• Basic Research •

17β-estradiol inhibits TGF-β-induced collagen gel contraction mediated by human Tenon fibroblasts *via* Smads and MAPK signaling pathways

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Abstract

● **AIM:** To investigate the impact of 17β-estradiol on the collagen gels contraction (CGC) and inflammation induced by transforming growth factor (TGF)-β in human Tenon fibroblasts (HTFs).

● **METHODS:** HTFs were three-dimensionally cultivated in type I collagen-generated gels with or without TGF-β (5 ng/mL), 17β-estradiol (12.5 to 100 μmol/L), or progesterone (12.5 to 100 μmol/L). Then, the collagen gel diameter was determined to assess the contraction, and the development of stress fibers was analyzed using immunofluorescence staining. Immunoblot and gelatin zymography assays were used to analyze matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) being released into culture supernatants. Enzyme-linked immunosorbent assay (ELISA) and reverse transcription-quantitative polymerase chain reaction (RT-PCR) were used to detect interleukin (IL)-6, monocyte chemoattractant proteins (MCP)-1, and vascular endothelial growth factor (VEGF) in HTFs at the translational and transcriptional levels. The phosphorylation levels of Sma- and Mad-related proteins (Smads), mitogen-activated protein kinases (MAPKs), and protein kinase B (AKT) were measured by immunoblotting. Statistical analysis was performed using either the Tukey-Kramer test or Student's unpaired *t*-test to compare the various treatments.

● **RESULTS:** The CGC caused by TGF-β in HTFs was significantly inhibited by 17β-estradiol (25 to 100 μmol/L), and a statistically significant difference was observed when comparing the normal control group with 17β-estradiol concentrations exceeding 25 μmol/L (P<0.05). The suppressive impact of 17β-estradiol became evident 24h after administration and peaked at 72h (P<0.05), whereas progesterone had no impact. Moreover, 17β-estradiol attenuated the formation of stress fibers, and the production of MMP-3 and MMP-1 in HTFs stimulated by TGF-β. The expression of MCP-1, IL-6, and VEGF mRNA and protein in HTFs were suppressed by 100 μmol/L 17β-estradiol (P<0.01). Additionally, the phosphorylation of Smad2 Smad3, p38, and extracellular signal-regulated kinase (ERK) were downregulated (P <0.01).

● **CONCLUSION:** 17β-estradiol significantly inhibits the CGC and inflammation caused by TGF-β in HTFs. This inhibition is likely related to the suppression of stress fibers, inhibition of MMPs, and attenuation of Smads and MAPK (ERK and p38) signaling. 17β-estradiol may have potential clinical benefits in preventing scar development and inflammation in the conjunctiva.

● **KEYWORDS:** Tenon fibroblasts; transforming growth factor-β; 17β-estradiol; fibrosis; wound healing

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INTRODUCTION

Contraction is an integral part of the wound healing process. However, excessive contraction can cause scarring. Scarring is related to the etiology or treatment failure in most blindness problems and plays a crucial role

in many systemic ailments. Subconjunctival scarring in the eye can impact not only surgery outcomes, such as glaucoma filtration surgery, strabismus, or pterygia, but also the prognosis of ocular surface sicknesses, including chemical burns and trauma[1-2]. This process is aided by the infiltration of inflammatory cells caused by various cytokines or chemokines. Thus, to explore novel pharmacological treatments, a deeper and more comprehensive understanding of the mechanisms of conjunctival healing and contraction, as well as the impact of various scarring modulators, is needed.

Sex hormones are steroid hormones that perform many functions in the regulation of body homeostasis. Progesterone and 17β-estradiol are the primary sexually active hormones in females. Meanwhile, the receptors that specifically interact with both hormones are also present in the eyes[3]. An imbalance in the relative levels of sex hormones in the circulation can cause inflammatory processes on the ocular surface and might contribute to ocular pathologies such as dry eye disease[4]. Our previous research has shown that retinal pigment epithelium (RPE) cell-mediated collagen contraction is significantly inhibited by female sex hormones[5]. The function of sex hormones in conjunctival wound healing *via* human Tenon fibroblasts (HTFs) remains unclear.

As a potential growth-promotive cytokine, transforming growth factor (TGF)-β can be produced by various cells in the inflammatory environment, such as fibroblasts and macrophages, and induce fibrosis by stimulating the migration and proliferation of fibroblasts[6-7]. Both TGF-β_1 and TGF-β_2 isoforms are involved in post-trabeculectomy scarring. However, *in vitro* investigations of HTFs have suggested that TGF-β_1 is significantly upregulated[8]. HTFs are important in subconjunctival wound healing and have become a significant focus for the development of antifibrotic treatments. As a model of wound contraction mediated by cells, collagen gels contraction (CGC) has been explored *in vitro*. We have previously shown that triptolide can effectively suppress the CGC caused by TGF-β in HTFs[9]. Herein, we explored the influence of progesterone and 17β-estradiol on the CGC induced by TGF-β using this model system. We analyzed the functions of 17β-estradiol in the synthesis of matrix metalloproteinases (MMPs), tissue inhibitors of metalloproteinases (TIMPs), stress fibers, the release of cytokines and chemokines, and the molecular pathways involved.

