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Original Article

Protective effect of estrogen against calcification in the cartilage endplate

Bin Sheng, Jinhua Zhou, Xiangyang Liu, Youchao Yuan, Yi Zhang, Hongzhe Liu, Shuai Peng, Bin Liu, Lei Chang

Department of Spine Surgery, Hunan Provincial People's Hospital, The First Affiliated Hospital of Hunan Normal University, Changsha, China

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Abstract: Clinical studies report that endogenous estrogen depletion is associated with disc degeneration. The present study aimed to investigate the effect and mechanism of estrogen on disc degeneration of the cartilage endplate. Three groups of mice with bilateral ovariectomy + 17β-estradiol injection (OVX + E2 Group), bilateral ovariectomy + vehicle injection (OVX + vehicle), or sham operation + vehicle injection (Sham Group) were included in this study. The mice were sacrificed at 12 weeks and the cartilage endplate (CEP) were harvested. The calcification status was evaluated by Alizarin red staining and RT-PCR, which demonstrated the calcification level of the CEP gradually developed from the Sham Group, OVX + E2, to the OVX + vehicle group. The CEP cells were isolated, cultured, and treated with IL-1β (75 ng/ml) for 24 h, with or without a pretreatment of 17β-E2 for 1 h. RT-PCR analysis of calcification-related genes ALP, OCN, RUNX2, and COL-I were analyzed, and calcification of CEP cells induced by IL-1β was reversed by pretreatment with 17β-E2, in a dose-dependent manner. The protective effect of 17β-E2 was abolished by estrogen receptor antagonist ICI182,780. These results suggest that decreased estrogen level may accelerate degeneration of the cartilage endplate by increasing calcification, which may be induced by IL-1β, in a dose-dependent manner.

Keywords: Estrogen, intervertebral disc, cartilage endplate, calcification

Introduction

The postmenopausal state is associated with the development of some gender-specific diseases such as osteoarthritis and lumbar degenerative spondylolisthesis, with similar characteristics of cartilage damage (the vertebral endplate, and the intervertebral disc). Clinical studies report that the incidence and severity of disc degeneration in elderly women are higher than those in age-matched men, which may because of the endogenous estrogen depletion [1, 2].

The intervertebral disc is a non-vascular structure consists of the cartilage endplate (CEP), nucleus pulposus (NP), and annulus fibrosus (AF). The cartilage endplate consists of the chondrocyte-like cells with low matrix-forming ability and no proliferative capacity [3]. The endplate also contains marrow contact channels (MCC) with the ability to provide nutrients and exchange metabolic wastes by diffusion and liquid flow, causing direct effects on growth, proliferation, metabolism, and apoptosis of NP cells [4-6]. Calcification of the cartilage endplate may be an important reason for the impairment of nutrient exchange and the speed of disc degeneration [7]. However, the possibility of the association between the estrogen level and the cartilage cells calcification is still unknown.

Cartilaginous tissues are estrogen-sensitive as estrogen receptors (ERs) are found in chondrocytes [8]. 17b-estradiol (E2) was also found to be able to enhance glycosaminoglycan synthesis in chondrocytes in cultured rabbit chondrocytes [9]. Cyclooxygenase-2 expression was also found to be suppressed by E2 in the chondrocytes and protect from reactive oxygen-induced damage [10, 11]. Furthermore, estrogen, especially E2 was found to have various effects on chondrocytes like proliferation and differentiation [12]. Consequently, the study aimed to investigate a possible relationship

between estrogen level and calcification of the cartilage endplate.

Experiments were performed to explore the possible mechanism. Pro-inflammatory cytokines were found to be able to induce degradation of skeletal tissues, especially cartilage extracellular matrices [13]. Ferreira et al. reported IL-1β was able to induce calcification of the bone marrow-derived mesenchymal stem cells, despite suppression of osteoblastic differentiation [14]. Another study also suggested that IL-1β suppressed not only the osteoblastic but also the negative regulators of soft-tissue calcification, which may contribute to mechanisms of heterotopic ossification in various disorders [15]. Moreover, previous studies also demonstrate that E2 can protect AF cells and NP cells against apoptosis induced by interleukin-1β (IL-1β) [16-18]. It is interesting whether the IL-1β can induce the calcification of CEP and whether the E2 could also protect the CEP against IL-1β. Therefore, we here hypothesized that estrogen can protect the EP from calcification induced by IL-1β.

