BMC Ophthalmology

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Efficacy of topical mesenchymal stem cell exosome in Sjögren's syndrome-related dry eye: a randomized clinical trial

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Abstract

Background Sjögren's syndrome (SS) is a chronic inflammatory autoimmune disorder affecting salivary and lacrimal glands, leading to distressing ocular symptoms. Existing therapeutic approaches for SS-associated dry eye syndrome (DES) show insufficient efficacy. This study investigates the use of topical MSC-derived exosomes in primary SS-related DES.

Methods In phase 1 and 2, triple-blinded, randomized trial, two vials of eye drops are given to each participant, one with code A and one with code B and only the supervisor knows the content of each vial. The treatment group ($n=8$ eyes) received 10 µg of MSC-derived exosomes twice daily for two weeks and the control group ($n=8$ eyes) received Phosphate buffered saline(PBS) for their respective eyes. Safety was assessed through ophthalmic exams, while DES assessment tests evaluated treatment effectiveness. Non-parametric tests employed to examine assumptions. The differences of ocular examination results between day 0 and month 3 were analyzed using a paired Student's t test. Measurement data that were not normally distributed are represented by the median (interquartile range), and comparisons were performed using the Wilcoxon rank-sum test.

Results The treatment showed promising results, with significant improvements observed in various indicators such as reduced Ocular Surface Disease Index scores, increased tear secretion, lower fluorescein scores, and longer tear-film break-up time before and after treatment and between the controls and the treated groups. Our results showed a significant increase in multifunctional proteins such as Epidermal Growth Factor and thrombospondin-1 and conversely, the levels of pro-inflammatory cytokines, including interleukin-6 and Matrix metalloproteinase-9 significantly decreased in tear of participant before and after treatment and between the controls and the treated groups.

Conclusions Our study underscores the safety and substantial therapeutic potential of using MSC-derived extracellular exosomes eye drops to treat SS-associated DES.

Trial registration IRCT20211102052948N1 The study protocol has been approved in Iranian Registry of Clinical Trials at 2022-04-20.

Keywords Mesenchymal stem cell, Exosomes, Sjögren's syndrome, Dry eye

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Background

Sjögren's syndrome, an enduring inflammatory autoimmune disease, primarily impacts the salivary and lacrimal glands. The persistent autoimmune inflammation of the lacrimal gland is caused by SS, leading to diminished tear production [1]. This results in hyperosmolarity and instability of the tear film, instigating the release of pro-inflammatory cytokines such as Interleukin-6 (IL-6), IL-22, tumor necrosis factor-α, and chemokines from immune cells [2, 3]. These cytokines and chemokines promote the production of matrix metalloproteinase-9 (MMP-9) and raise the levels of Th-1 and Th-17 cells. This sequence of events intensifies the symptoms of dry eye Syndrome (DES) by stimulating inflammation on the ocular surface, loss of epithelial cells, and decreased mucin secretion [4, 5]. As a result, DES, a protracted lack of moisture in the eyes, causes dysfunction on the ocular surface, giving rise to a range of distressing symptoms, including blurry vision, foreign body, burning sensation, hypersensitivity to light, eyestrain, and reddened eyes [6].

Despite the variety of existing therapeutic options, a significant number of individuals afflicted with SS continue to endure the distressing symptoms of DES. The current therapeutic approaches continue to rely on the symptomatic alleviation of sicca-related manifestations alongside the utilization of diverse immunosuppressive agents to address systemic manifestations. Common topical immunotherapy options for DES used to manage inflammation and improve symptoms include: corticosteroids, cyclosporine A [7]. In order to achieve an effective remedy to DES, novel treatment protocols that target the core pathophysiologic mechanisms should be designed.

MSCs are multipotent stem cells that can self-renew and differentiate into adipocytes, osteoblasts, chondrocytes. They can be extract from various tissues, including bone marrow, peripheral blood, adipose tissue, umbilical cord and dental pulp. MSCs are low immunogenic because they express low levels of MHC I and they don't express MHC II. The multipotent capacity of MSCs to undergo differentiation into diverse cellular lineages has sparked considerable interest in investigating their therapeutic potential for various immune-mediated disorders. This interest is fueled by their dual characteristics of immunoregulation and low immunogenicity [8]. However, the utilization of MSCs for therapeutic purposes has been accompanied by concerns about their potential risks of tumorigenesis and ectopic osteogenesis [9]. In this context, implementing cell-free therapies, including extracellular vesicles, is considered to exhibit a higher degree of safety. Among extracellular vesicles, exosomes have emerged as a particularly promising option, demonstrating positive effects in participants with SS [10, 11].

Exosomes, vesicles secreted by cells ranging from 30 to 150 nm, serve as a medium for intercellular communication. Exosomes carry a complex composition of proteins, peptides, nucleic acids (RNA, microRNAs (miRNAs), long noncoding RNA, and DNA), lipids, and metabolites [12, 13]. The composition of exosome cargos is influenced by the secretory cell type and the mechanism triggering exosome release. Interestingly, exosomes can both regulate and initiate inflammation. Stem cell-secreted exosomes, in particular, have been found to have immunosuppressive effects by inhibiting inflammasome activation [14]. The unique structure of the phospholipid bilayer enables exosomes to safeguard their contents effectively from degradation in the in vivo environment [15].

Because MSC-derived exosomes are nano-sized, they rapidly diffuse throughout the retina after intravitreal injection, significantly reduces inflammation and damage of retinal [16]. In terms of potential complications, such as vision loss resulting from hemorrhage or retinal detachment, it has been observed that the utilization of cell-derived exosomes presents a safer alternative to MSC-based treatments [17]. Furthermore, exosomes exhibit the advantage of prolonged storage capability under specific conditions without significantly impairing their RNA content [18]. Human Wharton's Jelly MSCs (HWJMSCs) are multipotent stem cells that can differentiate into various mesodermal cell lineages. Due to their low immunogenicity, robust self-renewal ability, and reduced ethical concerns compared to other types of stem cells, HWJMSCs present an advantageous choice for clinical implementation [19]. Exploring HWJMSC-derived exosomes as a potential therapeutic intervention for immune-mediated disorders has yielded promising outcomes [20–22].

Therefore, the current randomized controlled trial (RCT) was conducted to evaluate the efficacy and safety of topical ophthalmic HWJMSC-derived exosomes in treating DES associated with SS.

Methods

This study is a triple-blinded, randomized, controlled clinical trial (RCT) with the primary objective of assessing the effectiveness and safety of topical ophthalmic application of exosomes derived from HWJ-MSCs in treating DES among participant with SS.