MATERIALS AND METHODS

The Eagle's minimum essential medium (MEM), fetal bovine serum (FBS), 10×MEM, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPEs), phosphate-buffered saline (PBS), and trypsin-ethylene diamine tetraacetic acid (EDTA) (0.25%) were supplied by Invitrogen (USA). Bovine serum albumin (BSA) was purchased from Nacalai Tesque (Kyoto, Japan). The reconstitution buffer and the acid-solubilized native porcine type I collagen were supplied by Nitta Gelatin (Japan). Progesterone, 17β-estradiol, and protease inhibitor cocktail were supplied by Sigma (USA). The recombinant human TGF-β protein and antibodies for MMPs were supplied by R&D (USA). The bicinchoninic acid assay kit was obtained from Beyotime Biotechnology (China). Enzyme-linked immunosorbent assay (ELISA) kits for interleukin (IL)-6, monocyte chemoattractant proteins (MCP)-1, and vascular endothelial growth factor (VEGF) were sourced from BOSTER (Wuhan, China). TIMP-1 and TIMP-2 antibodies were supplied by BOSTER (Wuhan, China). Rabbit or mouse monoclonal antibodies specific for Sma- and Mad-related protein (Smad) 2, phospho-Smad2, Smad3, phospho-Smad3, c-Jun N-terminal kinase (JNK), phospho-JNK, p38, phospho-p38, extracellular signal-regulated kinase (ERK), phospho-ERK, protein kinase B (AKT), and phospho-AKT were bought from Cell Signaling Technology (Beverly, MA, USA). Antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was supplied by Proteintech (USA). The Rhodamine Phalloidin and 4'6-diamidino-2-phenylindole (DAPI) dyes were supplied by Cytoskeleton and Molecular Probes. All reagents and media used in this study for cell culture were endotoxin-free.

Isolation and Culture of Human Tenon Fibroblasts The HTFs were supplied by ScienCell Research Laboratories (USA). Cells were cultured using FBS (10%), streptomycin (100 μg/mL), and penicillin (100 U/mL)-contained MEM at 37°C and 5% CO_2. For experiments, HTFs were plated at a density of 5×10^5/well in 6-well culture plates or 5×10^3/well in 96-well culture plates, respectively. After 3d of incubation, the cells were washed twice using PBS. Then, after 24h of starvation with serum-free MEM, cells were cultured using serum-free MEM with or without the indicated concentration of 17β-estradiol for another 24h and followed by TGF-β (5 ng/mL) exposure for an indicated time. Finally, the total mRNA and proteins were extracted from the cells treated as above descriptions.

Collagen Gels Contraction Assay Relaxed HTFs-contained free-floating collagen gels were used to measure the CGC as previously described. Briefly, type I collagen (3 mg/mL), 10×MEM, reconstitution buffer and HTFs suspension (1.1×10^6 cells/mL in MEM) were mixed on ice in the volume ratio of 7:1:1:2. After 1h of coating using 1% BSA at 37°C, the mixture (0.5 mL) was added to each well of a 24-well plate. After the mixture gelated, the collagen gels were separated from the culture plate wells, then TGF-β (5 ng/mL), 17β-estradiol (12.5 to 100 μmol/L), or progesterone (12.5 to 100 μmol/L)-contained serum-free MEM (0.5 mL) was applied

to each gel. The amount of gel contraction was estimated every day using a ruler. Then, culture supernatants were collected for protein detection using ELISA.

Immunoblot Analysis MMP-1, MMP-3, TIMP-1, TIMP-2, as well as various phosphorylated signaling proteins in HTFs, were analyzed by immunoblotting[10]. Briefly, culture supernatants of collagen gels were used to assess the levels of MMP-1, MMP-3, TIMP-1 and TIMP-2. For the measurement of mitogen-activated protein kinases (MAPKs), Smads, and AKT, the medium was changed to the media devoid of serum for an additional 24h. Then, starved cells were incubated in serum-free MEM for 24h, with or without 17β-estradiol (100 μmol/L). Next, after 60-minute reaction with TGF-β, the cells were lysate using phenylmethylsulfonylfluoride (PMSF)-contained RIPA cell lysis buffer. After quantification, proteins were separated using a 10% SDS-PAGE gel, then separated protein bands were immunoblotted onto a 0.45 μm polyvinylidene difluoride (PVDF) membrane. After 1h of treatment using 5% fatty-free milk for blocking, the MMP-1, MMP-3, TIMP-1, TIMP-2, Smad2, Smad3, MAPKs, AKT, and phosphorylated Smad2, Smad3, MAPKs, and AKT antibodies were used for incubation with the membrane at 4℃ overnight. Next, after 1-hour incubation secondary antibody, the images were captured with a Tanon-5200 Multi-imaging System (Tanon Science and Technology Co., Ltd.). Target protein levels were evaluated using the Image J program.

