2 in condylar primary chondrocytes

As shown in Fig. 6, compared with the C group, E_2 significantly increased the protein and mRNA level of HIF2α in primary chondrocytes (Fig. 6B and 6C, both $P<0.01$). But the protein and mRNA level of HIF2α in the E_2+ICI group (oestrogen with pan-oestrogen receptor antagonist ICI182780 stimulation) were significantly lower than that in the E_2 group (Fig. 6B and 6C, both $P<0.01$). The stimulation of HIF2α translation inhibitor significantly decreased the elevated protein level of HIF2α caused by E_2 (Fig. 6B, $P<0.01$), while no significant difference of HIF2α mRNA level was found between E_2 and E_2+Meth group (oestrogen with HIF2α translation inhibitor Meth stimulation) (Fig. 6C).

The downstream OA-related factors of HIF2α (VEGF, MMP-13 and Col X) showed a similar pattern of change with HIF2α. As shown in Fig. 6, the protein and mRNA level of VEGF, MMP-13 and Col X were significantly higher in the E_2 group compared with the C group (Fig. 6B and 6C, VEGF: $P<0.05$, $P<0.01$; MMP-13: both $P<0.01$; Col X: both $P<0.01$). Likewise, compared with the E_2 group, the protein and mRNA level of VEGF, MMP-13 and Col X were significantly lower in the E_2+ICI

group (Fig. 6B and 6C, VEGF: $P<0.05$, $P<0.01$; MMP-13: both $P<0.01$; Col X: both $P<0.01$) and E_2+Meth group (Fig. 6B and 6C, VEGF: both $P<0.01$; MMP-13: both $P<0.01$; Col X: both $P<0.01$).

Interestingly, like the pan-ER antagonist ICI182780, the SERMs such as tamoxifen and endoxifen could also reverse the decreased mRNA level of aggrecan and Col II, and the increased mRNA level of IL-1, TNF-α, HIF2α and its' downstream OA-related factors VEGF, MMP-13 and Col X caused by oestrogen stimulation (supplemental Fig 2, all $P<0.05$).

3.8. E_2 caused degenerative changes and increased expression of HIF2α and its' downstream OA-related factors in condylar primary chondrocytes via ERβ

As shown in Fig. 7, compared with the C group, both E_2 and ERβ-specific agonist Erteberel significantly decreased the protein and mRNA level of aggrecan while increased that of HIF2α and its' downstream OA-related factor, VEGF, in the condylar primary chondrocytes (Fig. 7B and 7D, all $P<0.05$). Moreover, compared with the E_2 group, the protein and mRNA level of aggrecan were significantly higher while that of HIF2α and VEGF were significantly lower in the E_2+PHTPP group (Fig. 7D, aggrecan: $P<0.01$, $P<0.05$; HIF2α: both $P<0.01$; VEGF: both $P<0.01$). However, neither ERα-specific agonist Propyl pyrazole triol nor ERα-specific antagonist MPP significantly influenced the changed expression of aggrecan, HIF2α and VEGF compared with C and E_2 group respectively.

4. Discussion

TMJ is a load-bearing articulation with its load mainly from elevators, the function of which is adjusted by occlusion through feedback from mechanoreceptors in periodontal tissues. Occlusal abnormalities are considered as one possible factor contributing to TMJ OA [22]. As the most severe form of TMD, TMJ OA is characterized by loss of extracellular matrix, accelerated chondrocyte hypertrophy,

and overexpression of pro-inflammatory and cartilage-catabolic cytokines in condylar cartilage [5, 6]. In this study, UAC procedure induced OA-like lesions at 8 and 12 weeks after initiation, including chondrocyte derangement, decreased cartilage thickness (especially in pre-hypertrophic and hypertrophic layer), obvious loss of cartilage extracellular matrix (Col II, proteoglycan), up-regulation of pro-inflammatory (TNF-α) and cartilage-catabolic cytokines (MMP-13 and VEGF), and accelerated chondrocyte hypertrophy (up-regulation of Col X). These results are consistent with our previous studies [8, 21, 23]. It is also indicated that the UAC model is a useful animal model for evaluating the effect of different intervention factors on the prognosis of OA. Interestingly, the present results and our previous studies show that the OA-like or remodelling lesions in condylar cartilage usually appear in hypertrophic and pre-hypertrophic layer. It is different from OA in other appendicular joints (such as knee) in which OA lesions usually appear in fibrous layer firstly [24]. The distinct difference may be due to the different types of cartilage covered subchondral bone, with fibro-cartilage in condyle and hyaline-cartilage in other appendicular joints. It is suggested that TMJ possesses remarkable remodelling capacity due to the existence of fibro-cartilage [25].

