Umbilical mesenchymal stem cells attenuates corneal fibrosis induced by fungal keratitis through the TGFβ1/Smad2 signaling pathway

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Abstract

Background: Fungal keratitis (FK) is eye microbial infection disease which can leads to severe corneal blindness. Corneal scar formation is one of the major complications of fungal keratitis and is closely related to prognosis. The aim of the current investigation was to evaluate the anti-fibrosis effect of human umbilical cord mesenchymal stem cells (uMSCs) in FK model and further to explore underlying mechanisms.

Methods: FK mice model was made by corneal epithelial scratching and *F. solani* inoculation. The C57BL/6J mice were randomly divided into four groups, including control group, FK group, vehicleinj FK group and uMSCsinj FK group. After injury, antifungal drug natamycin eye drops were used topically to FK mice eyes 6 times per day for 7 days to inhibit fungi growth. Mice received repeated subconjunctival injection of uMSCs or veichle for 3 times including the 1d, 4d and 7d after wounding. At 14d, 21d and 28d post-injury, clinical observation, histological examination, second harmonic generation, immunofluoresence staining and molecular assays were performed.

Results: The uMSCs topical administration reduced corneal scar formation and corneal opacity, accompanying with decreased corneal thickness and inflammatory cell infiltration, following down-regulated fibrotic-related factors α-SMA, TGFβ1, CTGF, and COL□ and finally inhibited phosphorylation of TGFβ1/Smad2 signaling pathway, which indicating the potential anti-fibrotic and protective effect of human uMSCs against FK-induced corneal fibrosis.

Conclusion: Human uMSCs can evidently inhibit corneal fibrosis after FK wounding through TGFβ1/Smad2 signaling pathway regulation.

Keywords: Fungal keratitis, corneal fibrosis, umbilical cord mesenchymal stem cells

Background

Fungal keratitis (FK), known as keratomycoses, is severe eye infections caused by corneal trauma. FK is more common in agricultural corneal trauma (branch, straw, wheat straw, etc.) or after removal of corneal foreign bodies such as soil and sand. The disease is also seen in the long-term topical use of broad-spectrum antibiotics and corticosteroids, which will disturb the symbiosis of bacteria and fungi, and reduce the resistance of corneal tissue, thus promoting the proliferation and expansion of fungi in the cornea. Current antifungal drugs have low corneal permeability, it is difficult to achieve effective drug concentration in the cornea, and lack of a broad spectrum of antifungal drugs, making fungal keratitis difficult to treat [1]. The treatment of FK requires a combination application of multiple antifungal drugs, if necessary, surgery is needed. Pathologic corneal scar formation which leads to corneal blindness reminds to be the most common and serious complication of FK. Therapeutic corneal graft is required; however, it bears the risk of worldwide donor material shortage and allograft rejection [2]. Alternative therapies to corneal transplantation include tissue engineering [3], cell therapy [4], targeted drug and gene therapy [5-7]. Among all these novel and productive treatment modalities, cell therapy stands for a crucial

status not only because of its hypoallergenic but also its ability of immunomodulation and anti-fibrotic properties.

Umbilical cord-derived mesenchymal stem cells (uMSCs) are multipotent stromal cells derived from Wharton's jelly of umbilical cord tissue, and possess the capabilities of multipotent differentiations and immunomodulatory. Recently, studies have been focused on the anti-fibrosis effects of uMSCs in lung fibrosis [8], liver fibrosis [9]and renal fibrosis [10] which acquired positive effects. However, lots of studies focusing on the therapeutic effects of uMSCs in corneal epithelial defects and keratoplasty, few studies concentrating on the anti-fibrosis capacity of uMSCs in cornea diseases. It is reported that uMSCs can suppress scar formation and myofibroblast differentiation in a skin-defect mouse model [11]. Meanwhile, stromal thickness and collagen fibril defects in lumican null mice were restored by injection of human stromal stem cells [12]. Based on the anti-fibrosis effects of mesenchymal stem cells (MSCs) reported by numerous researchers, we conducted this research to explore potential mechanism and optimized utilization of MSCs therapy in the field of corneal fibrosis induced by infectious eye disease.

In the present study, we isolated and characterized human uMSCs, and investigated the role of uMSCs administration in the treatment of corneal fibrosis induced by *F. solani* keratitis through various technical means and further exploring the mechanism of TGFβ1/SMAD2 signaling pathway regulation during the healing period of FK, which provide a novel perspective and practice basis for cell therapy against corneal fibrosis induced by corneal microbial infections.

Methods

Male C57BL/6J mice were obtained from the Nanjing University Biomedical Research Institute (Nanjing, China). All mice were 6–8 weeks of age and 18–25g of weight. The mice were housed in an SPF-class animal laboratory at room temperature 20-25°C with appropriate humidity, automatic feeding, and 12:12-day cycle natural light. Intraperitoneal injection of 1%pentobarbital sodium (0.01ml/g; Sigma Aldrich, USA) was used in mice anesthesia and mice were sacrificed by cervical dislocation at different time points. 30 mice were involved in control group and 70 mice were involved in three experimental groups and 240 mice were involved in the study in total. All procedures used in this study were approved by the Henan Eye Hospital Ethics Committe in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Primary culture and identification of uMSCs

Human umbilical cords were acquired from the Henan Provincial People's Hospital. For the use of umbilical cord, written informed consent was obtained from the donors. The average age of donors is 26.33±2.68 years old and all the women donors were excluded of hepatitis B, hepatitis C, and syphilis, AIDS, cytomegalovirus infection, EB virus infection and other infectious diseases. Primary culture of uMSCs was established using standard procedures. In brief, fresh umibilical cords were washed with

0.01M pH7.2–7.4 phosphate buffer saline (PBS) supplemented with antibiotics (100 U/ml of streptomycin, 100 U/ml of penicillin) twice to remove blood and excess tissue. After treated with 70% ethanol, umbilical cords were then minced into small pieces and incubated with DMEM/F12 medium (1:1) (Hyclone Laboratories; Thermo Fisher Scientific Life Sciences, USA) supplemented with 10% fetal bovine serum(FBS; Hyclone Laboratories) in dishes at 37°C, 5% CO_2 supplement. Cells were trypsinized and collected for subculture when they reached 80% confluence. Only uMSCs in passages 2-5 were used in our study. Cultured cells were identified according to cell morphology and flow cytometry results. In brief, cultured cells were fluorescence marked with specific mensenchymal stem cell surface antigen CD29 (557332, BD biosciences, New Jersey, US) and CD44 (559942, BD biosciences, New Jersey, US), and hematopoietic stem cell marker CD34 (555821, BD biosciences, New Jersey, US) and CD45 (555484, BD biosciences, New Jersey, US).

FK model preparation

Mice were anesthetized, the beard was cut off, and the cornea was topically anesthetized with oxybuprocaine hydrpchloride eye drops (Santen Pharmaceutical Co., Ltd., Japan). The right eye of each mouse was selected as the experimental eye. The preparation of the model refers to the article published before by our research group [13]. Under the operating microscope (Topcon OMS-90, Japanese), the left hand was used to fully expose the cornea, and the right hand-held sterile blade (carbon steel, size 11, Shanghai Medical Suture Needle Factory Co., Ltd., China) was used to make a cross scratch in the center of the cornea. The scratch depth was to exceed Bowman's membrane and stop at the corneal stroma superficial layers. Use a sterile bamboo stick tip (tip diameter 0.30mm, length 1.10mm) to pick up a small amount of hyphae, inoculate the corneal scratches, repeatedly apply on the wound surface, so that the hyphae adheres evenly to the scratches, simulate the natural infection of the cornea process. After injury, antifungal drug natamycin eye drops were used topically to FK mice eyes 6 times per day for 7 days to inhibit fungi growth. The experimental fungus standard strain Fusarium oxysporum (No. 3.791) was purchased from the General Microbiology Center of the China Microbial Culture Collection Management Committee, Institute of Microbiology, Chinese Academy of Sciences, Beijing. Resuscitation and passage were performed according to standard methods, and well-grown fungi were used for the experiment. After 24 hours of modeling, observation under the slit lamp microscope showed that the formation of corneal fungal infection lesions and a small amount of empyema in the anterior chamber were successfully modeled. Cases of uninfected or perforation during observation were removed, and the number of deletions was substituted by subsequent experiments.