Gelatin Zymography For MMP-2 and proMMP-2 analyses, gelatin zymography was conducted on culture supernatants as previously reported[10]. Briefly, after mixing the nonreducing SDS sample buffer (2 μL) and the culture supernatants (4 μL), mixtures were separated by SDS-PAGE at 4℃ using gelatin (0.1%)-contained gel (10%). After 1h of washing using 2.5% Triton X-100-contained buffer, the gel was incubated with Triton X-100 (1%), $CaCl_2$ (5 mmol/L), and Tris-HCl (50 mmol/L, pH 7.5)-contained reaction buffer at 37℃ for 18h to restore protease activity. Finally, the coomassie brilliant blue staining was conducted to color the gel.

RNA Extraction and Reverse Transcription-Quantitative Polymerase Chain Reaction After 24h of exposure to TGF-β (5 ng/mL) with or without 17β-estradiol, cells were harvested for RNA extraction. For the detection of IL-6, MCP-1, and VEGF, RT-PCR was conducted as previously reported[11]. The MCP-1, IL-6, and VEGF primers used here were previously described[11-12]. Comparative cycle time (Ct) was used to establish fold-change differences between samples. The $2^{-\Delta\Delta CT}$ was used to normalize the relative expression of target mRNAs to GAPDH (Table 1).

Measurement of MCP-1, IL-6, and VEGF Secretion After 3d of exposure to TGF-β (5 ng/mL) of collagen gel with or without 17β-estradiol, supernatants were harvested, then centrifuged at 120× g for 5min. The resultant supernatant was stored at -80℃. MCP-1, IL-6, and VEGF released into the supernatant were quantified using ELISA kits.

Table 1 Sequences of primers

Genes	Nucleotide sequences (5′-3′)
GAPDH	
Forward	ACTCCTCCACCTTTGACGCT
Reverse	GGTCTCTCTCTTCCTCTTGTGC
MCP-1	
Forward	ATCAATGCCCCAGTCACCT
Reverse	TCCTGAACCCACTTCTGCTT
IL-6	
Forward	TTCGGTCCAGTTGCCTTCT
Reverse	GGTGAGTGGCTGTCTGTGTG
VEGF	
Forward	GAGCCTTGCCTTGCTGCTCTAC
Reverse	CACCAGGGTCTCGATTGGATG

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; MCP: Monocyte chemoattractant proteins; IL: Interleukin; VEGF: Vascular endothelial growth factor.

Fluorescence Microscopy For immunofluorescence staining, after 3d of TGF-β treatment with or without 17β-estradiol and fixation using 4% PFA, Triton-X-100 (0.5%)-contained buffer was used for cell permeabilization. Between each procedure, the coverslips were washed with PBST (0.1% Tween in PBS). Next, after blocking using 3% BSA, 30min of staining with rhodamine-phalloidin (1:140) for F-actin staining, and 10min of DAPI staining (1:500) for nuclei detection, cells were observed and images were captured using fluorescence microscopy (Oberkochen, Germany).

Statistical Analysis The GraphPad software 9.0 was used for data analyses. Values are expressed as means±standard deviations (SD). The Tukey-Kramer test or Student's unpaired *t*-test was conducted for comparison between different treatments. A $P<0.05$ was considered statistically significant.

RESULTS

Suppression of TGF-β-stimulated CGC by 17β-estradiol The incubation of HTFs-contained collagen gel with TGF-β and various concentrations of 17β-estradiol (12.5 to 100 μmol/L) significantly and dose-dependently suppressed the TGF-β-stimulated gel contraction (Figure 1A). The difference in suppression was significant from 25 μmol/L, and the greatest inhibitory effect was detected at 100 μmol/L. We also observed a time-dependent suppressive effect of 17β-estradiol (100 μmol/L) on gel contraction, and a significant difference was observed after 1d (Figure 1B).