In this study, the researcher (IAD) enrolled the participants and then she used two blocks A and B to assign the eye of participants to the different treatment groups. This process was randomized by using dice. (Details of the randomization procedure are described in the study protocol). In this three-blind study, two vials of eye drops are given to each participant, one with code A and one with code B. The researcher, participants, and clinical data analyzer are unaware and only the supervisor knows which vial contains stem cell exosomes. Shiraz University

of Medical Sciences Ethics Committee, Shiraz, Iran, approved the study protocol (IR.SUMS.REC.1400.852). The study protocol was also registered in the Iranian Registry of Clinical Trials (IRCT20211102052948N1) at 2022-04-20. Before their inclusion in the study, written informed consent was obtained from the WJ tissue donors and participants with SS. The trial was conducted in accordance with the guidelines for conducting clinical trials on human participants and the Declaration of Helsinki.

Materials

Serum-free Dulbecco's modified Eagle's medium (DMEM) (Gibco Invitrogen, Carlsbad, CA, USA), PBS, Fetal Bovine Serum (FBS) (Gibco Invitrogen, Carlsbad, CA, USA), Trypsin-Ethylene Diamine Tetra Acetic acid (EDTA), Glycerol Phosphate (Sigma, USA), Ascorbic Acid-2 Phosphate (AA2P) (Sigma, USA), Alizarin Red staining solution (Sigma, St. Louis, MO, USA), Sodium Dodecyl Sulphate (SDS-PAGE), Poly Vinylidene Fluoride (PVDF) membrane, Tris-Buffered Saline with Tween (TBST),

Cell culture and isolation of HWJMSCs

In this study, the umbilical cord samples were obtained from ten different donors at the Obstetrical Department of the Imam Hospital in Tehran, Iran. The umbilical cords were collected from healthy full-term women who underwent elective cesarean sections under sterile conditions. Umbilical cords blood serum was tested negative for various viruses and diseases, including hepatitis C virus (HCV), human immunodeficiency virus (HIV), hepatitis B virus (HBV), Epstein-Barr virus (EBV), cytomegalovirus (CMV), and syphilis. The culture conditions for WJ-MSCs were based on current Good Manufacturing Practice (cGMP) standards. The collected tissue samples were rinsed with PBS containing penicillin (1 U/ml) and streptomycin (100 mg/ml) (Invitrogen). After dissecting the blood vessels, the cords were fragmented into approximately 0.5-1 mm2 pieces using a sterile scalpel, rinsed with PBS and treated with type II collagenase (10 mg/ml; Invitrogen) for 1 h at 37 °C. These fragments were then placed in T75- culture flasks and immersed in DMEM F12 (Gibco Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS (Gibco Invitrogen) and 1% penicillin and streptomycin. The culture flasks were subjected to incubation at a temperature of 37 °C, 90% humidity and 5% CO2, during which shaking was omitted to facilitate tissue adherence. After ten days of incubation at 37 °C with 5% CO2, adherent cells reached 80–90% confluence. The MSCs were subsequently transferred to a new flask with the same culture medium for expansion and characterization during their 3rd-5th passage cycles, as discussed in subsequent sections.

Characterization and identification of HWJMSCs

Flow cytometry analysis

The isolated single cells were subjected to surface phenotype analysis using flow cytometry to validate their mesenchymal profile. A total of 1×10^6 cells were individually incubated for 1 h at 4 °C with an optimally diluted set of conjugated antibodies, specifically anti-CD45-FITC (ab27287), anti-CD90-APC (ab11155), and anti-CD105- PerCP-Cy5. 5 (ab91138), all sourced from Abcam (Cambridge, UK). Flow cytometry experiments were conducted using a BD FACS Calibur Flow Cytometer (BD Biosciences), and the resulting data were analyzed using Flowing software.

Mesodermal lineage differentiation

WJ-MSCs were cultured in 12-well plates at a density of 5×10^4 cells using DMEM supplemented with 5% FBS and 1% antibiotic, following the same culture conditions mentioned earlier. To assess multilineage differentiation potential, after 48 h, WJ-MSCs were cultured in the respective differentiation media as specified below.

For osteoblastic differentiation, WJ-MSCs were seeded in 6-well culture plates at a density of 1×10^5 cells/well. The cells were cultured in an HG-DMEM-F12 medium supplemented with 10 mM/L glycerol phosphate, 50 mg/ml AA2P, and 0.1 mM dexamethasone at 37 °C in a 5% CO2 incubator. The medium was refreshed every three days. After three weeks, the cells were fixed with 4% paraformaldehyde at room temperature for 30 min. The formation of mineralized nodules was detected by applying an Alizarin Red staining for 20 min.

For adipogenic differentiation, WJMSCs were seeded at 2×10^4 cells/well in 6-well culture plates. At 80% confluence, the medium was replaced with adipogenic medium and changed every 3–4 days over three weeks. After three weeks, the adipogenic medium was removed, and cells were washed with 1× PBS, fixed with 10% formaldehyde, rinsed with water, and stained with Oil Red O. The cells were subsequently incubated for 5 min at room temperature with Oil Red O solution. Following the incubation, the Oil Red O solution was removed, the cells were washed three times with distilled water, and were observed red lipid vacuoles using an inverted microscope.

Characterization of the isolated WJMSC-derived exosomes

Upon achieving a confluence of 80–90%, the growth medium was replaced and the cells were washed twice with PBS. The medium was then substituted with DMEM-F12 and supplemented with 10% exosome depleted FBS(Gibco) and 1% penicillin and streptomycin for 48 h at 37 °C. The cell culture supernatant was subsequently filtered and transferred to 50 ml sterile falcon tubes. The media was agitated with a vortex and

centrifuged at 400 x g for a duration of 10 min at 4 °C. In the next step, the supernatant media was subjected to centrifugation at 2500 x g for 30 min at the same temperature and the resulting supernatant was transferred to new sterile falcon tubes. To achieve high-purity exosomes, the supernatant was filtered using a 0.22 μm pore size filter (BIOFIL, China) to eliminate macrovesicles larger than 220 nm. The pre-purified supernatant was then transferred to an ultracentrifuge tube and subjected to two cycles of centrifugation at 110,000 x g for 120 min at 4 °C using an XL-90 Ultracentrifuge (Beckman Coulter, USA) equipped with an SW 28 Ti Swinging-Bucket Aluminum Rotor (Beckman Coulter, USA). After careful removal of the supernatant, the pellet containing the exosomes was homogenized by adding a small amount of PBS. Finally, the resultant exosomes were resuspended in PBS.