Thirty mice were randomly selected as blank controls. The remaining mice with similar degree of corneal lesions were weighed and sorted according to body mass. According to random number table, mice were randomly divided into three groups, including FK group, vehicle^{inj} FK group and uMSCs^{inj} FK group.

Control group referred to unwounded ones, whereas FK group referred to wounded ones without any injection but antifungal drug therapy, meanwhile vehicle^{inj} FK group referred to wounded ones with vehicle subconjunctival injection and antifungal drug therapy, finally uMSCs^{inj} FK group referred to wounded ones with uMSCs subconjunctival injection and antifungal drug therapy.

Topical administration of uMSCs

Mice in the uMSCs^{inj} FK group were anesthetized and the ocular surface was fully exposed with the help of left hand fixation. Topical anesthetic was applied onto the ocular surface. Under the operating microscope, the Hamilton micro-syringe (Hamilton, Switzerland) with a 30G needle on it was used to carefully insert into the conjunctiva sac and slowly injected 5×10^4 uMSCs in 5μl PBS. The injection operation was performed for 3 times including the 1d, 4d and 7d after wounding. Others in vehicle^{inj} FK group received a subconjunctive injection of 5μl PBS at the same time points using the same method. However, control group and FK group received no injection at all.

Observation and examination

The cornea was scored and photographed 14d, 21d and 28d after injury. A trained researcher blinded to the group was assigned to score and count corneal opacity and corneal area to prevent bias. The ocular surface pictures were taken by slit-lamp (SL-120; Zeiss, Jena, Germany) and the corneal opacity scoring criteria was as follows: grade 1 (mild corneal haze, pupil iris clearly visible), grade 2 (superficial corneal opacity, visible pupil and iris through the lesion), grade 3 (inhomogeneous full-thickness corneal opacity), and grade 4 (homogeneous and dense opacity [14]. The corneal scar formation areas (mm^2) in each group were analyzed by EyeStudio slit-lamp software according to the photographs taken by slit-lamp.

Pathlogical examination and hematoxylin-eosin (HE) staining

For the purpose of histological analysis, mice were sacrificed by cervical dislocation and eyeballs were dissected at 14d, 21d and 28d post-injury. The eyeballs were removed entirely along with some of the surrounding tissue and optic nerve to determine the location of the cornea. After a fixation in 4% paraformaldehyde for 24 hours, the tissues were dehydrated, dipped in wax, embedded, and sliced in sequence. The slices were under a routine operation of dewaxing and then HE staining was used for pathological examination. Photos were captured by Nikon 80i light microscope (Nikon, Sendai, Japan) and analyzed in terms of corneal thickness, histological structure, inflammatory cell infiltration and collagen destruction.

Whole mount corneal immunofluoresence staining

The mice were sacrificed by cervical dislocation at 14d post-injury. The intact eyeballs were removed and then fixed in 4paraformaldehyde for 90min at 4℃. Trim the eyeball, retain the cornea and limbus, and remove the iris, ciliary body and other tissues. Corneas were washed with 0.01 M PBS for 5 times (3 min each), and then dipped in 0.2Triton-2BSA 1:100 diluted anti-alpha smooth muscle actin antibody (ab5694, Abcam, Cambridge, US) overnight at 4℃. After conjugated with 1:500 diluted fluorescence-conjugated secondary antibody Alexa488 (A10042, eBioscience, US) in PBS overnight at 4℃, the corneas were then flatmounted with four radial cut, and sealed with mounting medium. Images were captured by tissue panoramic scanning microscopy (Pannoramic 250/MIDI, 3D HISTECH, Hungary).

Second harmonic generation (SHG)

At 14d, 21d and 28d post-injury, mice were under deep anesthesia and fixed on a designated plate, with the ocular surface faced up. According to a previously described method[15], a modified plastic bowl containing sterile PBS was fixed on the ocular surface sealed by erythromycin eye ointment to ensure the use of water immersion objective. SHG imaging was performed using an inverted two-photon excitation fluorescence microscope (NLO780, Zeiss). The laser was tuned to 780 nm and a 20× water immersion objective (numerical aperture =1.0) was used to focus the excitation beam and to collect backward signals. The Z-stack (Z=5 μm) layer-by-layer scan was used, and the obtained images were three-dimensionally reconstructed using Imaris software (×64, version 7.4.2, Bitplane, Zurich, Switzerland) to calculate the average signal intensity of the image. The stronger the image signal intensity, the more regular the corneal matrix collagen fibers are arranged, the corneal matrix structure is intact, and the weaker the image signal, the corneal matrix collagen fiber structure is disordered or degraded. Collagen SHG signals were visually represented with grey-scale images.

Quantitative real-time PCR

The mice were sacrificed by cervical dislocation at 14d, 21d and 28d post-injury and the corneas were trimmed as described above. Corneas were cut into small pieces and grinded for RNA extraction. RNeasy Mini Kit (Qiagen, US) was used to extract total RNA according to the manual and cDNA was generated by reverse transcription (TIANScript RT Kit, TRANSGEN BIOTECH, Peking, China). Real-time amplification was performed using TransStart Top Green qPCR Supermix (AQ131, TRANSGEN BIOTECH, Peking, China) in ABI 7500 Real Time PCR System (Applied Biosystems/Life Technologies) for the following molecules: α-Smooth muscle actin (α-SMA), forward sequencing: TCAGACCTGTGTGTTCCCTA, reverse sequencing: AGACGTGCTTCTTTTCCTTG, transforming growth factorβ1 (TGFβ1), forward sequencing: AAGCCCGAGGACCACATTTT, reverse sequencing: GGGGCCTTTGGCTCAGAAAT, connective tissue growth factor (CTGF), forward sequencing: CATCTCCACCCGAGTTACCA, reverse sequencing:

TGCACTTTTTGCCCTTCTTA, collagen Ⅰ(COLⅠ), forward sequencing: TCAGACCTGTGTGTTCCCTA, reverse sequencing: AGACGTGCTTCTTTTCCTTG, and housekeeping gene β-actin, forward sequencing: GGCACCACACCTTCTAC, reverse sequencing: CTGGGTCATCTTTTCAC. Gene expression was standardized to housekeeping gene expression in respective samples. Each individual experiment was done in triplicate.

Enzyme-linked immunisorbent assay (ELISA)

The mice were sacrificed by cervical dislocation at 14d post-injury and the corneas were trimmed as described above. Total protein quantitative detection Kit (E-BC-K318, Elabscience, Wuhan, China) was used to extract total protein. The protein levels of α-SMA and COLⅠ in corneal homogenates were assayed using Mouse ACTα2 (Actin Alpha 2, Smooth Muscle) ELISA Kit (E-EL-M2434c, Elabscience, Wuhan, China) and Mouse COL1 (Collagen Type Ⅰ) ELISA Kit (E-EL-M0325c, Elabscience, Wuhan, China) according to the manual instructions. Meanwhile, standard curves were prepared for calculating the cytokine concentration. The analysis was performed with the pooling of the corneas with duplicates.