Absence of Progesterone Impact on TGF-β-stimulated CGC in HTFs Further, the impact of progesterone on the

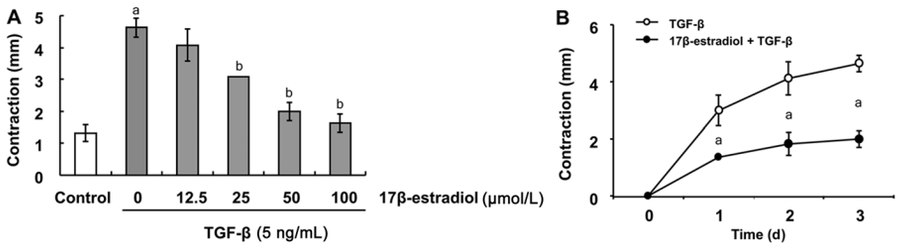

Figure 1 The dose- and time-dependent effects of 17β-estradiol on the CGC induced by TGF-β in HTFs A: Cells were cultured for 3d in collagen gels with or without 5 ng/mL TGF-β and with 17β-estradiol (12.5 to 100 μmol/L), and then the CGC was measured; [a]*P*<0.05 *vs* Control; [b]*P*<0.05 *vs* cells cultured with TGF-β only. B: Cells were cultured in collagen gels plus 5 ng/mL TGF-β with or without 17β-estradiol (100 μmol/L) and the contraction of the gel was measured; [a]*P*<0.05 *vs* cells cultured without 17β-estradiol. Data were presented with means±SDs. CGC: Collagen gels contraction; HTF: Human Tenon fibroblasts; TGF: Transforming growth factor.

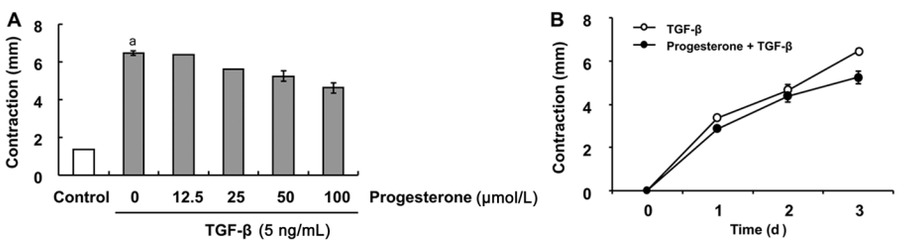

Figure 2 Absence of the impact of progesterone on the CGC caused by TGF-β in HTFs A: Cells were cultured for 3d in collagen gels with or without 5 ng/mL TGF-β and with progesterone (12.5 to 100 μmol/L), and then the CGC was measured; [a]*P*<0.05 *vs* Control. B: Cells were cultured in collagen gels plus 5 ng/mL TGF-β with or without progesterone (100 μmol/L) and the contraction of the gel was measured. Data were presented with means±SDs. CGC: Collagen gels contraction; HTF: Human Tenon fibroblasts; TGF: Transforming growth factor.

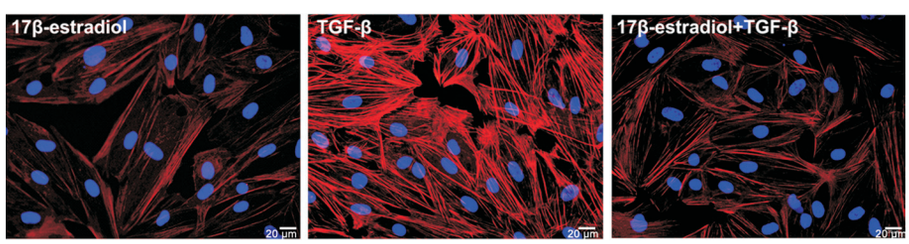

Figure 3 17β-estradiol impairs the formation of actin stress fibers in HTFs Cells were visualized with F-actin antibodies (red fluorescence) and DAPI (blue, nuclei) under a Zeiss fluorescence microscope. The TGF-β-stimulated production of F-actin was reduced by pre-incubation with 17β-estradiol (100 μmol/L). Scale bar, 20 μm. HTF: Human Tenon fibroblasts; TGF: Transforming growth factor.

CGC in HTFs stimulated by TGF-β was analyzed. HTFs were incubated with different doses of progesterone (12.5 to 100 μmol/L; Figure 2A) or for the indicated time (Figure 2B). These results showed that progesterone had no impact on the CGC stimulated by TGF-β in HTFs.

Suppression of Stress Fiber Formation in HTFs by 17β-estradiol Given the importance of the cytoskeleton to cell motility, the production of F-actin in HTFs was determined after 17β-estradiol treatment. The formation of actin stress fibers was detected after TGF-β induction. However, the formation of actin stress fibers stimulated by TGF-β was significantly impaired after 17β-estradiol treatment (100 μmol/L; Figure 3).