The morphology of exosomes was examined through transmission electron microscopy (TEM, HITACHI, Japan), and Dynamic Light Scattering (DLS). For the assessment of exosome nanoparticle size, nano Zetasizer (SZ-100) was utilized. The protein concentration of exosomes obtained from the cultured medium of HWJMSCs was determined using the bicinchoninic acid (BCA) protein quantification kit (Thermo Fisher Scientific, Waltham, MA). Western blot analysis was performed to evaluate the expression of exosome protein markers, including CD9, CD63, and CD81.

Western blot

The WJMSC-Exos labeling was detected using Western blot analysis. The procedure involved preparing an SDS-PAGE gel (Solarbio, Beijing, China), calculation of sample volume based on the concentration of protein, and electrophoresis of each group of markers and samples. The gel was subsequently sectioned based on molecular weight, with proteins transferred onto a PVDF membrane. After blocking the membrane with 5% non-fat milk powder for a duration of 2 h, the PVDF membrane was exposed to appropriately diluted primary antibodies (rabbit anti-human CD81, CD63, and CD9 antibodies) (Abcam, Cambridge, UK) and incubated at 4 °C for 16 h. Following the primary antibody incubation, the PVDF membrane was exposed to a diluted solution of goat anti-rabbit IgG antibody (Abcam) solution for 1 h at 4 °C, followed by thorough washing with TBST. The horseradish peroxidase-enhanced chemiluminescence method facilitated the attainment of color development. he intensity of the protein band was assessed by quantifying the gray value using Image J analysis software; to determine the relative protein expression level in the samples, the gray value was compared to that of the internal reference.

Preparation of exosome eye drop

To formulate the drops, after collecting and purifying the exosomes using ultracentrifuge, pellets were dissolved in PBS with concentration of 100 μg/mL and pH between 7.2 and 7.4, packed in sterile vials and stored the refrigerator. All the above steps were performed under GMP condition.

Experimental design

Study design and participant

This study is a randomized, parallel-group, three-way blind, phase 1–2 clinical trial involving eight participants. Demographic and clinical characteristics of primary Sjögren syndrome(pSS) participants included in the study are presented in Table 1. Randomization of treatment assignment was performed using the rand function in Excel software. The processes of enrollment and allocation are depicted in Fig. 1, utilizing a CONSORT flow diagram. A total of 10 eligible participants (20 eyes) were recruited for the study and were randomly distributed into two groups at a 1:1 ratio. However, two participants discontinued their enrollment due to COVID-19 infection within the first month of the study. To monitor compliance, a detailed registration of participants was carried out at the beginning of the study and basic information was collected from them. Before the start of the intervention, regular meetings were held with the participants to

Table 1 Demographic and clinical characteristics of pSS participant included in the study

participant ID	Age (years)	Gender	anti-Ro (SS-A) Ab	anti-La (SS-B) Ab	OSDI	Schirmer's test***	DES	TBUT (s)	TMT
pSS1	55	F	+	+	35	+	+	1.79	0.1–0.3
pSS2	50	F	+	+	23	+	+	3.74	< 0.1
pSS3	45	F	+	+	43	+	+	2.93	0.1–0.3
pSS4	37	F	+	+	33	+	+	2.28	0.1–0.3
pSS5	34	F	+	+	35	+	+	8.24	0.1–0.3
pSS6	56	F	+	+	25	+	+	6.45	0.1–0.3
pSS7	34	F	+	+	14	+	+	6.19	0.1–0.3
pSS8	38	F	+	+	35	+	+	4.33	0.1–0.3

Abbreviation: pSS, primary Sjögren syndrome, F, Female, OSDI, ocular surface disease index, DES, Dry Eye Syndrome, TBUT (s), Tear break-up time (sec), TMT, Tear meniscus thickness, Ab, Antibody