Western blot assay

The mice were sacrificed by cervical dislocation at 14d post-injury and the corneas were trimmed as described above. 0.1g tissue was fully grinded in liquid nitrogen, and then transferred to 1ml RIPA protein lysate. Determination of sample protein concentration was by Coomassie Brilliant Blue Kit. Protein separation was performed by electrophoresis in different concentrations of gels. Polyvinylidene fluoride membranes (Bio-Rad Laboratories, Hercules, CA, USA) were used to transfer the samples. Block the membranes with a blocking solution (PBST solution containing 5% skim milk powder) for 2 hours at room temperature and then incubated in a 1:1000 dilution of primary rabbit polyclonal anti-Smad2 antibody (bs-0718R, Bioss Antibodies, Peking, China) and primary rabbit polyclonal anti-phospho-Smad2 antibody (bs-5618R, Bioss Antibodies, Peking, China) at 4℃ overnight. The membranes were washed in TBST (10 mM Tris-HCl [pH 7.5], 150 mM NaCl, and 0.05% Tween-20) for 3 times (5min each), and then incubated in a 1:3000 dilution of goat-anti-rabbit secondary antibody (bs-0295G, Bioss Antibodies, Peking, China) for 1h at room temperature. Chemiluminescent detection was performed with ECL luminol reagent (SC2048, Santa Cruz, USA). After the X-ray film is photographed and fixed, ImageJ software (National Institutes of Health, USA) was used for grey value calculating.

Statistical analysis

Each part of the experiment was repeated at least 3 times, and the statistical analysis is based on the summary of the results. Data were expressed as the mean ± S.D. and analyzed using GraphPad Prism

software (GraphPad Prism7, GraphPad software company, San Diego, California, US). Corneal opacity score, corneal scar area, corneal thickness and mRNA level fibrosis-related factors were 3 or 4 grouped data at 3 time points, which were analyzed with regular two-way ANOVA (not repeated measures) along with multiply comparison corrected by Turkey test. As for SHG, ELISA and westernblot analysis, nonparametric one-way ANOVA was performed along with multiply comparison corrected by Turkey test as well. $P < 0.05$ was considered to be statistically significant.

Results

Primary cell culture and identification of uMSCs

The morphology of the cultured cells is characterized according to their adherence to plastic surfaces with a fibroblast-like morphology (Fig. 1C). Cultured cells are positive for mensenchymal stem cell surface antigen CD29 and CD44 (Fig. 1B), whereas negative for hematopoietic stem cell marker CD45 and CD34 (Fig. 1A). According to the results of flow cytometry, cell number of CD45-CD34-CD29+CD44+ counts for 96.30% among total number of cells, which demonstrate the cultured cells belong to a member of mesenchymal stem cells.

uMSCs prevented corneal scar formation and enhanced slit-lamp observation and clinical score

Corneal scar formation is one of the major complications of FK and is closely related to prognosis. 12 mice of each group were involved in ocular surface examination. At 14d, 21d and 28d post-injury, $uMSCs^{inj}$ FK group showed decreased corneal scar formation, and enhanced corneal transparency, compared with $vehicle^{inj}$ FK group and FK group (Fig. 2a). The comparison of corneal opacity scores and scar formation area (mm^2) between $uMSCs^{inj}$ FK group and the other two groups were statistically significant differences both at the 14d, 21d, and 28d post-injury (Fig. 2b and c).

Corneas from $uMSCs^{inj}$ FK group exhibited reduced corneal thickness and inflammatory cell infiltration after FK in histological examination.

Histological examination was performed on 14d, 21d and 28d after injury. 6 mice of each group were involved. The $vehicle^{inj}$ FK group and FK group exhibited corneal thickening, multiply layers of irregularly aligned, excessive collagen deposition and extensive inflammatory infiltrates. In contrast, corneas from $uMSCs^{inj}$ FK group showed less corneal thickness, relative normal structural arrangements, fewer inflammatory cell infiltrations (Fig. 3a-i). The comparison of average corneal thickness (μm) in $uMSCs^{inj}$ FK group and the other two groups had statistically significant difference at 14d, 21d and 28d post-injury (Fig. 3m). Thus, histological examination provided further evidence of the therapeutic effects of uMSCs application on FK.

Collagen destruction was restored by uMSCs treatment in SHG

SHG pictures were taken by two-photon confocal microscopy in vivo at 14d, 21d and 28d post-injury. 6 mice of each group were involved. The mice corneas of control group exhibited high intensity transient signals due to regular arrangement of corneal collagen fibers (Fig. 4a, e, and i). However collagen degradation and abnormal collagen deposition caused by infection and inflammation in FK group and vehicle^{inj} FK group exhibited weak signals excited by confocal microscopy (Fig. 4b, f, and j). Of note, collagen destruction was restored by uMSCs treatment with an average optical intensity (AOD) 69.97±7.09 at 14d post-injury, which had statistically significant differences compared with FK group (26.09±1.27) and vehicle^{inj} FK group (28.98±3.32) (Fig. 4m). Light scattering were also markedly reduced in uMSCs treatment group compared with the other two groups.

uMSCs inhibited α-SMA production in mice corneal after FK

The α-SMA is a biochemical marker for myofibroblast. The corneal fibroblasts grown in presence of TGFβ1 expressed α-SMA and acquired fibroblastic phenotype [16]. Since α-SMA has been identified as a key mediator in corneal fibrogenesis, we determined the effect of uMSCs on α-SMA production during corneal fibrosis by immunofluorescence at 14d post-injury. 4 mice of each group were involved. Mice corneas of control group could only be found of α-SMA expression at the pericorneal vascular region due to vascular wall smooth muscle cells staining, but was not observed in the central area of cornea (Fig. 5a). The α-SMA production was up-regulated in the lesion area of corneas 14d post-injury in FK group and vehicle^{inj} FK group (Fig.5c and d), whereas was found down-regulated in uMSCs^{inj} FK group (Fig. 5b), which was consistent with the results of clinical manifestations. The results demonstrated that uMSCs application could inhibit keratocyte-to-myofibroblast transition during the healing period of FK.

Fibrosis-related factors were down-regulated by uMSCs administration

Relative mRNA expression of fibrosis-related factors α-SMA, TGFβ1, CTGF and COL□ in per cornea from FK group, vehicle^{inj} FK group and uMSCs^{inj} FK group were investigated by quantitative PCR at 14d, 21d and 28d post-injury. 6 mice of each group were involved. The results demonstrated that uMSCs administration significantly inhibited the expression of pro-fibrogeneic genes α-SMA (1.72-fold decrease at 14d, 2.35-fold decrease at 21d, 3.2-fold decrease at 28d; $p<0.05$), TGFβ1 (2.37-fold decrease at 14d, 5.46-fold decrease at 21d, 8.21-fold decrease at 28d; $p<0.05$), CTGF (2.3-fold decrease at 14d, 2.76-fold decrease at 21d, 4.18-fold decrease at 28d; $p<0.05$) and COL□ (1.53-fold decrease at 14d, 2.41-fold decrease at 21d, 3.16-fold decrease at 28d; $p<0.05$) mRNA levels compared with vehicle^{inj} FK group (Fig.6a-d). No statistically significant differences were detected in the α-SMA, TGFβ1, CTGF, and COL□

mRNA expression between the FK group and vehicle^{inj} FK group. Protein concentration of α-SMA and COL□ were detected by ELISA kits at 14d post-injury, and the results revealed up-regulation of α-SMA and COL□ after FK. Meanwhile, the detection of the two cytokines in uMSCs^{inj} FK group was down-regulated 1.24±1.35−fold decrease and 1.16±1.86−fold decrease compared with vehicle^{inj} FK group at 14d post-injury (Fig.6e and f). Of note, there was a statistically difference between control group and FK group in α-SMA protein expression, indicating a up-regulation of α-SMA during the healing period of FK, which was a key regulator in corneal fibrogenesis. However, topical application of uMSCs reduced α-SMA production and attenuated corneal fibrosis during the healing period of FK.