It has been suggested that oestrogen plays an important role in maintaining condylar cartilage homeostasis and influencing the progression of TMJ OA. Several *in vitro* studies of condylar chondrocytes or condylar cartilage have shown that the treatment of supra-physiological E_2 (10^{-6} M) could significantly inhibit the chondrocyte proliferation, DNA and proteoglycan synthesis of mandibular condylar chondrocytes, decrease the expression of extracellular matrix (proteoglycan) and cartilage thickness, and up-regulate the expression of pro-inflammatory cytokines (IL-1β, IL-6 and IL-8) and hypertrophic factors (Col X) [26-29]. These results suggest that excessive oestrogen has the potential to cause degenerative changes of condylar cartilage. However, lack of oestrogen induced by ovariectomy increases condylar cartilage thickness, but decreases the bone volume of subchondral bone [12]. In the

present study, our *in vivo* experiment applied the OVX procedure and E_2 supplement after OVX to the female rats in OVX+UAC and E+UAC group respectively, causing a continuous lack of oestrogen and high plasm concentration of oestrogen. Compared with the UAC or Sham+UAC group, high level of oestrogen significantly aggravated cartilage degradation in TMJ OA induced by UAC, which was characterised by further decreased cartilage thickness, increased loss of the extracellular matrix (Col II and proteoglycan), increased expression of pro-inflammatory (TNF-α), catabolic (MMP-13 and VEGF) and hypertrophic (Col X) factors. While lack of oestrogen significantly alleviated the UAC-induced cartilage degradation, which was characterised by increasing the cartilage thickness and expression of extracellular matrix (Col II and proteoglycan), but decreasing the expression of pro-inflammatory (TNF-α), catabolic (MMP-13 and VEGF) and hypertrophic (Col X) factors. These results are consistent with our previous study which have revealed that high-physiological oestrogen aggravated condylar cartilage degradation induced by UAC in gonad-intact female rats [9], and the study of Wang XD et al in which high level of oestrogen aggravated cartilage degenerative changes induced by monosodium iodoacetate injection [30]. Also, the present *in vitro* study demonstrated that high concentration of E_2 (10^{-6} M) led to loss of extracellular matrix (proteoglycan and Col II), up-regulation of pro-inflammatory (TNF-α and IL-1) and increased expression of HIF2α and its' down-stream OA-related factors (VEGF, MMP-13 and Col X) in primary chondrocyte, which could be significantly reversed by pan-ER antagonist ICI182780, SERMs, and ERβ-specific antagonist PHTPP. Meanwhile, the ERβ-specific agonist Erteberel could also individually lead to the destructive effect (decreased expression of proteoglycan) on the primary chondrocytes as E_2. It is indicated that the effect of oestrogen on degenerative changes of condylar cartilage play in a biphasic manner, and the effect of oestrogen is mediated mainly by ERβ.

Hypoxia is another important factor influencing the homeostasis of condylar cartilage and the progression of OA. In recent years, some researchers have focused

on the interaction between oestrogen and hypoxia in the neovascularization which is also an important pathological basis of OA progression [16, 31-33]. George AL et al have demonstrated that exogenous oestrogen could increase the expression of HIF1α in breast tissues, and then up-regulate the level of VEGF to promote angiogenesis, which could be blocked by both ER antagonist (ICI182780) and HIF1 inhibitor (YC-1) [20]. Another study of endometrial cancer has also shown that at normoxic condition, oestrogen could not increase the expression of VEGF due to the degradation of HIF1α caused by pVHL, but the oestrogen could trigger the nuclear translocation of HIF1α to elevate the level of VEGF when HIF1α was stabilized by $CoCl_2$ [33]. Specific deletion of pVHL of mice could continuously stabilize the activity of HIF1α and HIF2α, which could reverse the dysfunction of neovascularization and bone formation caused by OVX procedure [19]. Furthermore, it has been found that anti-ERα IgG could coimmunoprecipitate FLAG-HIF2αDM with ERα both in the presence and absence of E_2, and that GST pull-down assays showed that GST-HIF2αDM (396–823) physically interacted with ERα-E and that E_2 increased their interaction, which indicates that HIF2α might form a protein complex with ERα [34]. These results suggest that there is obvious interaction between oestrogen and HIF in the progression of neovascularization. Coincidentally, both oestrogen and HIF2α are related to the degenerative changes of osteoarthritic cartilage which is related closely to neovascularization. In the present *in vivo* study, with the progression of OA, high level of oestrogen could further up-regulate the UAC-induced elevated expression of HIF2α and its down-stream OA-related cytokines (VEGF, MMP13 and Col X), and aggravate the destructive changes of condylar cartilage at the same time. On the contrary, lack of oestrogen induced by OVX could significantly down-regulate the UAC-induced elevated expression of HIF2α and its down-stream OA-related cytokines (VEGF, MMP13 and Col X) with alleviating the degenerative changes of condylar cartilage. Furthermore, in the present *in vivo* study, the ICI182780, PHTPP, tamoxifen, endoxifen and HIF2α translation inhibitor could significantly reverse the