Materials and methods

Animals

Twenty-four female Sprague Dawley rats (weighing 180-240 g) were used in the study. The rats were equally assigned into 3 groups randomly. Two groups of mice underwent bilateral ovariectomy (OVX Group) and the remaining group underwent a sham operation (Sham Group). One of the OVX mice groups (OVX + E2) received subcutaneous injections of 17β-estradiol (Sigma, St. Louis, MO) with the dose of 10 μg/kg 3 days post-ovariectomy (5 days/week for 8 weeks). The other two groups (OVX + vehicle, Sham) were treated with vehicle (0.9% NS). The mice were sacrificed at 12 weeks following the standard protocol, and the cartilage endplate were harvested and fixed in 10% buffered formalin. The animal study was approved by the Ethics Committee of the First Affiliated Hospital of Soochow University.

Cell culture and drug treatment

Endplate cartilage from rats was carefully collected with all AF and NP tissues removed. The CEPs were prepared with PBS and cut into pieces, then digested with 0.02% collagenase type II (Invitrogen, USA) and cultured in DMEM supplemented with 5% FBS overnight. Subsequently, the cell suspension was filtered through a 70 μm mesh and resuspended in DMEM/F-12 medium, then cultured at 37°C, with 5% CO_2 and 20% O_2. The CEP cells were passaged for three times and then collected for use.

Six groups of CEP cells were included, consisting of one control (no E2, IL-1β, and ICI182,780), one induction (75 ng/ml IL-1β, but no E2 and ICI182,780) and four treatment groups (0.1 μmol/l 17β-E2 + 75 ng/ml IL-1β, no ICI182,780; 1 μmol/l 17β-E2 + 75 ng/ml IL-1β, no ICI182,780; 10 μmol/l 17β-E2 + 75 ng/ml IL-1β, no ICI182,780; 10 μmol/l 17β-E2 + 10 μmol/l ICI-182,780 + 75 ng/ml IL-1β). The CEP cells were seeded at a density of 1.5×10^4 cells/well with IL-1β (75 ng/ml) for 24 h, with or without a pretreatment of 17β-E2 for 1 h.

Histological staining

Samples were decalcified by 5% formic acid, embedded in paraffin and cut into 5-um sections along the median sagittal plane. The decalcified sections or cultured cell were stained with 1% Alizarin red and counterstaining in hematoxylin. The sections were observed with the Digital Image Analyzer (Ni-E, Nikon, Japan).

RT-PCR analysis

Freshly collected CEP tissues of T12/L1 and L1/2 intervertebral discs or CEP cells were stored in RNAstore reagent and in liquid nitrogen. Total RNA was isolated with TRI REAGENT ® RNA (Molecular Research Center) according to manufacturers' instructions. One μg of total RNA was reverse-transcribed using SuperScript III first-strand synthesis kit (Life Technologies). The PCR end products and GAPDH were analyzed and the DNA bands were visualized with ethidium bromide.

Statistical analysis

Graphpad Prism 7 software was employed in this study. The data was presented as the mean ± standard deviation (SD), and compared by one-way analysis of variance (1 way-ANOVA). *P* values less than 0.05 were considered to be significant.