Inhibition of MMPs and TIMPs Secretion Induced by TGF-β in HTFs by 17β-estradiol Next, the production of MMPs and TIMPs in HTFs with or without 17β-estradiol treatment were evaluated. HTFs were cultivated for 3d on collagen gels containing TGF-β and different doses of 17β-estradiol. The immunoblotting of culture supernatants demonstrated that pre-incubation with 17β-estradiol dose-dependently decreased the levels of MMP-1 and MMP-3, which were increased in the presence of TGF-β. The gelatin zymography showed that the 17β-estradiol treatment did not affect the increased the TGF-β-stimulated proMMP-2 and active MMP-2 expression (Figure 4). Furthermore, the expression of TIMP-2 in HTFs was significantly decreased by TGF-β treatment and 17β-estradiol induced a reduction of TIMP-2 in a dose-dependent pattern in HTFs. However, pre-incubation with 17β-estradiol did not affect the increased levels of TIMP-1 induced by TGF-β in HTFs (Figure 4).

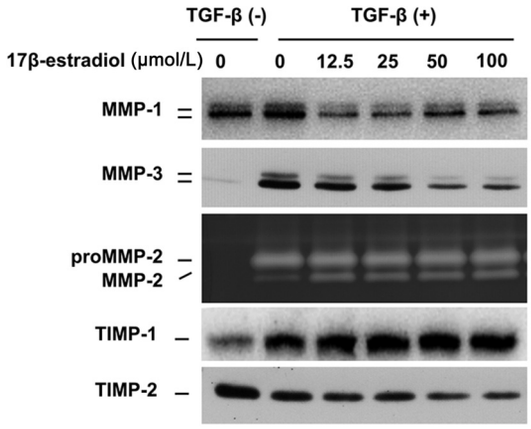

Figure 4 17β-estradiol inhibits the production of TIMPs and MMPs in HTFs Representative graphs showing the production of MMP-1, MMP-3, proMMP-2, active MMP-2, TIMP-1, and TIMP-2 in the supernatants of HTFs after the treatment of indicated concentration of 17β-estradiol based on immunoblot and gelatin zymography. MMP-1 and MMP-3 in the supernatants but not proMMP-2 and active MMP-2 were decreased following the pre-incubation with 17β-estradiol. In addition, 17β-estradiol induced a concentration-dependent reduction in the level of TIMP-2 but not TIMP-1 in the supernatants of HTFs exposed to TGF-β (5 ng/mL). HTF: Human Tenon fibroblasts; TGF: Transforming growth factor; MMP: Matrix metalloproteinases; TIMPs: Tissue inhibitors of metalloproteinases.

Suppression of IL-6, MCP-1, and VEGF Expression Stimulated by TGF-β in HTFs by 17β-estradiol Next, the IL-6, MCP-1, and VEGF secretions were examined in HTFs using ELISA (Figure 5A, 5B) and RT-PCR (Figure 5C, 5D). TGF-β induction dramatically elevated the secretion and mRNA expression of IL-6 and MCP-1 in HTFs. However, TGF-β-enhanced IL-6 and MCP-1 expression were greatly inhibited by pre-incubation with 17β-estradiol. Additionally, 17β-estradiol reduced the protein and mRNA levels of VEGF that were upregulated by TGF-β (Figure 6).

Impairment of Smads and MAPKs phosphorylation Induced by TGF-β in HTFs by 17β-estradiol Since Smads, AKT, and MAPKs (ERK, p38, and JNK) signaling pathways are involved in TGF-β-related fibrosis responses in various disorders, we used immunoblotting to analyze how 17β-estradiol suppressed their phosphorylation induced by TGF-β in HTFs. Results showed that TGF-β induced the phosphorylation of Smads, AKT, and MAPKs. After 24h of pretreatment with 17β-estradiol, the phosphorylation of Smad2, Smad3, ERK, and p38 was significantly inhibited. However, JNK and AKT were not affected. Neither TGF-β nor 17β-estradiol affected the overall abundance of Smads, AKT, and MAPKs (Figure 7).