elevated expression of HIF2α and its down-stream OA-related cytokines (VEGF, MMP13 and Col X) of condylar chondrocytes induced by high concentration of E_2 (10^{-6} M), and then maintain the stability of extracellular matrix. Furthermore, the ERβ-specific agonist Erteberel only could also significantly enhance the expression of HIF2α, VEGF, MMP13 and Col X, and disturb the stability of extracellular matrix as E_2. It is indicated that oestrogen could regulate the expression of HIF2α and its down-stream factors, and then regulate the homestasis of condylar cartilage via oestrogen-ERβ-HIF2α pathway. In 2015, nicotinamide phosphoribosyltransferase (NAMPT or visfatin), a direct target of hypoxia-inducible factor-2α, was proved to be an essential catabolic regulator of osteoarthritis [35]. Given that ICI182780 is also known to promote bone destruction [36], in the future, SERMs or commercial drugs directly targeting HIF2α or its downstream effectors such as NAMPT, may be introduced into clinic for therapeutic strategies to abrogate cartilage destruction in osteoarthritis.

In conclusion, during TMJ OA progression, high level of oestrogen could aggravate the degenerative changes of condylar cartilage, while lack of oestrogen could alleviate it via oestrogen-ERβ-HIF2α pathway, which may be a new therapeutic target in future OA treatment.

Competing interests: The authors do not have any conflicts of interest to report.

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Figure Captions

Fig. 1 (A). Experimental schedule and grouping design. (B). Uterus and body weights of the different groups (n=12). #: *P*<0.05 vs Sham+UAC subgroup, ##: *P*<0.01 vs Sham+UAC subgroup.