****Values are in mm/5 min; normal flow > 10 mm/5 min

. '+' indicates dryness and tear secretion ≤ 5 mm/5 min

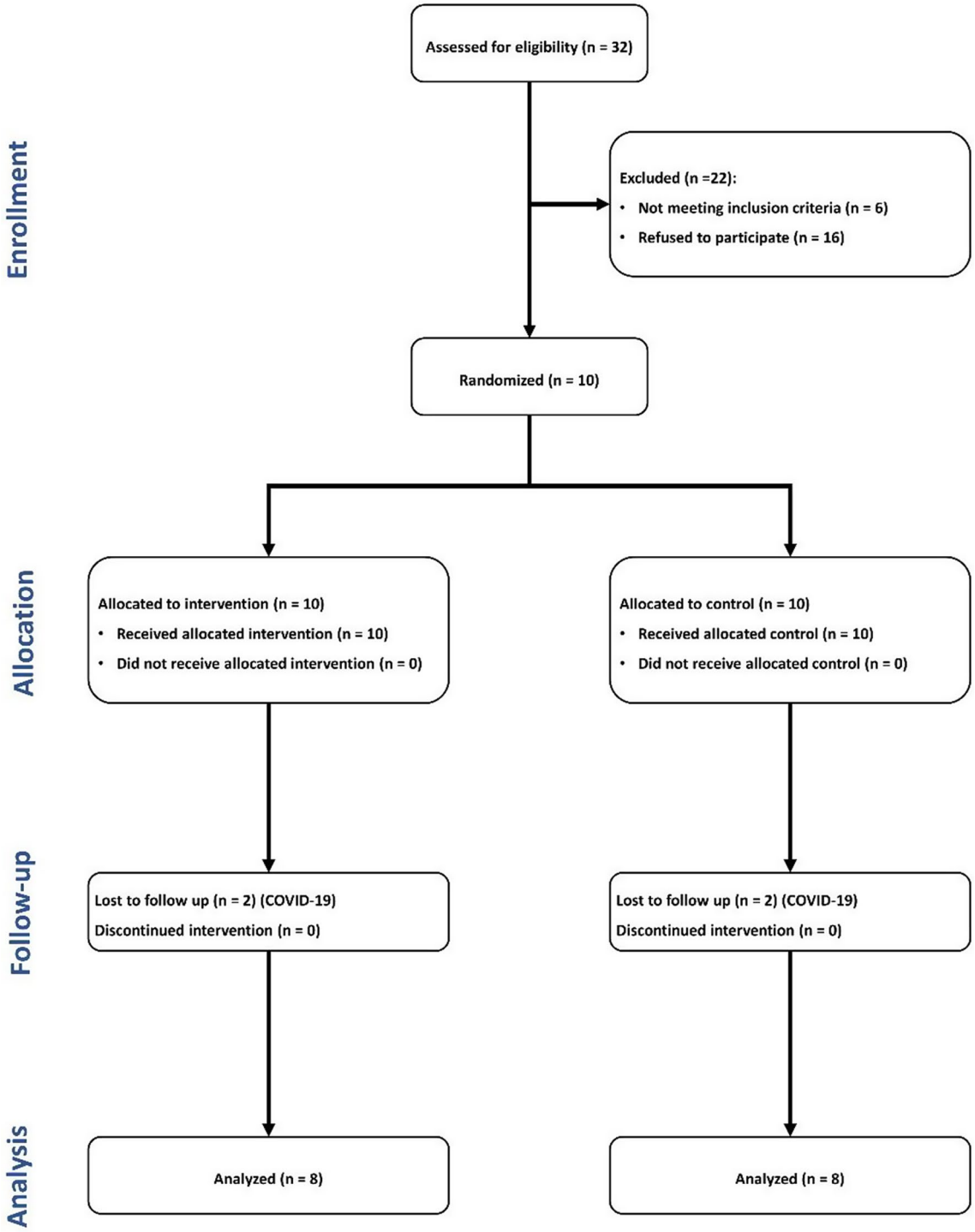

Fig. 1 The CONSORT flow diagram shows how participants were assigned to groups and followed up during the clinical trial. CONSORT: Consolidated Standards of Reporting Trials

provide necessary training about the study protocol, the importance of compliance, emphasize correct adherence to treatment protocols, and assess their condition. Contact with participants and monitoring of side effects were also carried out daily.

This study was conducted from April 2022 to September 2022. The treatments that the participants were using prior to their enrolment in the trial included hydroxychloroquine, prednisolone and artificial tear. when they started the trial, they only stopped using artificial tear

drops and their disease was well controlled during the study.

The inclusion criteria for participants in the article are as follows:

1. Female participants.
2. The age range is 30–55 years.
3. Confirmed SS diagnosis based on the ACR-EULAR 2016 classification [23].
4. Presence of evident dry eye symptoms, including dryness, burning sensation, discomfort in the ocular surface, visual fatigue, or foreign body sensation. (OSDI Score assessment)
5. Resistance to treatment with standard treatments.
6. Tear Film Break Up Time (TFBUT) < 10 s. (A single drop of fluorescein hydrochloride, 2%, was injected into the conjunctiva's inferior bag for evaluation of tear film stability. For observation, cobalt blue light was used. Three measurements were taken, and the average was taken, and the time it took for the first dry spot to appear after blinking was recorded).
7. Corneal Fluorescein Staining score (FLCs) < 6. (Commercially available strips were used to stain the cornea with fluorescein. The participant's eye is greeted with a piece of blotting paper that contains the dye. Participants are asked to blink. The tear film covering the cornea's surface is covered in the dye as a result of blinking. The ophthalmologist then shines a blue light at participant's eye. Any problems that the dye may cause on the cornea's surface will appear green in blue light).
8. Schirmer's test result of ≤ 10 mm / 5 min in at least one eye (after testing both eyes) for tear secretion. (Schirmer's I test was performed on participants 'eyes without using any anesthesia and consisted of 5 minutes of sterile Whatman 41 paper to observe the strip getting wet. The ophthalmologist will insert the end of a piece of sterile Whatman 41 paper inside each eye's lower eyelid. At the same time, both eyes are tested. For five minutes, the eyes are closed. The doctor examines the paper and assesses how much of it has drained after five minutes).

They were selected during their routine visits at the Poostchi Ophthalmology clinic in Shiraz, Iran. Participants were deemed ineligible for the study if they were:

1. Pregnant or lactating females;
2. Known cases of severe systemic diseases;
3. presenting with active cases of fungal, viral, or bacterial keratitis or conjunctivitis;
4. manifesting hypersensitivity reactions to any medications included in this study;
5. wearing contact lenses;
6. Participants demonstrating pre-existing untreatable ocular conditions, such as glaucoma or uveitis;
7. unable to comply with the investigator's requirements;
8. Subjects who have undergone recent ocular surgery within the past three months;
9. have applied eye drops that may potentially interfere with the study objectives within the past 24 h.

Intervention

A total of eight eligible participants were randomly assigned to two parallel treatment groups. In each participant, one eye received Wharton's Jelly MSC-derived exosomes at a dosage of 10 ug/drop as an intervention, and the other eye, as a control, received PBS twice daily for two weeks. The primary outcome measure was the assessment of changes in the Ocular Surface Disease Index (OSDI), while secondary outcome measures included evaluating changes in the Schirmer's I test, Tear Meniscus Thickness (TMT), conjunctiva redness score, subjective improvement of ocular symptoms, TFBUT, corneal fluorescein staining test, and alterations in the expression of inflammatory genes.

Data collection

Data collection was carried out by a single ophthalmologist. The participant's evaluation encompassed four stages: the initial assessment of DES, the evaluation after completion of treatment, and the follow-up assessments at 1 and 3 months' post-treatment completion. During the follow-up, careful surveillance was conducted to monitor the clinical indicators and document any side effects that may relate to the topical application of HWJMSC-derived exosomes eye drops.

The severity of DES was evaluated by the following tests: OSDI, TMT measurement, TFBUT, corneal fluorescein staining, and Schirmer's I test. OSDI is a validated 12-item questionnaire, with scores ranging from 0 to 100 to evaluate DES. The results were categorized into four groups: normal (0–12), mild (13–22), moderate (23–32), and severe DES (> 33) [24]. A TMT ranging from 0.2 to 0.5 mm was considered normal, while values below 0.2 mm indicated the presence of DES [25]. TFBUT measures dryness in the tear film after a participants' blink. Fluorescein hydrochloride (2%) is applied, participants blink multiple times, and observations under cobalt blue light note the time for the first dry spot. A TFBUT of less than 10 s indicated dry eye, while a TBUT of 10 s or more was considered normal [26]. Corneal fluorescein staining was conducted using commercially available strips, and the severity of staining was assessed using the SICCA ocular staining form, which assigned a maximum score of 12 for each eye. Severity grades were defined as follows: grade 0 (0–9 dots), grade 1 (10–32 dots), grade 2 (33–100

dots), and grade 3 (>100 dots) [27]. An additional tool utilized to evaluate DES is the Schirmer's I test, a commonly employed method for evaluating tear production, which involves the non-anesthetic application of sterile Whatman 41 paper to participants' eyes. Severity classifications for DES were determined as follows: normal (>10 mm), moderate (5–10 mm), and severe DES (≤5 mm) [28].Collection of Tear Samples.