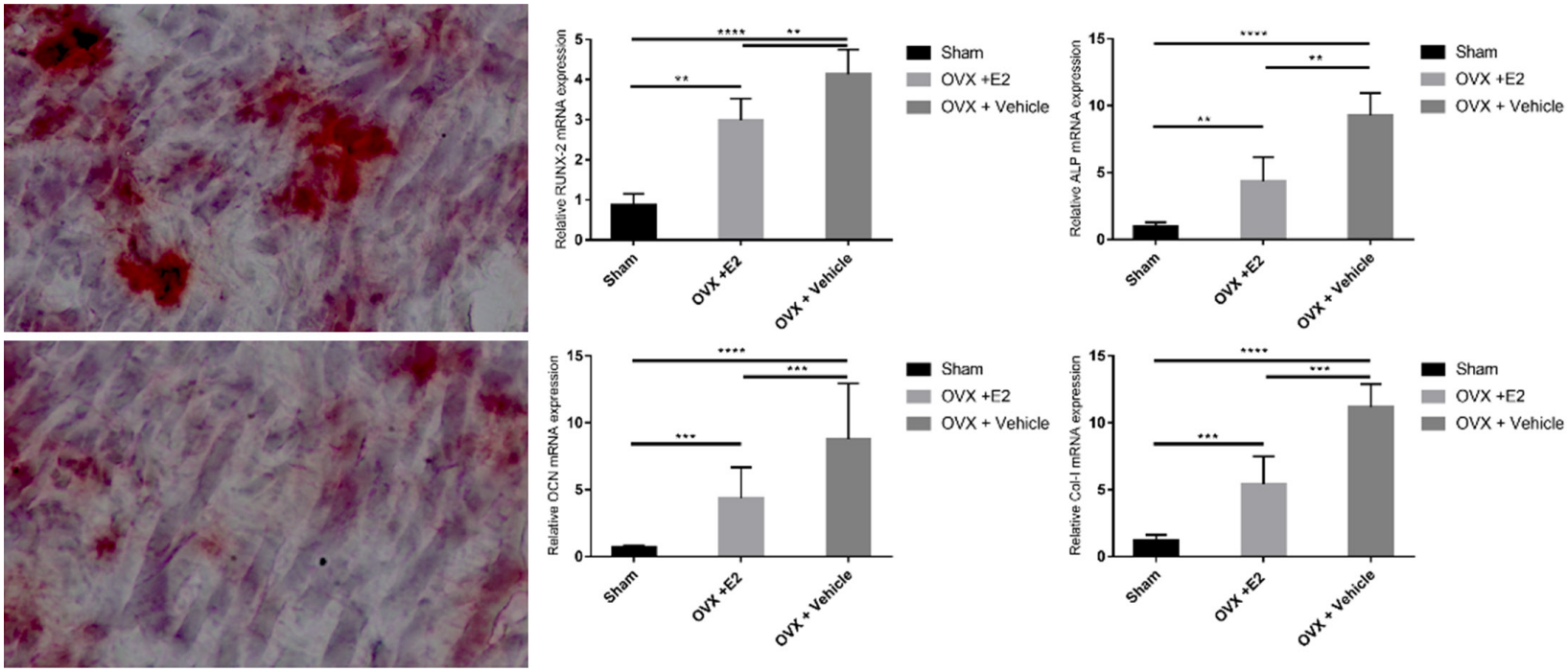

Figure 1. RT-PCR analysis of the mRNA levels of calcification-related genes ALP, OCN, RUNX2, and COL-I in samples of CEP. The expression of calcification-related genes ALP, OCN, RUNX2, and COL-I was significantly higher in the OVX + vehicle group, with the least in the Sham group.

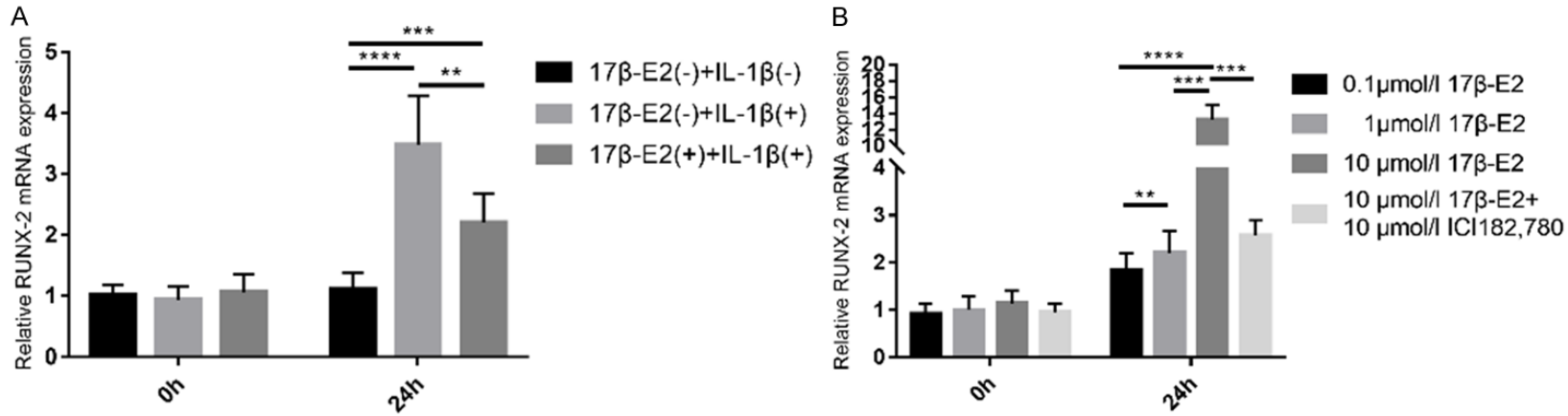

Figure 2. RT-PCR analysis of the mRNA levels of calcification-related genes RUNX2 in CEP cells. A. The group without additional 17β-E2 and IL-1β showed no significantly increased expression of RUNX2 after 24 h of culture. But for the two groups with additional IL-1β, the expression of RUNX2 increased significantly at 24 h compared with 0 h, furthermore, the group without additional 17β-E2 showed significant increased expression of RUNX2 in comparison with the group added with 1 μmol/l 17β-E2. B. Expression of RUNX2 seems to confer a dose-department increase in coordination with the increased concentrations of 17β-E2. Furthermore, expression of RUNX2 decreased significantly with treatment of ICI182,780, an inhibitor of the estrogen receptors. The expression manner of other genes like ALP, OCN, and COL-I was similar and not shown here again. *indicates $P < 0.05$, **indicates $P < 0.01$, and ***indicates $P < 0.001$ ****indicates $P < 0.0001$ based on one-way ANOVA.