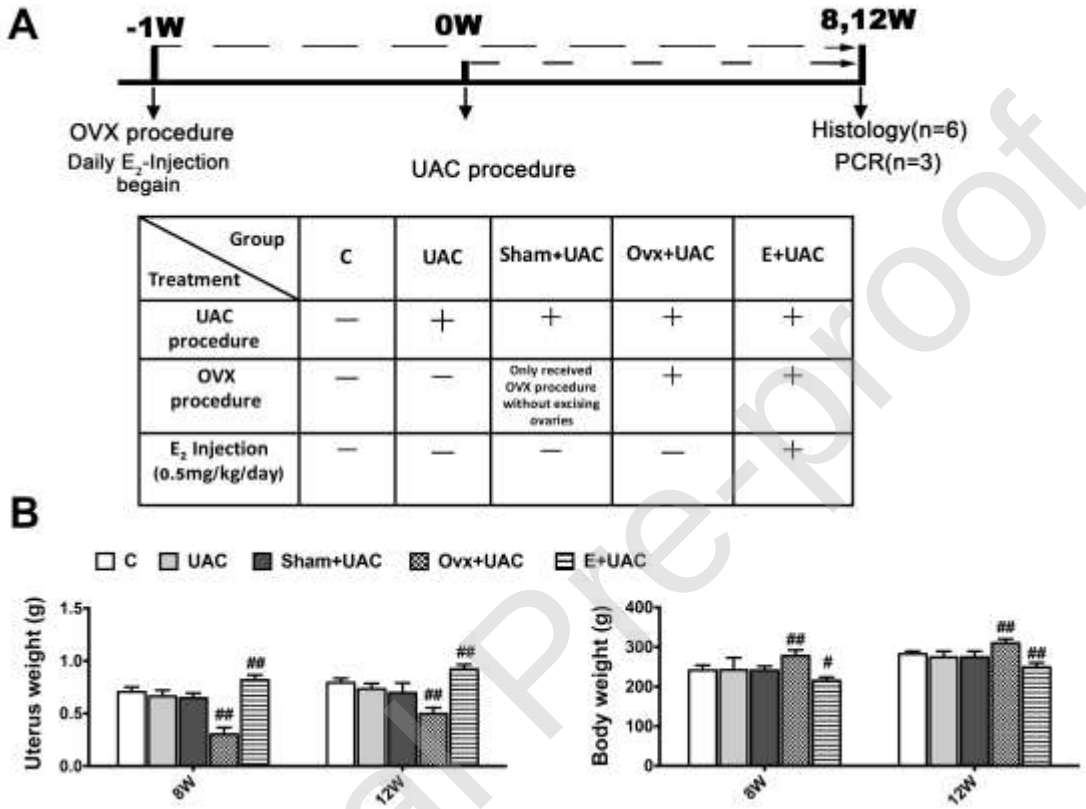

Fig. 2 (A). H&E, Safranin O and Col II-immunohistochemical staining of condylar cartilage. (B). Comparisons of cartilage thickness in the centre and posterior thirds of the TMJ condylar cartilage among different groups (n=6). (C). Comparison of the percentage of Safranin O and Col II-positive area among different groups (n=6). *: *P*<0.05 vs C subgroup, **: *P*<0.01 vs C subgroup; #: *P*<0.05 vs Sham+UAC subgroup, ##: *P*<0.01 vs Sham+UAC subgroup.