DISCUSSION

Herein, we demonstrated that 17β-estradiol could dose-

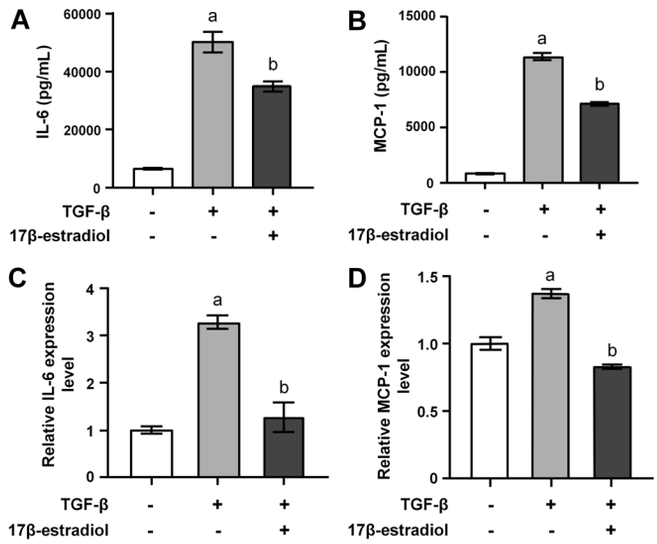

Figure 5 17β-estradiol suppresses the IL-6 and MCP-1 expression in HTFs after indicated treatment A, B: ELISA showed that 17β-estradiol (100 μmol/L) inhibited the TGF-β-induced IL-6 (A) and MCP-1 (B) secretion in HTFs; C, D: RT-PCR showed that 17β-estradiol (100 μmol/L) inhibited the TGF-β-induced upregulation of mRNA expression of IL-6 (C) and MCP-1(D) in HTFs. Means±SDs are presented. [a]*P*<0.05 *vs* cells cultured without TGF-β; [b]*P*<0.05 *vs* cells cultured with TGF-β only. IL: Interleukin; MCP: Monocyte chemoattractant proteins; HTF: Human Tenon fibroblasts; TGF: Transforming growth factor; ELISA: Enzyme-linked immunosorbent assay; RT-PCR: Reverse transcription-quantitative polymerase chain reaction.

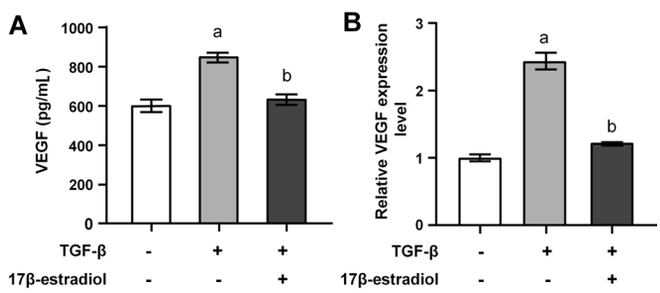

Figure 6 Effects of 17β-estradiol on VEGF in TGF-β-induced HTFs A: ELISA showed that 17β-estradiol (100 μmol/L) suppressed the release of VEGF into the culture medium induced by 5 ng/mL TGF-β in HTFs; B: RT-PCR showing the expression of VEGF in HTFs stimulated by TGF-β was inhibited by 17β-estradiol (100 μmol/L). Means±SDs are presented. [a]*P*<0.05 *vs* cells cultured without TGF-β; [b]*P*<0.05 *vs* cells cultured with TGF-β only. VEGF: Vascular endothelial growth factor; HTF: Human Tenon fibroblasts; TGF: Transforming growth factor; ELISA: Enzyme-linked immunosorbent assay; RT-PCR: Reverse transcription-quantitative polymerase chain reaction.

and time-dependently suppress the TGF-β-stimulated CGC induced, whereas progesterone had no impact. Moreover, 17β-estradiol markedly inhibited the TGF-β-stimulated formation of stress fibers and secretion of MMP-1 and MMP-3 in HTFs. Additionally, TGF-β increased the mRNA expression and protein translation of MCP-1, IL-6 and VEGF in HTFs,