Results

Effect of estrogen on calcification of CEP

As shown in the Alizarin red stain (**Figure 1**), the OVX + vehicle group showed more ectopic bone tissue in CEP than the other two groups with histological examination. Next, we used RT-PCR to analyze mRNA levels of calcification-related genes, namely, the ALP, OCN, RUNX2, and COL-I. Similarly, expression of calcification-related genes was significantly higher in the OVX + vehicle group, with the least in the Sham group (**Figure 1**). These results are consistent with the hypothesis that estrogen depletion can accelerate degeneration of CEP by inducing calcification of it.

The protection effect of Estrogen against IL-1β induced calcification is shown in **Figure 2A**. The addition of Il-1β successfully promoted calcification of CEP, as compared between the control and induction Groups. Moreover, estrogen protected the CEP from calcification induced by IL-1β in a dose-dependent manner, as less calcification was observed when more E2 was added to the medium. The protective effect of 17β-E2 was also abolished by estrogen receptor antagonist ICI182,780 (**Figure 2B**).

Discussion

The present study demonstrates that the calcification level of the CEP gradually developed from the Sham Group, OVX + E2, to the OVX + vehicle group. The CEP cells were protected by estrogen from calcification induced by IL-1β in a dose-dependent manner. These results suggested that decreased estrogen level may accelerate the degeneration of the cartilage endplate by increasing calcification, which may be induced by IL-1β, in a dose-dependent manner.

Aged females appear to bear a higher incidence and severity of disc degeneration, when compared with the age-matched males. Similarly, untreated post-menopausal women seem to have a lower intervertebral disc height in comparison with the estrogen replacement treatment females. Taken together, it seems the estrogen level is closely associated with the degeneration of the intervertebral disc [1, 19].

The intervertebral disc is a non-vascular structure consists of cartilage endplate, nucleus pulposus, and annulus fibrosis. The nutrients of the IVD are mainly supplied from two sources. First, the peripheral micro-circulatory system, which only provides the nutrients for the periphery of the IVD. Second, the marrow contact channel of the CEP, which is the most important mechanism that provided most nutrients for the avascular tissues inside the IVD, roles by diffusion and liquid flow to exchange wastes and nutrients [7, 20, 21]. Previous studies demonstrated that loss of the chondrogenic phenotype of the CEP is an important part of the degeneration of IVD, as the number and volume of capillaries or medullary sinuses decreases, which affects the nutrient supply and finally accelerates disc degeneration [22]. As a result, the degeneration of the cartilaginous endplate plays an important role in the degeneration process of IVD. The present study successfully demonstrates that decreased estrogen level may accelerate the degeneration of the cartilage endplate by increasing calcification, which may be an important part of the degeneration of IVD.

IL-1β is a multifunctional inflammatory cytokine in the progression of IVD cell degeneration, which is able to increase the synthesis of matrix-degrading enzymes, decrease the synthesis of proteoglycan, collagen I and collagen II [23, 24]. Previously, Le Maitre et al. identified that a local concentration of IL-1β was present in human IVD degeneration tissues [23]. Similar results were found that the enzyme activity was upreg-

ulated by IL-1β and reduced by its inhibitor, which suggests that the key role of IL-1β is in regulating matrix enzyme activity [25]. In another study, IL-1β was also found able to induce apoptosis of NPCs, and is resisted by E2 [18, 26-28]. Similar results were found as E2 was able to induce NP cell proliferation and protect against IL-1β-induced apoptosis [17].

Estrogen has various effects all around the body, including the reproductive system, neurotransmitter release, bone structure, cognitive function, and blood vessels [29, 30]. Estrogen has been proved to have various functions on chondrocytes, from metabolism, proliferation, to differentiation [12, 31-33]. For example, previous studies have demonstrated that chondrocytes can be stimulated to synthesize proteoglycan and glycosaminoglycan by additional estrogen, which is necessary for cartilage function [9, 12, 34, 35]. It was also found able to protect the chondrocytes from reactive oxygen-induced damage by suppressing cyclooxygenase-2 expression [10, 11]. Moreover, the ERa/GRIP1 pathway may have protective roles on cartilage metabolism via regulating the extracellular matrices metabolism [36]. Similarly, 17b-estradiol has been detected to promote human AF cells proliferation and to exert anti-apoptotic effects [6, 16, 37].

Conclusion

In conclusion, the results of the current study have shown that decreased estrogen level may accelerate degeneration of the cartilage endplate by increasing calcification, which may be induced by IL-1β, in a dose-dependent manner.

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Disclosure of conflict of interest

None.

Address correspondence to: Bin Sheng, Department of Spine Surgery, Hunan Provincial People's Hospital, The First Affiliated Hospital of Hunan Normal University, 61 Jiefang West Road, Changsha, China. E-mail: ayej47938320035@126.com

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