A volume of 100 µl of tear samples was collected using a sterile capillary tube without any additives. The collected samples were transferred into sterile vials and stored at -80 ºC for gene expression analysis.

Real-time reverse transcription polymerase chain reaction (RT-PCR)

RNA extraction from human tears was carried out following a previously established protocol [29]. Total RNA was extracted using the EX6101-RNX Plus Solution (Sinaclon Bio Science) in accordance with the manufacturer's instructions. Reverse transcription and cDNA amplification were conducted using the AddScript cDNA synthesis kit (addbio). The primers for human MMP9, Epidermal Growth Factor (EGF), lactoferrin (LTF), IL6, IL22, and beta Actin (ACTB were synthesized by Germany's Metabion company. Quantitative real-time PCR experiments were performed using SYBR Green I on a Thermal Cyclers 96 instrument (Applied Biosystems, Germany). The primer sequences can be found in Table S1. ACTB was employed as a reference gene to normalize and determine relative expression levels. The expression levels were determined using the $2-\Delta\Delta CT$ method.

Statistical analysis

Due to the scarcity of data, non-parametric tests were utilized to scrutinize assumptions. The Wilcoxon Rank-Sum test was employed for pairwise median comparisons, while the Chi-Square test was used to investigate differences among categorical variables. The Friedman test was applied to identify variations in treatments across multiple testing attempts. Numerical data are presented as median and interquartile range (IQR), and proportions are expressed as frequency (%).

For the assessment of the HWJMSC-derived exosomes effects on eye clinical parameters, hierarchical cluster analysis was done using representation of data by log2-transformed signals. Also, to evaluate the gene expression profiles in tear samples Pearson correlation heatmap served as the distance measure for clustering and this analysis was executed using R software (version 4.3.1).

Results

Characterization of HWJMSCs

Assessment of the proliferation kinetics and characteristics of the HWJMSCs during culture was meticulously monitored using an inverted microscope. The microscope showed the presence of elongated and slender fibroblast-like cells exhibiting a remarkable plastic adherence, aligning with classical morphological features of MSCs. Morphological images of HWJMSCs under the inverted microscope on days 7, 14, and 21 are displayed in Fig. 2a.

Surface phenotype analysis was then conducted using flow cytometry to validate the mesenchymal identity of these fibroblast-like cells. Flow cytometry demonstrated that most of this cell population expressed the mesenchymal markers of CD90 (88.8%) and CD105 (85.4%), while there was insignificant expression of hematopoietic-specific cell markers, such as CD45 (0.20%) (Fig. 2b).

Using specific differentiation media, multilineage differentiation of these cells was then attempted, and after approximately three weeks, the capacity for differentiation into osteogenic or adipogenic lineages was evaluated. Oil Red-O and Alizarin staining revealed the presence of intracellular lipid droplets and calcium oxalate deposition, indicating successful abiogenic and osteogenic differentiation of the HWJMSCs in their corresponding media, respectively, whereas undifferentiated cells did not exhibit these features. These results collectively validate the MSC phenotype of these cells, as evidenced by the staining assays presented in (Fig. 2c).

Characterization of HWJMSC-derived exosomes

We utilized various analytical techniques to characterize the properties and identity of the purified nanoparticles derived from HWJMSCs. The BCA method was utilized to analyze the protein concentration, yielding a result of 100 µg/µL. TEM was used to examine the morphology of the extracted exosomes. The TEM photograph displayed round-shaped exosomes with bilayer lipid membranes, as illustrated in (Fig. 3a). Their size was found to be within the range of 30 to 150 nm (Fig. 3b). These purified nanoparticles were also shown to have the characteristic exosomal surface markers, being positive for makers such as CD9, a tetraspanin protein commonly found on the surface of exosome/EVs, and CD63 and CD81, two transmembrane markers (Fig. 3c).

Therefore, the results of these analytical techniques affirm the successful isolation of HWJMSCs and the successful extraction of exosomes from cells.

Characteristics of the participants

Eight female participants diagnosed with primary SS-associated DES were enrolled in this clinical trial. The enrollment and allocation processes are presented in Fig. 1 using a CONSORT flow diagram. The participants' ages ranged from 34 to 56 years, with a median of 41.5 years. The DES severity varied among these participants. Within both the control and treatment groups, there was