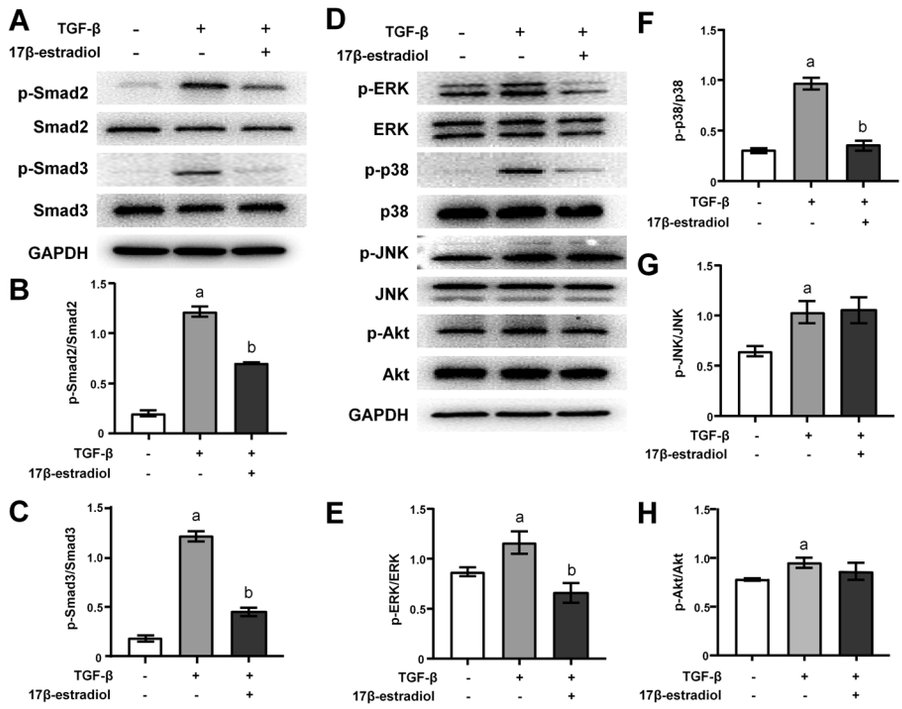

Figure 7 17β-estradiol impairs TGF-β-induced activation of Smads and MAPK signaling pathways in HTFs HTFs were exposed to 17β-estradiol (100 μmol/L) for 24h before further stimulation with TGF-β (5 ng/mL) for 1h. Representative blots (A, D) showing the expression of Smad2, p-Smad2, Smad3, p-Smad3, AKT, p-AKT, ERK, p-ERK, p38, p-p38, JNK, p-JNK, and GAPDH in cells after indicated treatment based on Western blotting. Summarized results of the blots are displayed in the bar graphs based on greyscale analysis (B, C, E, F, G, H). Means±SDs are presented. [a]*P*<0.05 *vs* cells cultured without TGF-β; [b]*P*<0.05 *vs* cells cultured with TGF-β only. HTF: Human Tenon fibroblasts; TGF: Transforming growth factor; Smad: Sma- and Mad- related proteins; MAPK: Mitogen-activated protein kinases; AKT: Protein kinase B; ERK: Extracellular signal-regulated kinase; JNK: c-Jun N-terminal kinase; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

which were also suppressed by 17β-estradiol. Then, we showed that 17β-estradiol might exert its influence by attenuating the phosphorylation of Smads, p38, and ERK signaling pathways.

The steroid hormone 17β-estradiol, also known as estrogen, has a role in maintaining and regulating the body's equilibrium in various physiological conditions[13]. Numerous studies have shown that 17β-estradiol also has pharmacological effects on different disorders. However, there are contradictory findings regarding the regulatory function of 17β-estradiol and progesterone on the conjunctiva and little is known about how these female-specific sex hormones function in the eye[14]. Herein, we showed that 17β-estradiol suppressed the CGC caused by TGF-β in HTFs, but progesterone had no effects. Additionally, 17β-estradiol inhibited the synthesis of MCP-1, IL-6 and VEGF in HTFs. These results suggested the anti-inflammatory and anti-fibrosis benefits of 17β-estradiol, which is also reported in other illness models[15-16]. An imbalance of steroid hormones has been demonstrated in thyroid-related orbitopathy[17], where inflammation and fibrosis play a contributory role in the pathogenesis of the disease[18]. The anti-inflammatory and anti-fibrotic effects of 17β-estradiol suggest its potential utility in the treatment of thyroid-related orbitopathy. Further investigation into the effects of 17β-estradiol on thyroid-related orbitopathy is warranted.

Conjunctival scar prevention or modification remains a therapeutic concern in ophthalmology. TGF-β, IL-6[19], MCP-1[20], VEGF[21], connective tissue growth factor (CTGF) [22], and the metalloproteinase family[23] are all implicated in the etiology of conjunctival scarring. The regulation of HTFs through growth factors[21-22], inflammatory processes[24], and/or fibroblast proliferation[2] is commonly used for the prevention of tissue fibrosis. Scarring development is critically impacted by TGF-β. TGF-β can cause fibrosis by activating the proliferation of local fibroblasts, as well as their migration to injured tissues[23]. A robust intracellular cytoskeletal framework mediates fibroblast contraction to close wounds throughout the wound healing process[25]. The CGC caused by the development of actin stress fibers is regulated by fibroblasts[9]. A previous study has also shown that TGF-β-induced cell contractility is enhanced by the production of actin stress fibers. Consistent with the results of previous observations, we found that TGF-β induced CGC and the development of stress fibers in HTFs. Additionally, 17β-estradiol markedly inhibited the TGF-β-stimulated formation of stress fibers[26]. Decreasing the production of actin stress fibers can play a crucial role in the inhibition effect of 17β-estradiol on CGC induced by TGF-β in HTFs.

Furthermore, MMPs are a wide class of enzymes that degrade extracellular matrix (ECM) components during the wound-healing process to reconstruct the ECM[23]. TIMPs are tissue inhibitors of metalloproteinases that are naturally produced by the body and regulate the activity of MMPs. Unbalanced levels of MMPs and TIMPs can lead to an excessive breakdown or accumulation of the ECM, leading to inadequate wound healing at the ocular surface[27]. TGF-β promotes fibrosis by inducing trans-differentiation of cells as well as increasing the MMP-1, MMP-2, and MMP-3 expression[10]. Research on animals has found that inhibiting MMPs might lessen the contraction of the matrix and avoid the formation of subconjunctival scars[28]. Moreover, 17β-estradiol has shown potential for decreasing the buildup of collagens and other ECM components by reducing the expression of MMP-3[29]. Additionally, 17β-estradiol can inhibit TGF-β_2-stimulated CGC and MMP-9 and MMP-2 secretion by RPE cells[5]. Herein, we found that 17β-estradiol inhibited MMP-1 and MMP-3 secretion in TGF-β-induced HTFs. Altogether, these results demonstrated that the inhibitory effects of 17β-estradiol on TGF-β-induced CGC might also be driven by comparable processes.