uMSCs attenuates phosphorylation of TGFβ1/Smad2 signaling pathway in the regulation of corneal fibrosis

As we know, TGFβ is critical for the differentiation of the multiply layers of the cornea, and the Smads family is one of the most important signaling pathways for TGFβ. [9]. To validate the role of TGFβ1/Smad2 signaling pathway in the regulation of corneal fibrosis in mice FK model, we conducted westernblot analysis to detect relative protein levels of Smad2 and phosphorylated Smad2 in corneas at 14d post-injury (Fig.7a). 6 mice of each group were involved. Data revealed that the Smad2 expression was up-regulated by FK 1.93±1.57−fold increase compared with control group and had no statistically differences between FK group, vehicle^{inj} FK group and uMSCs^{inj} FK group (Fig.7b). Phosphorylation of Smad2 was hard to detect in control group mice corneas whereas was up-regulated 4.73±1.01−fold and 4.62±1.58−fold increase in FK group and vehicle^{inj} FK group respectively (Fig.7c). Samples from uMSCs^{inj} FK group exhibited partially inhibited phosphorylated Smad2 expression with a 2.08±0.8−fold decrease compared with vehicle^{inj} FK group, indicating potentially down-regulation capacity of TGFβ1/Smad2 signaling pathway in FK mice corneas.

Discussion

FK leads to corneal fibrosis. The keratocyte-to-myofibroblast transition plays crucial role during the process of corneal scar formation and TGFβ is the key regulatory factor during this period. At the early stage of injury, damaged epithelium and basement membrane release inflammatory cytokines, mainly IL-1 (α and β), which leads to the necrosis and activation of keratocytes[17]. Quiescent keratocytes become activated fibroblasts, and keratocytes proteins such as cystallins and keratin sulfate proteoglycans are down-regulated [18].α-SMA was up-regulated due to myofibroblast transdifferentiation, which leads to excessive extracellcular matrix deposition and lamella disorganization. It is also confirmed by studies that myofibroblast transformation is trigged by TGFβ in vivo and in vitro [19, 20]. However, only TGFβ1 and TGFβ2 are active in this process, except for TGFβ3[21]. CTGF is downstream regulator of TGFβ1/Smad2 signaling pathway and participates in myofibroblasts transformation and collagen synthesis. Down-regulation of TGFβ1, TGFβ2, and CTGF expression by effective triple siRNA combination application reduced corneal fibrosis and scarring [22]. Human corneal keratocytes secret extracellular

matrix (ECM) ,mainly consisting of type I, V, and VI collagens, which constitute highly regular collagenous lamellae in corneal stroma [23]. Proteoglycans in fibrotic extracellular matrix is characterized with altered glycosaminoglycans, which contribute to the reduced transparency of scarred tissue [24]. In our study, the expressions of scar formation associated factors α-SMA, TGFβ1, CTGF, and COL□was up-regulated in FK model, which indicating keratocyte-to-myofibroblast transition and ECM deposition.

MSCs therapy is widely used in the field of ophthalmology, especially ocular surface diseases. Studies have shown that MSCs can reduce the inflammatory response of the dry eye syndrome (DES), increase the secretion of tears, and improve symptoms [25]. Corneal limbal stem cell deficiency (LSCD) leads to delayed healing of the corneal epithelium and formation of corneal neovascularization. In vitro experiments have shown that MSCs can stimulate cell proliferation when co-cultured with limbal stem cells and corneal epithelial cells [26]. In addition, the combination of MSCs and bioengineering materials for ocular surface damage is better than MSCs alone [27, 28]. MSCs have also been shown to attenuate the immune rejection of corneal transplantation by regulating immune cells [29]. It is well known that corneal allograft rejection is mainly mediated by CD4+ T cells and macrophages [30]. The regulation of MSCs on these immunoregulatory cells makes it an ideal anti-rejection treatment. As for MSCs in restoring corneal transparency, intrastromally transplantation of uMSCs in cornea had been verified to restore the dendritic and hexagonal morphology of host keratocytes and improve corneal transparency [31]. Liu [32] revealed that corneal transparency and stromal thickness of lumican null mice were significantly improved by uMSCs transplantation. Similarly, our results indicate that the corneal opacity, corneal thickness and collagen destruction can be suppressed by uMSCs treatment in mice FK model and fibrogenesis-related genes can be reduced through TGFβ1/Smad2 signaling pathway, which is consistent with the results of several in vivo and in vitro studies.

Compared with other available sources of MSCs, the uMSCs have the advantages of lower immunogenicity, higher capacity in proliferation, conveniently available, and less ethical controversy [11, 33]. The mechanisms involved in the therapeutic effects exerted by uMSCs, mainly focused on paracrine effects to suppress inflammation and myofibroblast differentiation. Researchers demonstrated that mRNA quantification of TSG-6 in MSCs predicted their efficacy in sterile inflammation models for corneal injury [34]. Exosomes are closed nanoscale membrane vesicles (30-150 nm) that transport active substances between different cells and identified as a new kind of major paracrine factor released by uMSCs [35]. Yu[36] reported that intravitreally injection of uMSCs-derived exosomes could reduce damage, inhibit apoptosis and suppress inflammation responses in laser-induced retinal injury. uMSCs-exosomal microRNAs has been found to play key roles in suppressing myofibroblast differentiation by inhibiting excess α-SMA and collagen deposition[11].

Allogeneic transplantation often results in graft versus host response or host rejection. Since uMSCs do not express MHC class II molecules on the surface and can inhibit lymphocyte proliferation by producing some soluble cytokines, uMSCs can induce host immune tolerance and reduce transplant rejection[37]. In this experiment, the transplanted animals did not see significant immunological rejection, the eye showed normal corneal transparency, no corneal neovascularization, clear pupil and iris texture, no ocular

reaction, such as uveal reaction. Studies suggest that uMSCs transplantation did not result in host anti-graft rejection.

Of note, the anti-fibrosis effect of uMSCs mainly occurs in cases with mild symptoms, and it is ineffective for those cases with severe infection, strong inflammatory response and perforation. This suggests that for the FK, a kind of infectious keratopathy, the use of antifungal drugs is essential at early stage, and further in combination with anti-inflammatory agent to reduce the inflammatory response to tissue damage, which is also our next research direction.

Conclusion

The current study suggests that human uMSCs can evidently inhibit corneal inflammation and corneal fibrosis after FK wounding, the corneal opacity, scar formation area and corneal thickness can be reduced accompanying with down-expression of α-SMA, TGFβ1, CTGF, and COL□ through TGFβ1/Smad2 signaling pathway regulation. Our study has an implication for further exploration of MSCs as a novel therapy for patients with infectious eye disease and other inflammatory and fibrosis ocular diseases. However, further studies should be established to explore the in-depth mechanism underlying mesenchymal stem cells regulation in corneal fibrosis for new therapies.

Abbreviations

FK: fungal keratitis; uMSCs: umbilical cord mesenchymal stem cells; SHG: second harmonic generation; α-SMA: α-smooth muscle actin; TGFβ1: transforming growth factorβ1, CTGF: connective tissue growth factor, COL□: collagen □.