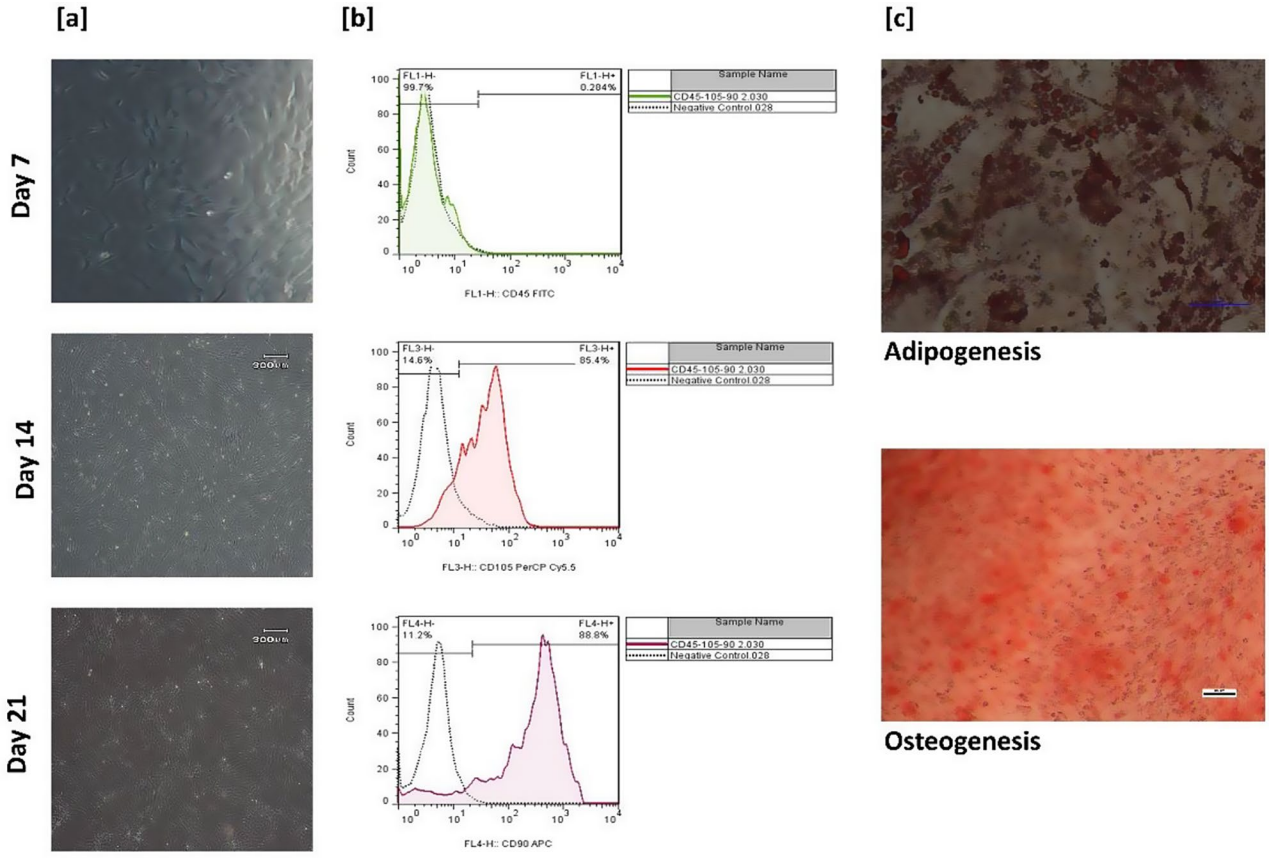

Fig. 2 (**a**) Microscopic images of HWJMSCs morphology on days 7, 14, and 21. Scale bar: 300 µm. (**b**) Flow cytometry results for mesenchymal markers expression of CD90, CD105, and CD45. (**c**) Alizarin red and Oil Red O staining for osteogenic and adipogenic differentiation. HWJMSCs: Wharton's jelly-derived mesenchymal stem cells

one eye with mild DES, two with moderate DES, and five with severe DES.

Evaluation of the effect of HWJMSC-derived exosomes on clinical parameters

Table S2 provides comprehensive details regarding the clinical outcomes, lacrimal function, and ocular examination for the control and treatment groups at the baseline (before treatment), 2nd weeks (2 weeks' post-treatment), 4th weeks (4 weeks' post-treatment), and 12th weeks (12 weeks' post-treatment). During our study no local or systemic complications related to the administration of HWJMSC-derived exosomes have been reported by the participants. Redness was significantly improved in the other clinical index assessed in this study. Although the number of eyes with very mild, mild, moderate, and severe redness was the same in both groups at the beginning, significant differences emerged between the control and treatment eye groups at two weeks and 12 weeks (with P values of 0.015 and 0.046, respectively) and at these times, the treatment groups has improved more than the control groups. (Fig. 4a)

The OSDI values exhibited varying trends in the control and treatment eye groups. There was a significant increase in OSDI values (P value = 0.011) in the control group from the baseline to the 12th week, indicating an overall worsening of the condition. Conversely, there was a significant decrease in OSDI values (P value = 0.011) in the treatment group, suggesting an overall improvement. Additionally, the OSDI remained consistently lower in the eyes receiving HWJMSC-derived exosomes at all three time points after two, four, and 12 weeks. (Fig. 4b)

Evaluation of the effect of HWJMSC-derived exosomes on lacrimal function

Tear secretion, TFBU, and TMT were measured in this study to assess the changes in the lacrimal function. Tear secretion significantly increased in the treatment group from the baseline to the 2nd week and 4th week; there were, however, no significant differences in tear secretion between the baseline and the 12th week in the treatment group. Conversely, tear secretion remained stable in the control group across different time points. Notably, the difference in tear secretion between these two groups was only significant in the 2nd week (P value = 0.010).

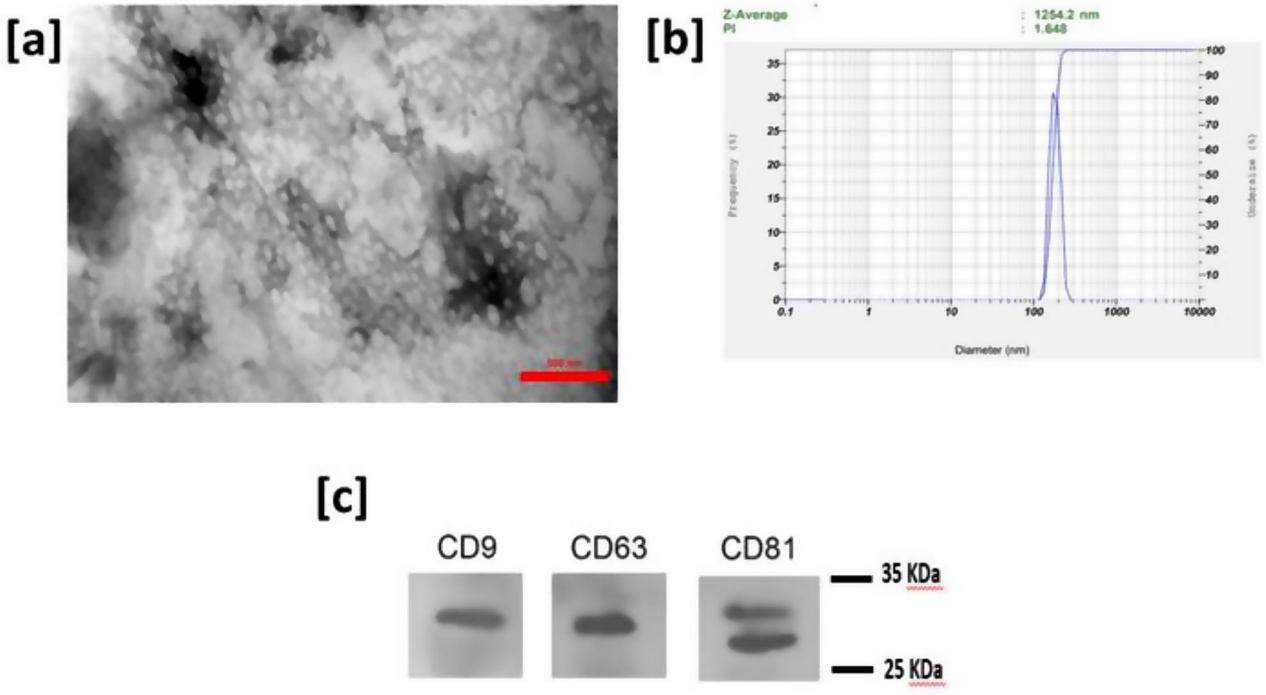

Fig. 3 (**a**) TEM image of HWJMSC-derived exosomes morphology. Scale Bar: 300 nm. (**b**) DLS-determined size distribution of HWJMSC-derived exosomes. (**c**) Western blotting illustrates the expression of HWJMSC-derived exosome markers (CD9, CD63, and CD81). HWJMSCs: Wharton's jelly-derived mesenchymal stem cells; DLS: Dynamic Light Scattering