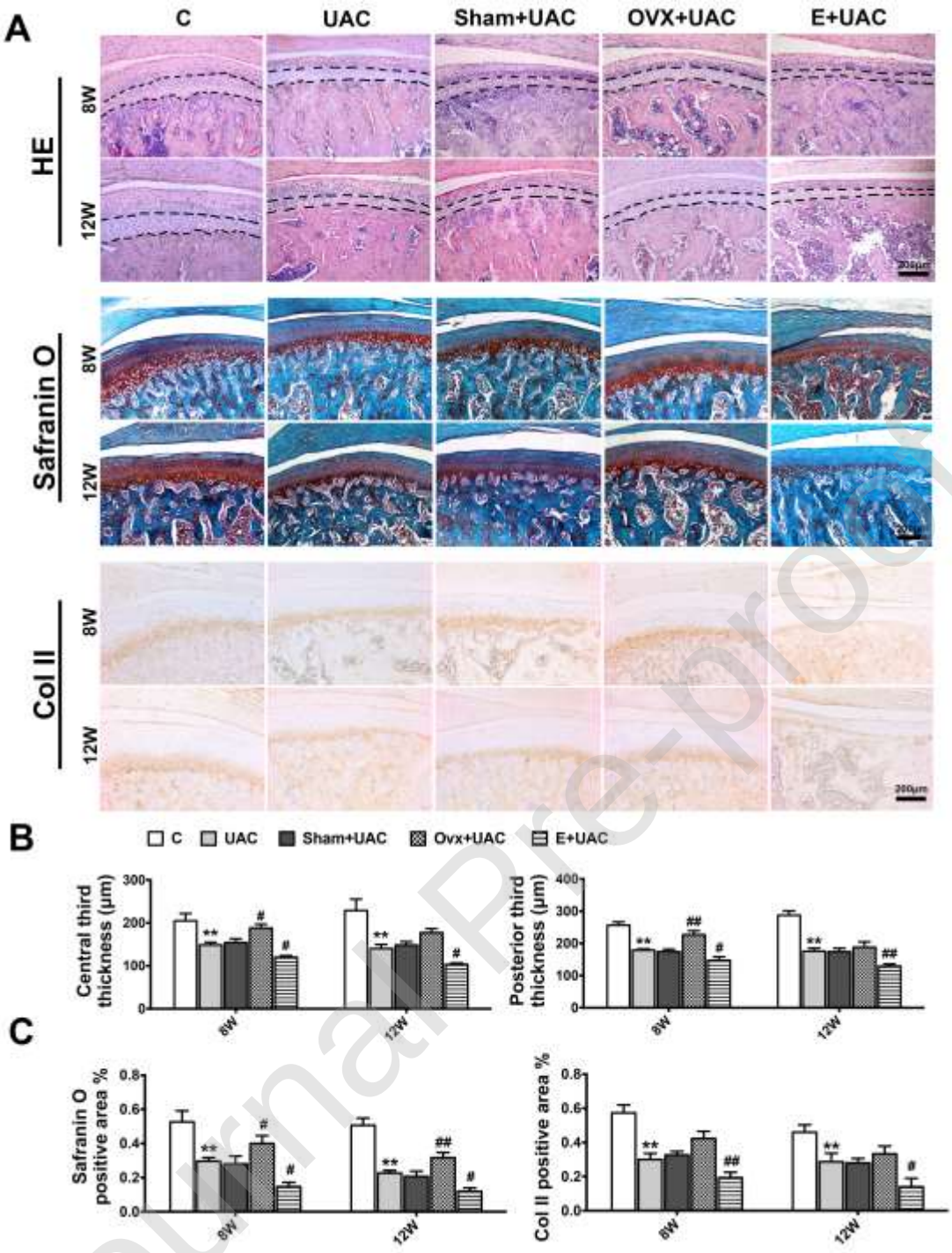

Fig. 3 (A, B). TNF-α immunohistochemical staining of condylar cartilage and comparisons of the percentage of TNF-α positive chondrocytes among different groups (n=6). (C). Comparisons of the TNF-α mRNA level of condylar cartilage among different groups (n=3). *: P<0.05 vs C subgroup, **: P<0.01 vs C subgroup; $^{\#}$: P<0.05 vs Sham+UAC subgroup, $^{\#\#}$: P<0.01 vs Sham+UAC subgroup.

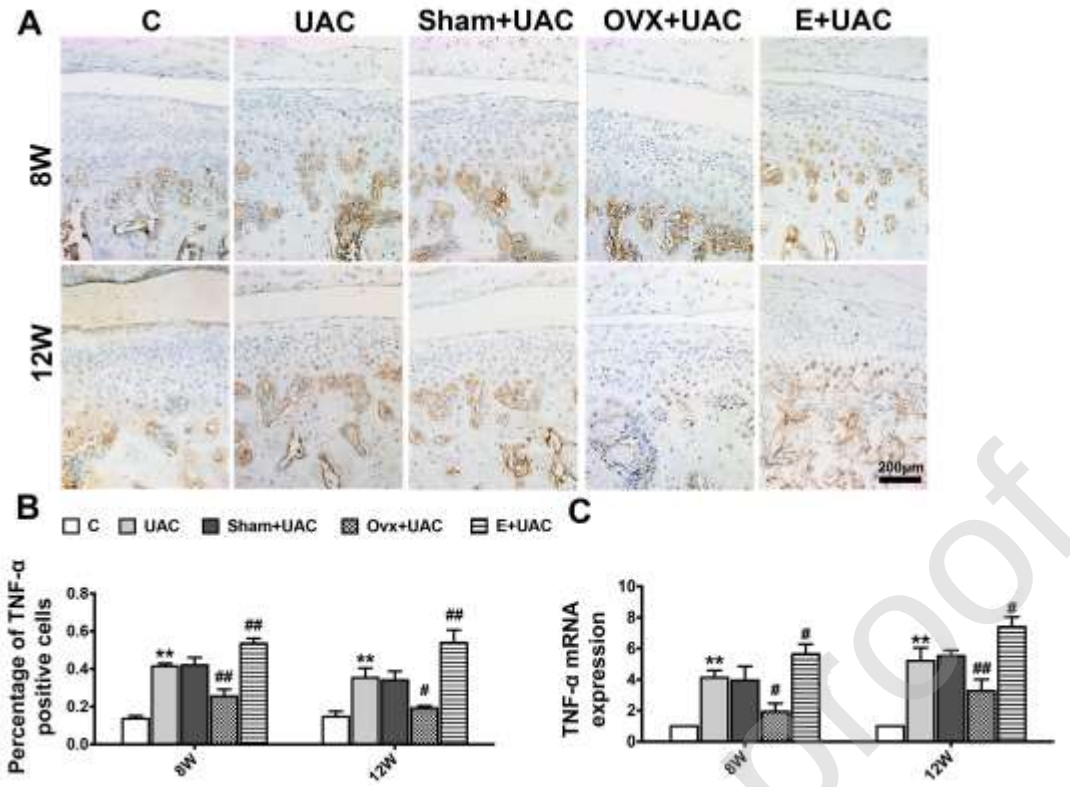

Fig. 4 (A). HIF2α and VEGF immunohistochemical staining of condylar cartilage. (B). Comparisons of the percentage of HIF2α and VEGF positive chondrocytes among different groups (n=6). (C). Comparisons of the HIF2α and VEGF mRNA level of condylar cartilage among different groups (n=3). *: P<0.05 vs C subgroup, **: P<0.01 vs C subgroup; $^{\#}$: P<0.05 vs Sham+UAC subgroup, $^{\#\#}$: P<0.01 vs Sham+UAC subgroup.