Declarations

Ethics approval and consent to participate

Mice were maintained in a SPF-class animal laboratory in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. For the use of umbilical cord, written informed consent was obtained from the donors. All procedures used in this study were approved by Henan Provincial Eye Hospital Ethics Committe in compliance with the National Institutes of Health guidelines.

Consent for publication

Not applicable

Availability of data and material

All the data supporting the findings was contained within the manuscript.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

LYW and YZ designed the research, YZ, YQC, SYW and FYQ conducted the experiments, LYW and YZ analyzed the results, YZ drafted the manuscript. All authors read and approved the final manuscript.

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Figures

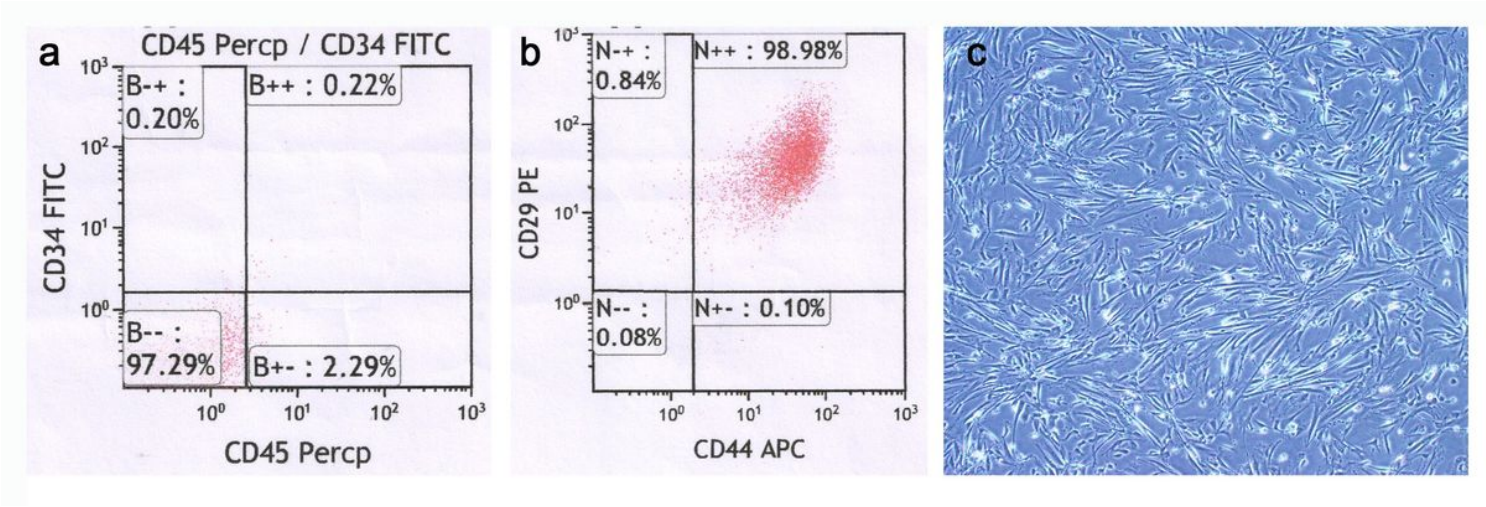

Figure 1

Human uMSCs culture and identification. Cultured cells were negative for CD45 and CD34 (a), whereas positive for CD29 and CD44 (b) and cell number of CD45-CD34-CD29+CD44+ counted for 96.30% among total number of cells. (c) The morphology of the cultured cells was characterized according to their adherence to plastic surfaces with a fibroblast-like morphology.

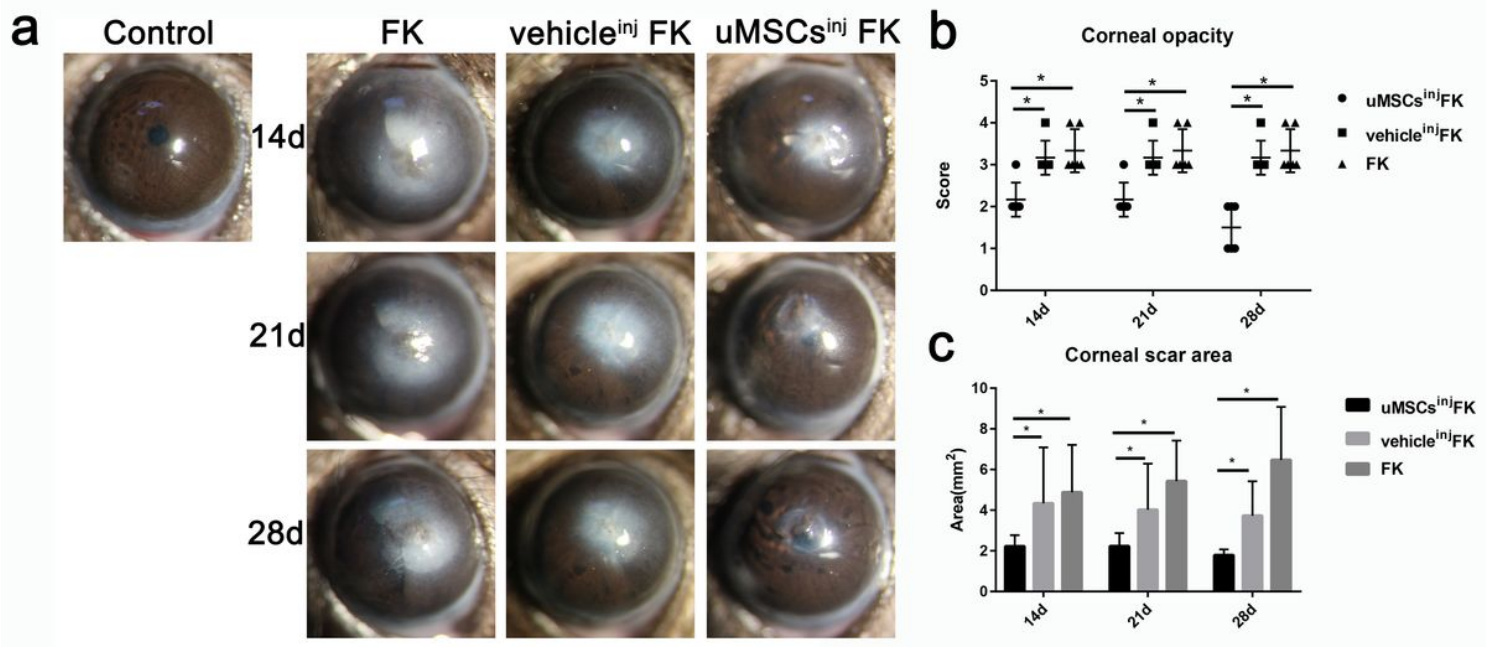

Figure 2

Ocular surface observation and examination. (a) Slit-lamp microscope observation and photograph of control group, FK group, vehicleinj FK group and uMSCsinj FK group. The uMSCsinj FK group showed decreased corneal scar formation, and enhanced corneal transparency, compared with vehicleinj FK group and FK group at 14d, 21d, and 28d post-injury. The comparison of corneal opacity scores (b) and scar formation area (mm2) (c) between uMSCsinj FK group and the other two groups were statistically significant differences both at the 14d, 21d, and 28d post-injury. Corneal opacity and scar formation area

were significantly down-regulated by uMSCs administration. Magnification of ocular surface photos: 25×. Values are presented as means ± SD, n=12, Mean values with asterisks are significantly different (P < 0.05).