(Fig. 4c) Similarly, there were no significant differences between the baseline and various time points in the study for TFBUT in the control group. However, TFBUT significantly increased in the treatment group within the first two weeks (P value = 0.012), and there was a statistically significant difference between these two groups at this specific time point. (Fig. 4d) For TMT, no significant changes were found, either within the control group or the treatment group, across various time points. Additionally, the TMT value was not significantly different between these two groups at any time point. (Fig. 4e)

Evaluation of the effect of HWJMSC-derived exosomes on the ocular surface

The effect of HWJMSC-derived exosomes on the ocular surface was measured using fluorescein staining. Initially, both groups had similar numbers of very mild, mild, moderate, and severe staining. However, in the second week, the treatment group showed significant improvement, with an increased number of normal staining, leading to a considerable difference compared to the other group (P value = 0.010). This improvement continued in the 4th week and 12th week, with the treatment group consistently being superior to the control group (P value = 0.026 and P value = 0.038, respectively). (Fig. 4f)

A heatmap of indications obtained from participants in each cluster with the hierarchical clustering dendrogram is demonstrated in Fig. 5. Based on the hierarchical cluster analysis, correlation was observed in terms of OSDI indication before treatment and 3 months after treatment as well as TMT indication. Also, OSDI indication showed correlation between the data of treatment after 2 weeks and 1 month with the same findings about tear secretion and TMT indications. Additionally, data of the redness score revealed correlation 1 and 3 months after treatment as well as fluorescein staining. (Fig. 5)

Evaluation of gene expression profiles for multifunctional proteins in tear samples

The tear samples were collected from the participants before and after intervention at different time points to assess the alterations in gene expression levels of IL-6, IL-22, LTF, thrombospondin-1 (THBS1), EGF, and MMP-9. In the treatment group, a significant increase in EGF gene expression was observed from the baseline to the 2nd and 4th week (with P values of 0.003 and 0.011, respectively). However, by the 12th week, EGF gene expression showed no significant differences between the control and the treatment group. Likewise, THBS1 gene expression in the treatment group significantly rose from the baseline to the 2nd week (P value = 0.020), but no significant differences were found in THBS1 gene expression between the control and the treatment group at the 4th and 12th week. Contrary to the observed improvements in other immunomodulatory parameters, exosomes did not affect the levels of LTF.

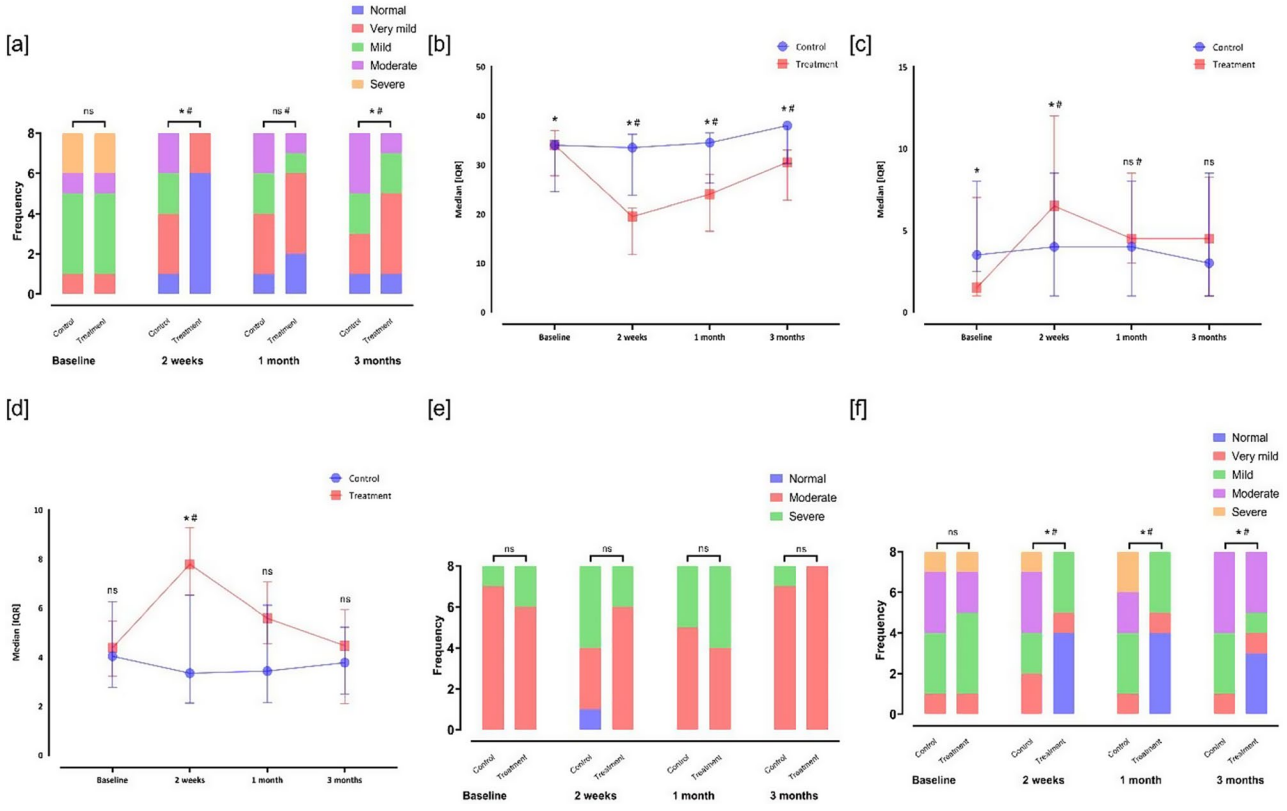

Fig. 4 Ocular surface and lacrimal function tests including (**a**) Redness, (**b**) OSDI, (**c**) Tear Secretion, (**d**) TFBUT, (**e**) TMT, (**f**) Fluorescein staining. In the statistical analysis, each eye was considered separately. The data are presented as Median [IQR] ($n = 8$ for each group). The symbol ∗ represents a statistically significant difference compared to the control group, while # represents a statistically significant difference compared to the baseline. TFBUT: Tear Film Break Up Time; OSDI: Ocular Surface Disease Index; TMT: Tear Meniscus Thickness