IL-6 is a cytokine with pleiotropic effects that contributes to the control of lymphocyte activity and regulates the acute-phase response[19]. Macrophages also play a crucial role in wound healing and scar formation[30]. MCP-1 is an effective chemoattractant for monocytes and macrophages. MCP-1 and IL-6 play an essential role in the ocular inflammatory process by modulating the proliferation, infiltration, and activation of immunocompetent cells in the conjunctiva[31]. For example, glaucoma patients with elevated levels of MCP-1 and IL-6 in their aqueous humor have poor outcomes after glaucoma filtration surgery[32]. Furthermore, during experimental glaucoma filtration surgery, targeting MCP-1 results in longer bleb survival and less cellular damage compared to mitomycin-C[33]. A previous study has shown that 17β-estradiol can reduce pro-inflammatory cytokines in other parts of the human body[16]. In SV40-immortalized human corneal epithelial cells, 17β-estradiol greatly inhibits the expression of tumor necrosis factor (TNF)-α, IL-6, and IL-1 induced by hyperosmolarity[34]. Additionally, 17β-estradiol inhibits lipopolysaccharide (LPS)-stimulated MCP-1 in adipocytes[35]. Similarly, 17β-estradiol substantially inhibited the mRNA transcription and protein translation of IL-6 and MCP-1 in response to TGF-β stimulation in our current investigation, suggesting that 17β-estradiol has a protective effect on conjunctival inflammation.

Angiogenesis is a crucial stage during wound healing. It is facilitated by VEGF, which allows inflammatory cells and fibroblasts to migrate to the injury site[36]. Scar development in cutaneous wounds is induced by increased levels of VEGF *via* collagen deposition[37]. VEGF is also increased in the aqueous humor after glaucoma filtration surgery and its inhibition leads to a reduction in scar formation at the trabeculectomy bleb and improves the overall success of glaucoma surgery[21]. Recent studies have illuminated the therapeutic potential of sodium hyaluronate[2] and exosome-mediated aptamer S58[25] in impeding sub-conjunctival scar formation *via* the inhibition of fibroblast proliferation and collagen deposition. Our investigation has revealed that 17β-estradiol has the capability to inhibit the contraction of HTFs, while also suppressing inflammation and the production of VEGF. As a consequence of its multi-target effects, 17β-estradiol may impede conjunctival scarring.

The traditional signaling pathway of TGF-β includes Smad2/3 regulation of transcription of target genes. TGF-β can also promote many signaling cascades in HTFs, including the well-known Smads cascade as well as other signaling pathways, such as MAPKs. Recent research has shown that both Smads and MAPKs are involved in the TGF-β-induced production of stress fibers[6]. Moreover, MAPK signaling inhibitors can significantly inhibit collagen gel contraction caused by TGF-β in HTFs, indicating the crucial role of MAPKs in this TGF-β-induced effect[38]. 17β-estradiol has been demonstrated to prevent the collagen contraction stimulated by TGF-β_2 by suppressing the phosphorylation of Smad2[5]. Additionally, estradiol drastically represses TNF-α-induced phosphorylation of MAPKs but not AKT in human brain vascular pericytes[39]. Therefore, we hypothesized that 17β-estradiol might be an effective anti-fibrosis drug that regulates the TGF-β/Smads, MAPKs, and AKT signaling pathways. To validate this hypothesis, the Smads, MAPKs, and AKT phosphorylation in TGF-β-stimulated HTFs were measured. We found that 17β-estradiol pretreatment inhibited the TGF-β-caused phosphorylation of Smads (Smad2 and Smad3) and MAPKs (ERK and p38) in HTFs. These results indicated that the inhibition of Smad2, Smad3, ERK, and p38-mediated pathways is significantly associated with the inhibitory effects of 17β-estradiol on the contractility and inflammation of HTFs. In conclusion, we demonstrated for the first time that 17β-estradiol inhibits the contractility and inflammation of HTFs induced by TGF-β. These effects of 17β-estradiol might be mediated through the inhibition of Smad2, Smad3, ERK, and p38 signaling pathways. Thus, 17β-estradiol might help inhibit the production of scar tissues during the healing process of a subconjunctival wound. Nevertheless, *in vivo* evaluation of the effects of 17β-estradiol on Tenon fibroblasts and conjunctival wound healing should be addressed in the future.

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