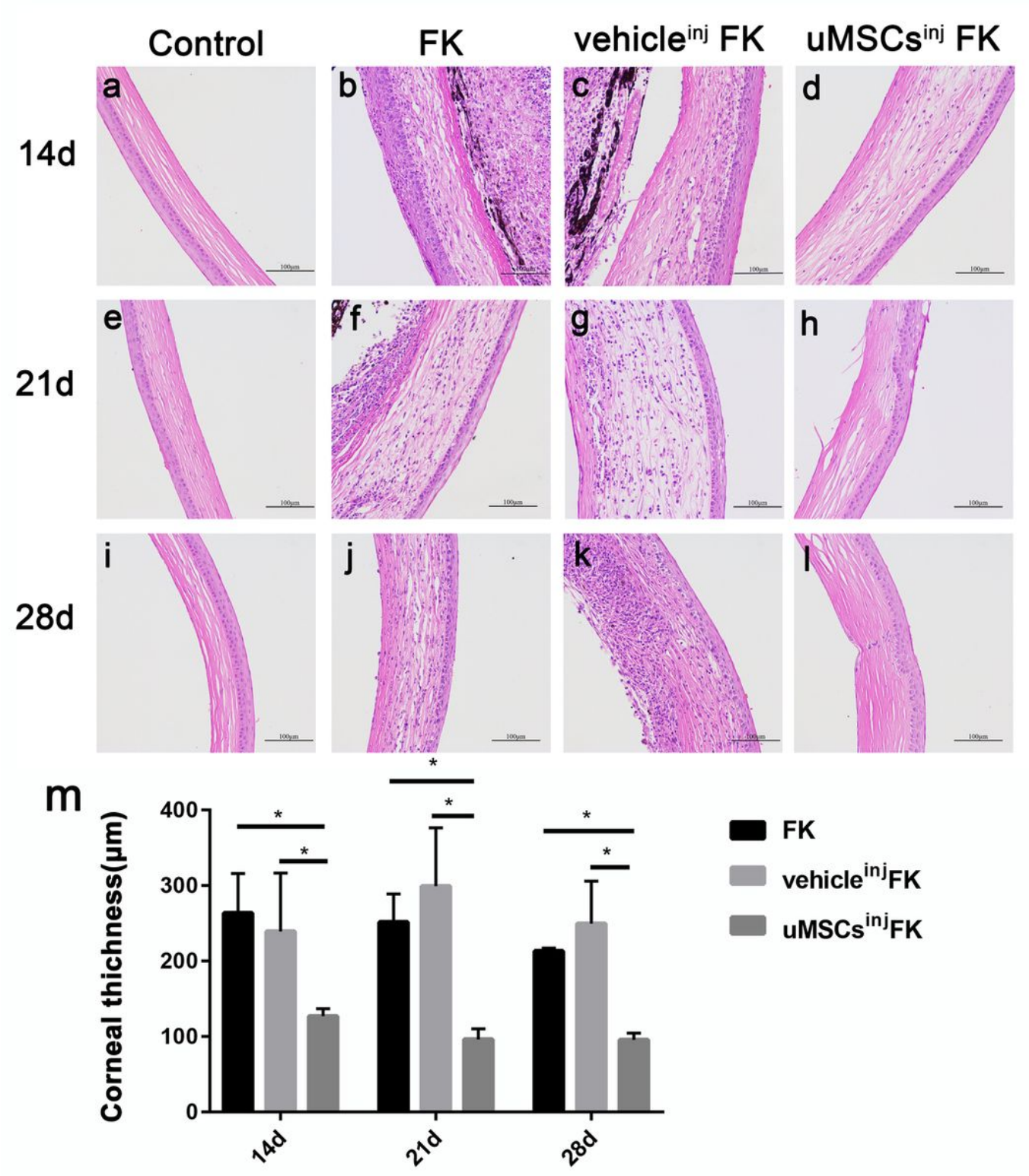

Figure 3

Histological examination and corneal thickness evaluation between groups. (a, e and i) Corneas from control group mice with no injuries. Corneas from FK group mice (b, f and j) and vehicleinj FK group mice

(c, g and k) exhibited corneal thickening, multiply layers of irregularly aligned excessive collagen deposition and extensive inflammatory infiltration. In contrast, corneas from uMSCsinj FK group (d, h and i) showed less corneal thickness, relative normal structural arrangements, and fewer inflammatory cell infiltrations. (m) The comparison of average corneal thickness (μm) in uMSCsinj FK group and the other two groups had statistically significant difference at 14d, 21d and 28d post-injury. Thus, histological examination provided further evidence of the therapeutic effects of uMSCs application on FK. Values are presented as means ± SD, n=6, Mean values with asterisks are significantly different ($P < 0.05$). Scale bar, 100μm.