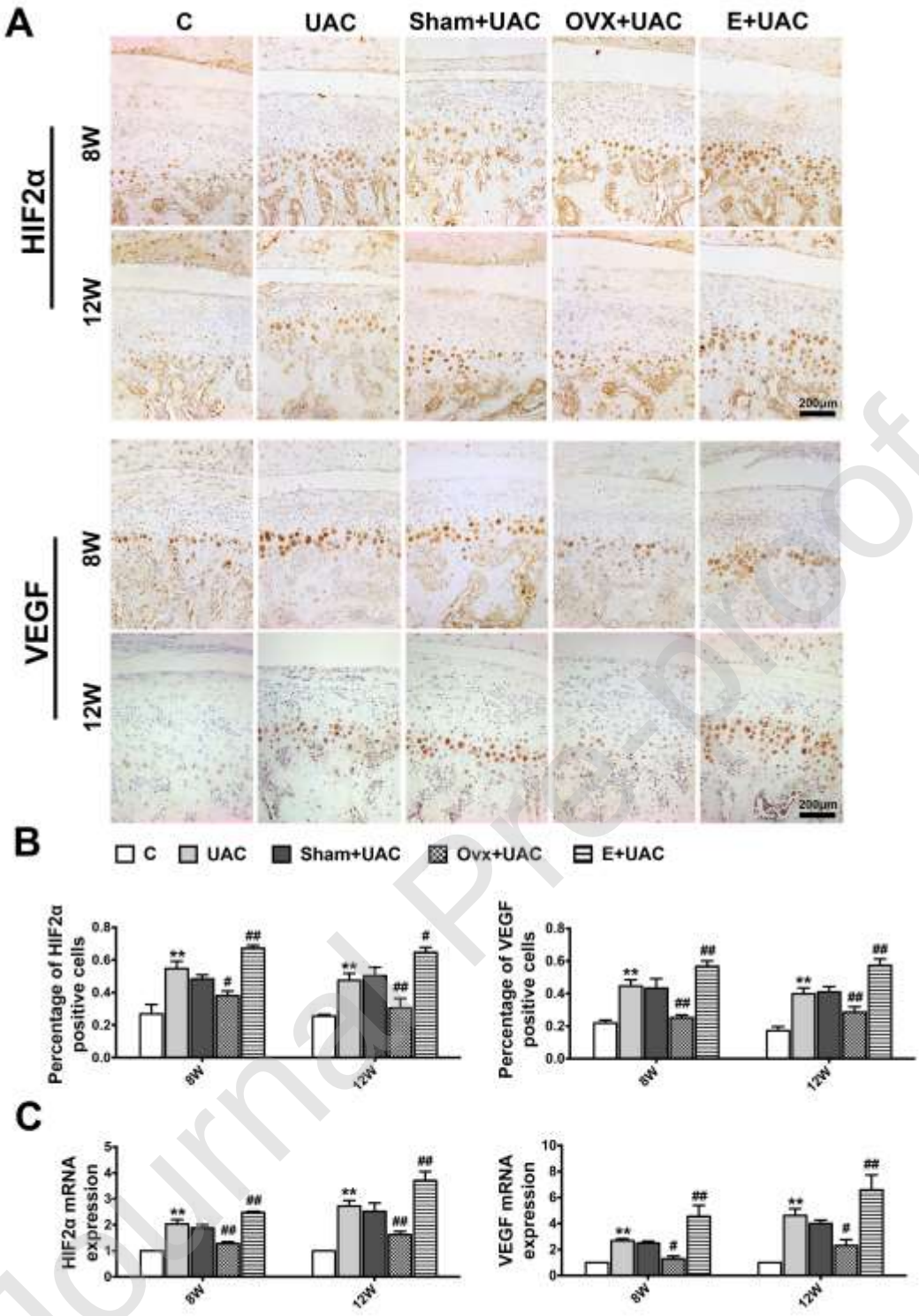

Fig. 5 (A). MMP-13 and Col X immunohistochemical staining of condylar cartilage. (B). Comparisons of the percentage of MMP-13 and Col X positive chondrocytes among different groups (n=6). (C). Comparisons of the MMP-13 and Col X mRNA level of condylar cartilage among different groups (n=3). *: $P<0.05$ vs C subgroup, **:

P<0.01 vs C subgroup; #: *P*<0.05 vs Sham+UAC subgroup, ##: *P*<0.01 vs Sham+UAC subgroup.