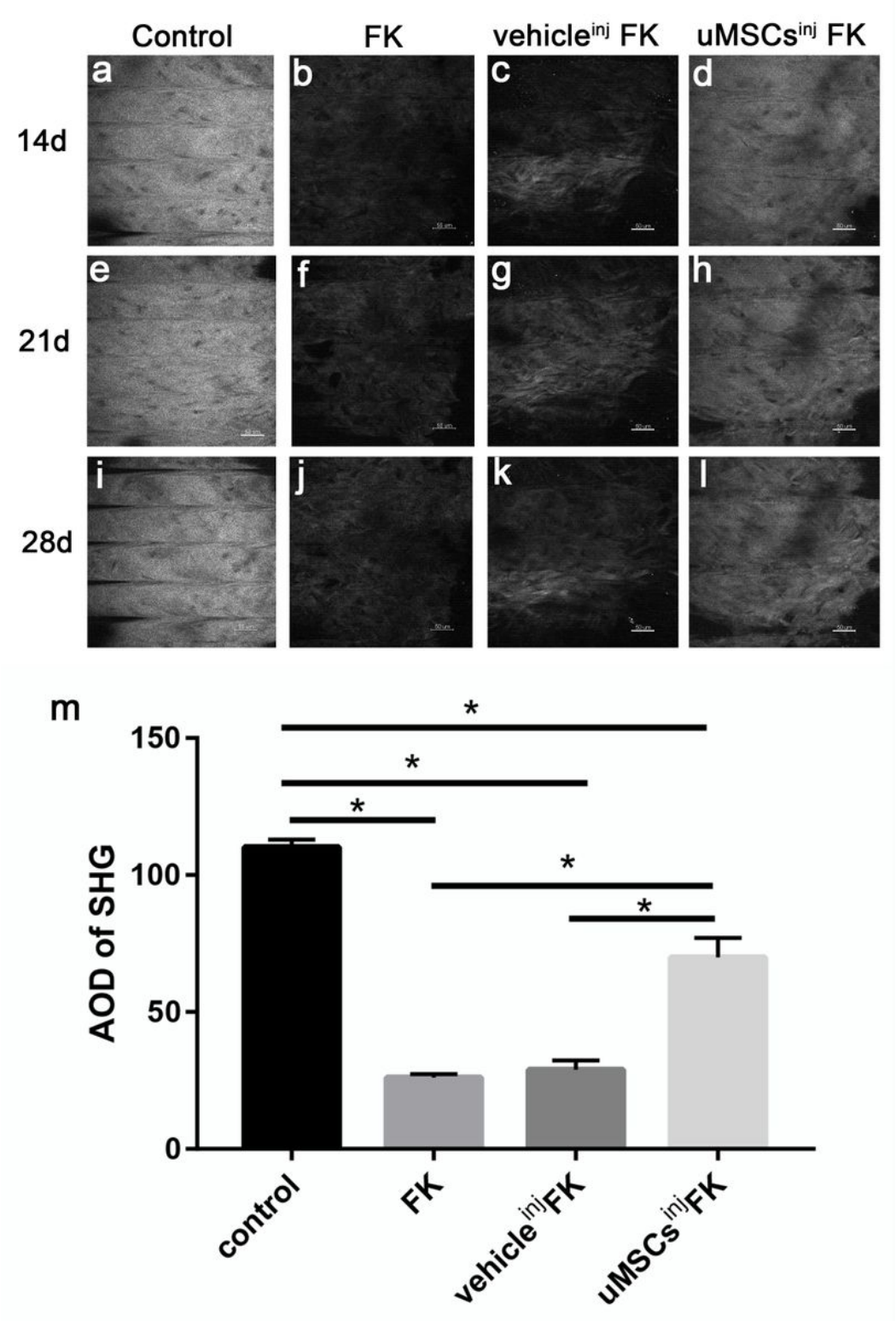

Figure 4

Second harmonic generation (SHG) taken by two-photon confocal microscopy in vivo. (a, e and i) Corneas from control group mice exhibited high intensity transient signals, indicating normal and regular arrangement of corneal collagen fibers. Corneas from FK group (b, f and j) and vehicleinj FK group mice (c, g and k) exhibited weak signals excited by confocal microscopy, indicating collagen degradation and abnormal collagen deposition caused by infection and inflammation. The uMSCsinj FK group mice

corneas (d, h and i) exhibited relative high intensity transient signals indicating relative regular collagen fibril arrangements compared with the other two groups. (m) Statistical chart of average optical intensity (AOD) of SHG between groups on 14d post-injury. Collagen destruction was restored by uMSCs treatment with an AOD of 69.97±7.09 at 14d post-injury, which had statistically significant differences compared with FK group (26.09±1.27) and vehicleinj FK group (28.98±3.32). Values are presented as means ± SD, n=6, Mean values with asterisks are significantly different ($P < 0.05$). Scale bar, 50μm.

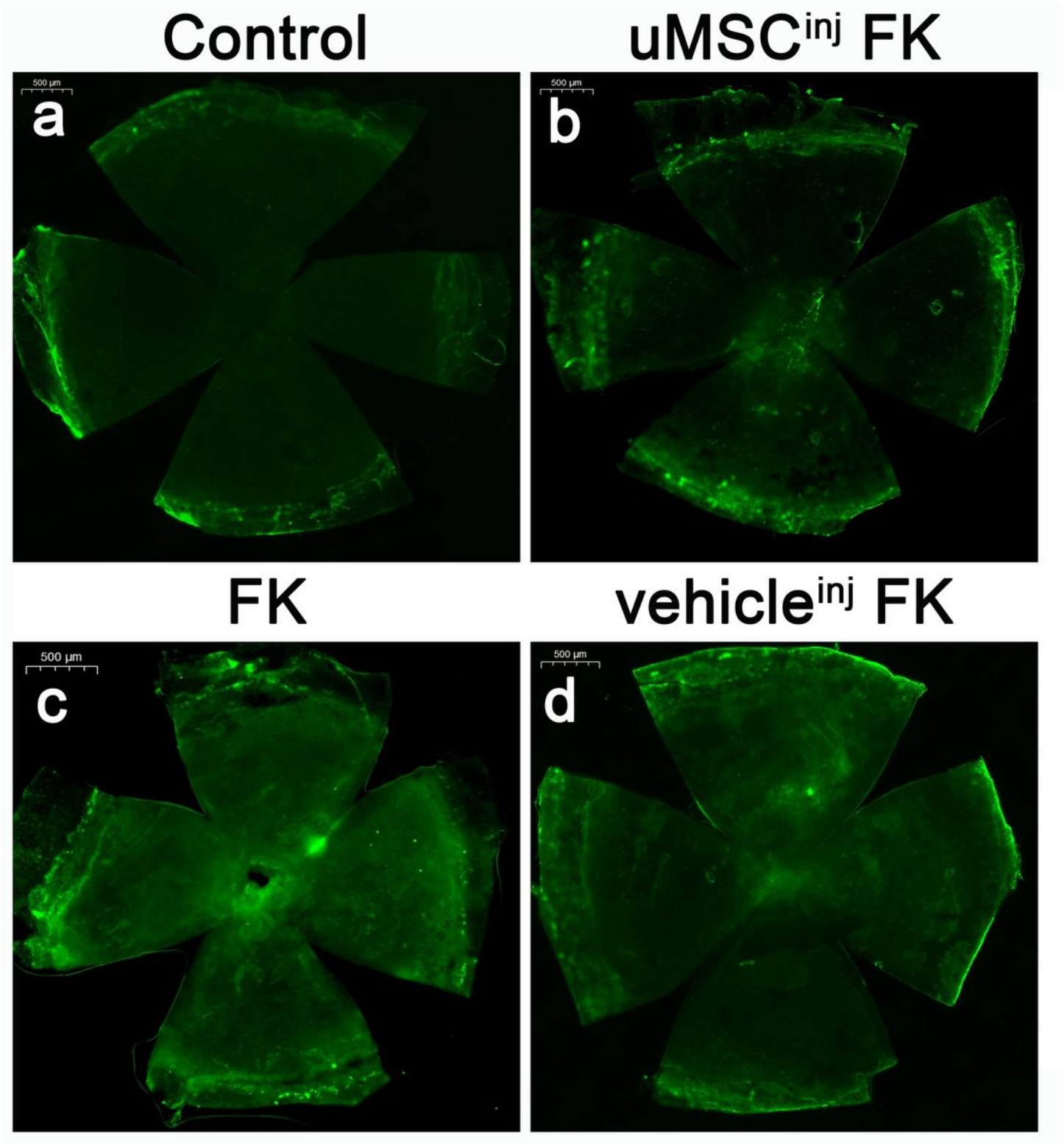

Figure 5

The α-SMA detected by immunofluorencence. Mice corneas of control group (a) could only be found of α-SMA expression at the pericorneal vascular region due to vascular wall smooth muscle cells staining, but was not observed in the central area of cornea. The α-SMA production was up-regulated in the lesion area of corneas 14d post-injury in FK group (c) and vehicleinj FK group (d), whereas was found down-regulated in uMSCsinj FK group (b), which was consistent with the results of clinical manifestations. The results demonstrated that uMSCs application could inhibit keratocyte-to-myofibroblast transition during the healing period of FK. The α-SMA was labeled by FITC, n=4. Scale bar, 500μm.

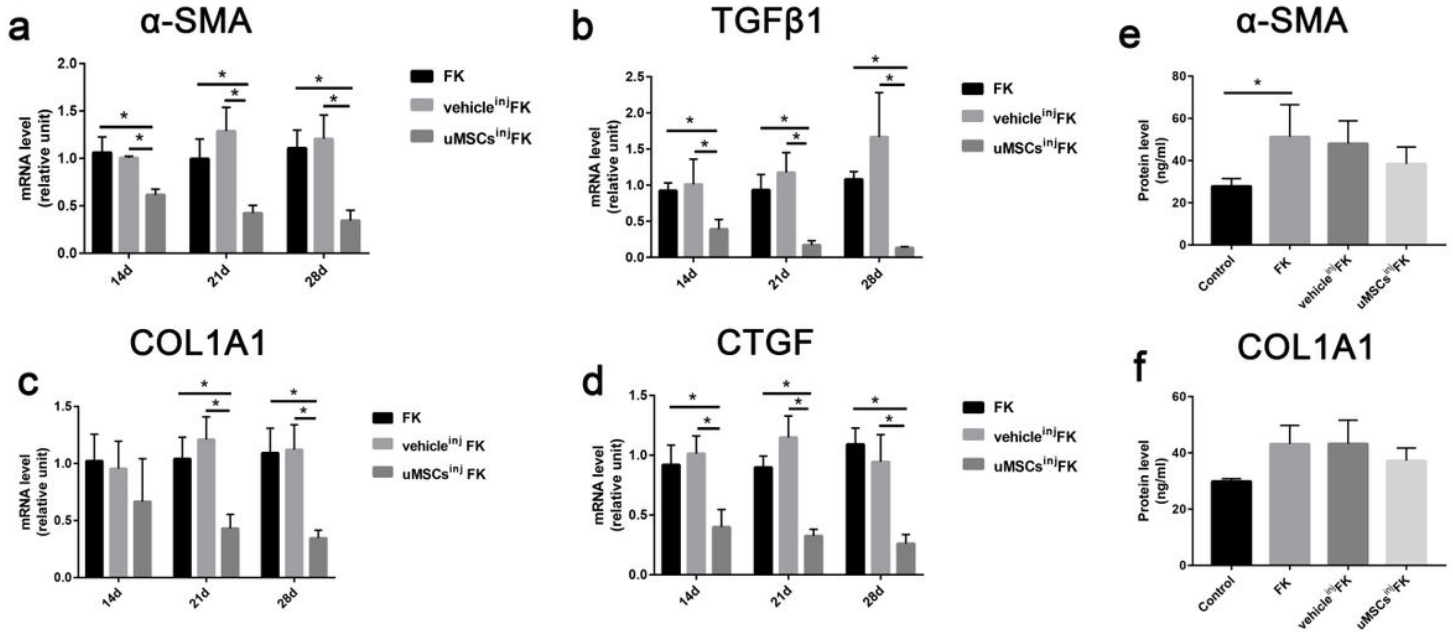

Figure 6

Fibrosis-related factors α-SMA, TGFβ1, CTGF and COL□were down-regulated by uMSCs administration. (a-d) uMSCs administration significantly inhibited the expression of pro-fibrogeneic genes α-SMA, TGFβ1, CTGF and COL□ and had a statistically difference compared with vehicleinj FK group and FK group. No statistically significant differences were detected in the α-SMA, TGFβ1, CTGF, and COL□ mRNA expression between the FK group and vehicleinj FK group. Protein concentration of α-SMA (e) and COL□ (f) were detected by ELISA kits at 14d post-injury, and the results revealed up-regulation of α-SMA and COL□ after FK. Meanwhile, the detection of the two cytokines in uMSCsinj FK group was down-regulated compared with FK group and vehicleinj FK group at 14d post-injury. Values are given as means ± SD, n=6, Mean values with asterisks are significantly different ($P < 0.05$).

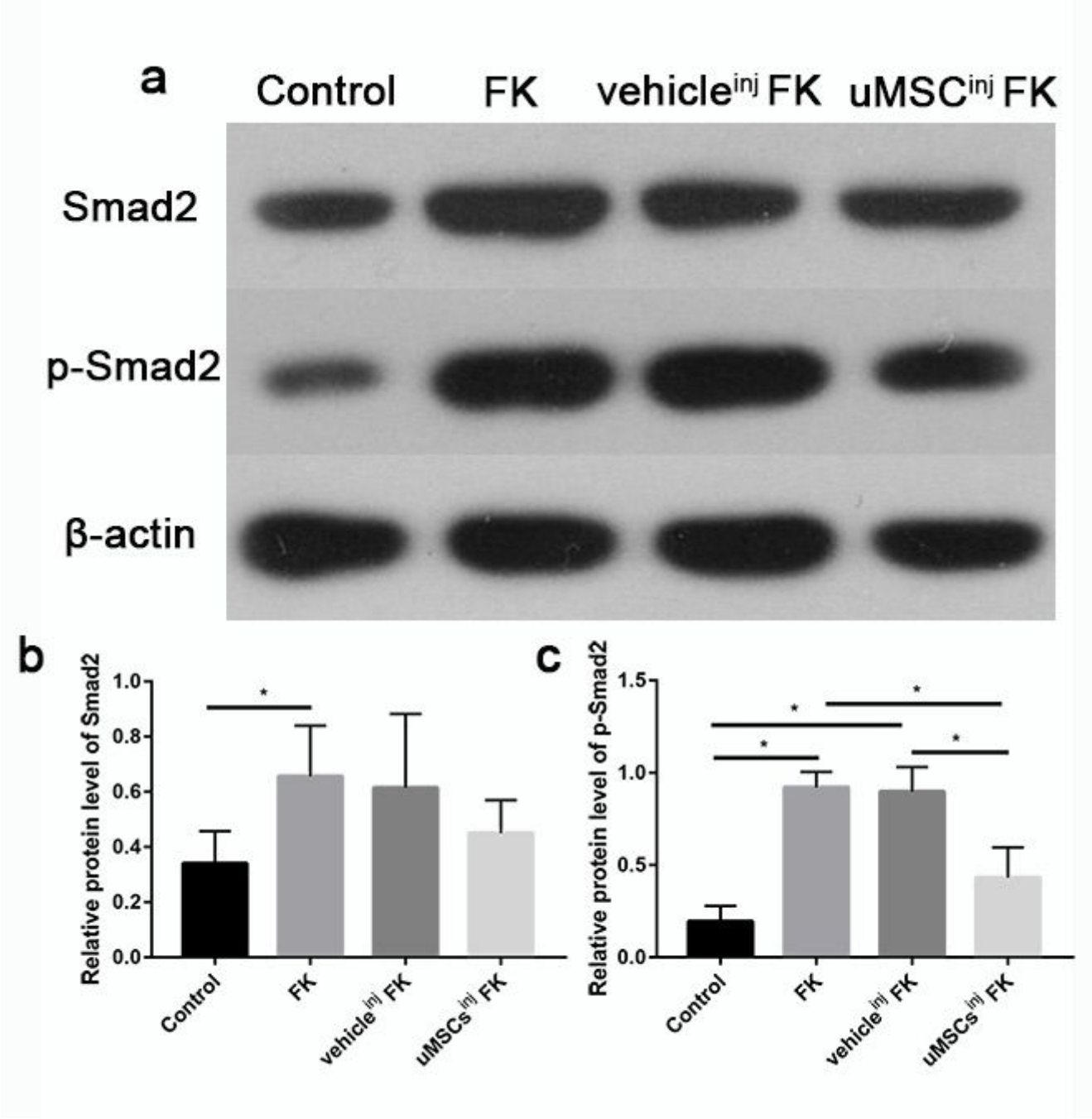

Figure 7

uMSCs attenuates phosphorylation of TGFβ1/Smad2 signaling pathway in the regulation of corneal fibrosis. (a) To validate the role of TGFβ1/Smad2 signaling pathway in the regulation of corneal fibrosis in mice FK model, we conducted westernblot analysis to detect relative protein levels of Smad2 and phosphorylated Smad2 in corneas at 14d post-injury. (b) The Smad2 expression was up-regulated by FK compared with control group and had no statistically differences between FK group, vehicleinj FK group and uMSCsinj FK group. (c) However, phosphorylation of Smad2 was hard to detect in control group mice corneas whereas was up-regulated in FK group and vehicleinj FK group respectively. Samples from uMSCsinj FK group exhibited partially inhibited phosphorylated Smad2 expression compared with vehicleinj FK group, indicating uMSCs potentially hold the capacity of down-regulated TGFβ1/Smad2

signaling pathway in mice FK. Values are given as means ± SD, n=6, Mean values with asterisks are significantly different (P < 0.05).

Supplementary Files

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- supplement1.pdf