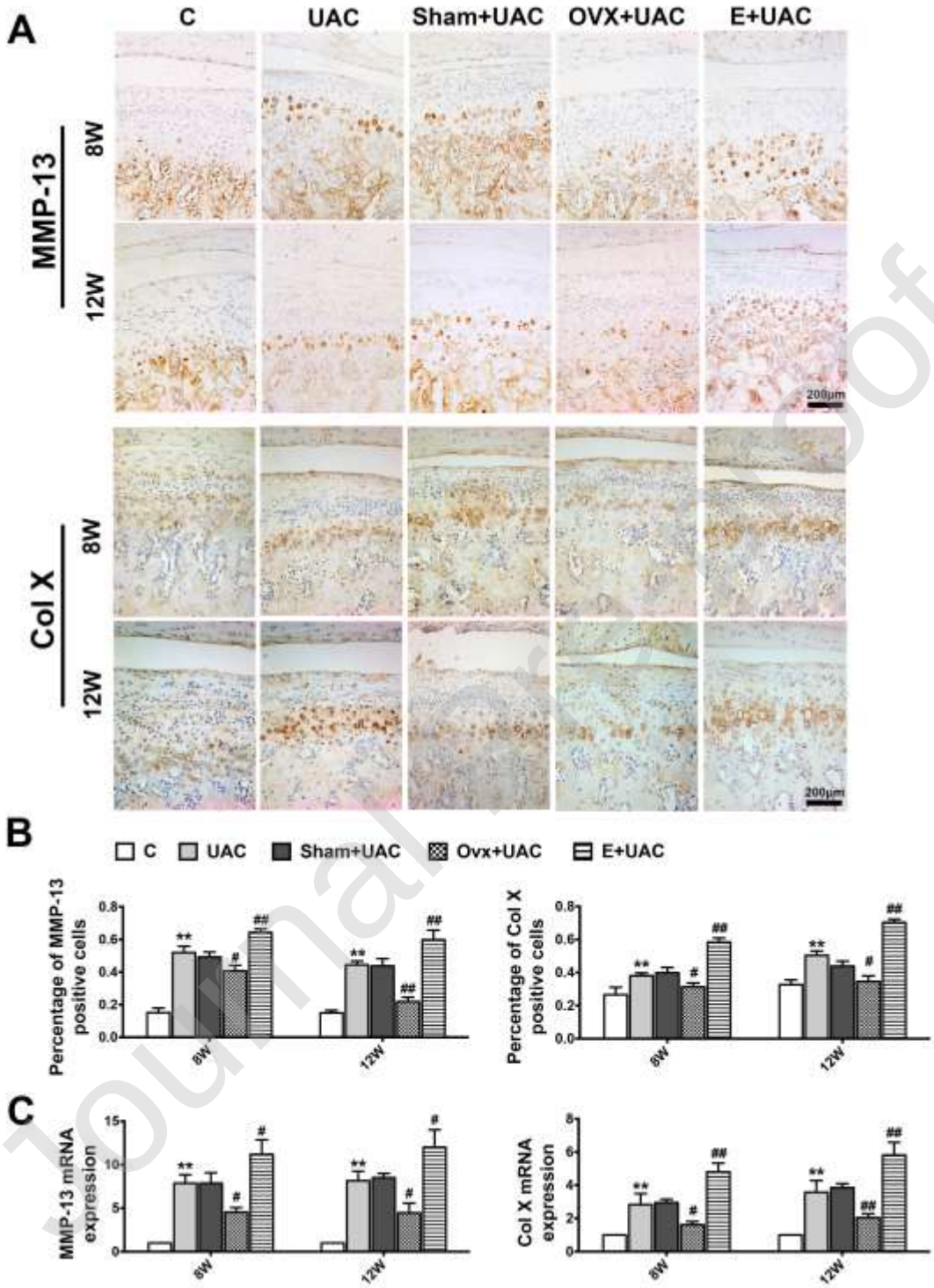

Fig. 6 (A). The protein level of aggrecan, HIF2α, VEGF, MMP-13 and Col X in

condylar primary chondrocyte indicated by western blot. (B). Comparisons of the protein level of aggrecan, HIF2α, VEGF, MMP-13 and Col X in condylar primary chondrocyte among different groups (n=3). (C). Comparisons of the mRNA level of aggrecan, Col II, TNF-α, IL-1, HIF2α, VEGF, MMP-13 and Col X in condylar primary chondrocyte among different groups (n=3). *: P<0.05 vs control group, **: P<0.01 vs control group; $^{\#}$: P<0.05 vs E_2 group, $^{\#\#}$: P<0.01 vs E_2 group.

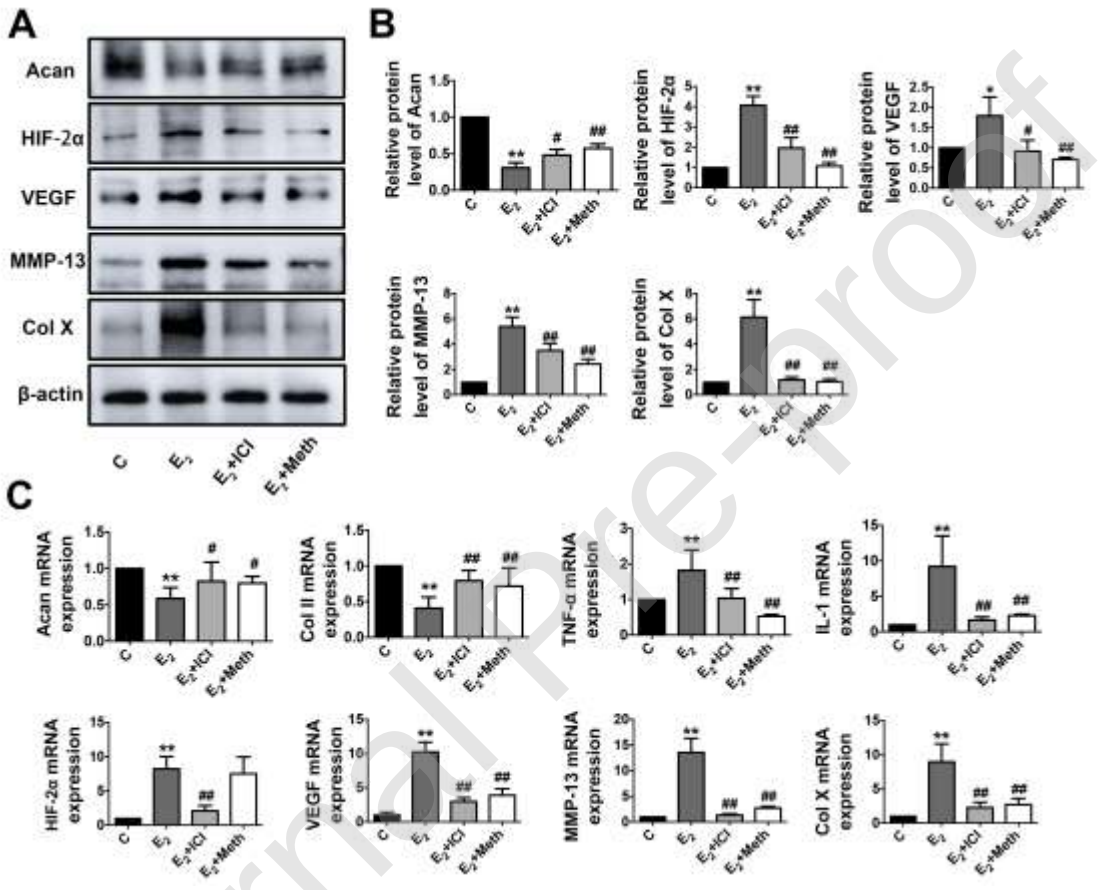

Fig. 7 (A) and 7(C). The protein level of aggrecan, HIF2α, VEGF in condylar primary chondrocyte indicated by western blot. (B) and (D). Comparisons of the protein and mRNA level of aggrecan, HIF2α, VEGF in condylar primary chondrocyte among different groups (n=3). *: P<0.05 vs control group, **: P<0.01 vs control group; $^{\#}$: P<0.05 vs E_2 group, $^{\#\#}$: P<0.01 vs E_2 group.