In the treatment group, a significant decrease in IL-6 gene expression was noted from the baseline to the 2nd and 4th week (with P values of 0.020 and 0.011, respectively). However, by the 12th week, IL-6 gene expression showed no significant differences between the control and the treatment group. Similarly, MMP-9 gene expression in the treatment group significantly declined from the baseline to the 2nd and 4th week (with P values of 0.011 and 0.035, respectively). Yet, by the 12th week, no significant differences were observed in MMP-9 gene expression between the control and the treatment group. Contrary to the decrease observed in other pro-inflammatory factors, exosomes did not alter the levels of IL-22. (Fig. 6)

The heatmap correlation displays a representation of the direction and strength of association between the expression of individual genes. The correlation heatmap reveals which genes are positively correlated and which are negatively correlated. Strong positive correlations can be understood with gene pairs IL-6: MMP-9, IL-6:IL-22, LTF: EGF, EGF: THBS-1, and IL-22: MMP-9, whereas strong negative correlations can be realized in gene pairs EGF: IL-6, EGF: MMP-9, IL-22: LTF, IL-6: THBS1, IL-6: LTF, MMP-9: LTF, and THBS1: MMP-9. (Fig. 7a)

Overall, as depicted in Fig. 7b, correlation value of|r| demonstrates two distinct group of highly related genes expression including, IL-6, IL-22, and MMP-9 and also THBS1, EGF, and LTF.

Discussion

This investigation represents the first attempt to employ exosomes from umbilical cord-derived HWJMSCs as a therapeutic agent in the form of topical ophthalmic drops for treating SS-associated DES in human participants. In this phase 1–2 clinical trial, which was constrained by a limited sample size, we postulated the potential ameliorative effects of topical HWJMSC exosomes on the clinical manifestations of DES. Our findings indicate that the OSDI, tear secretion, TFBUT, corneal fluorescein staining, and conjunctival redness were generally improved in the eyes that received HWJMSC exosome treatment.

While currently used agents can alleviate immune inflammation in advanced participants, the long-term use of these drugs can cause irrecoverable side effects such as infection, cataracts, and glaucoma [30, 31]. MSC-based therapy has extensively gained interest during the last decade for treating autoimmune and inflammatory

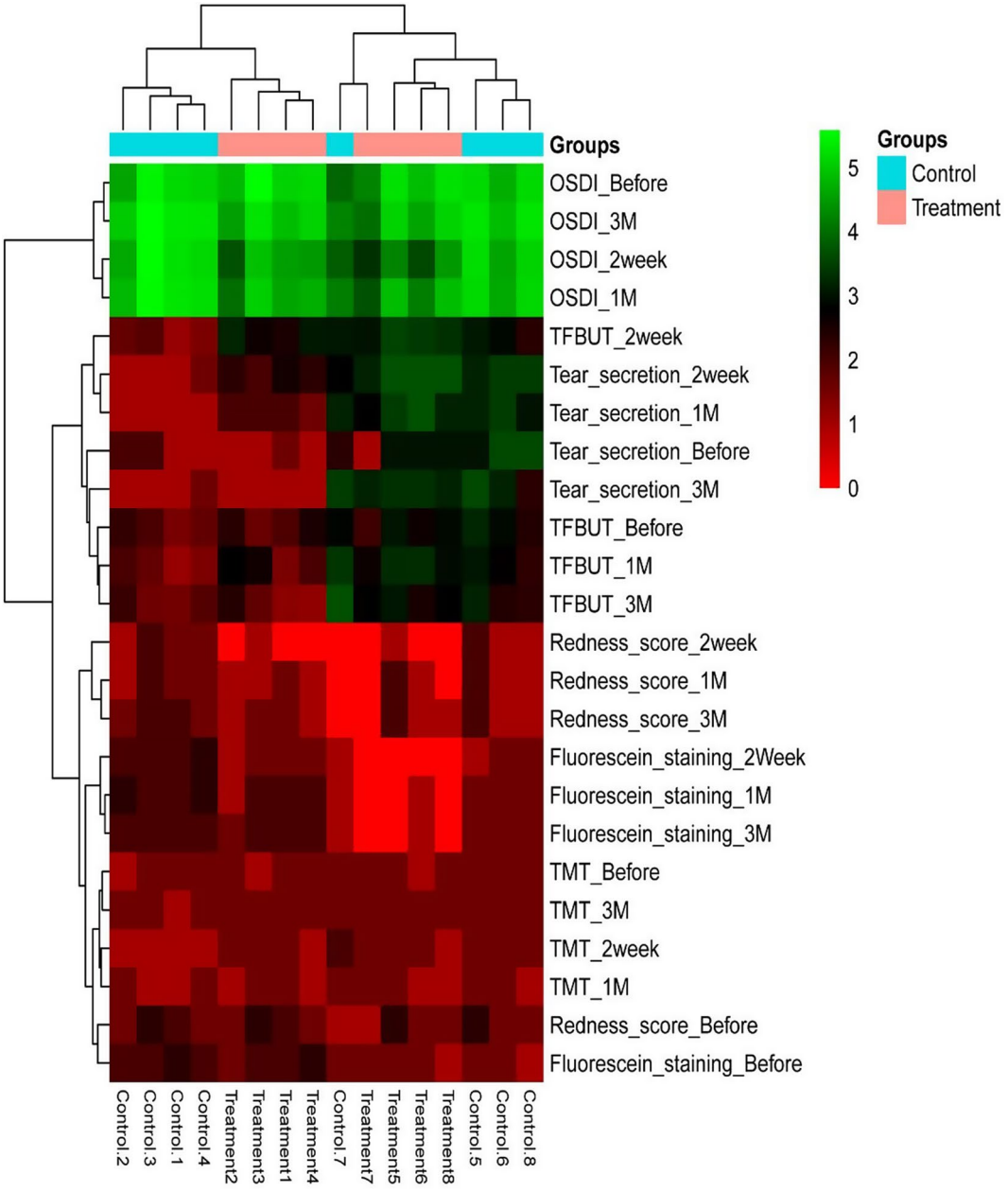

Fig. 5 Hierarchical cluster analysis (HCA) using log2-transformed signals of the effect of HWJMSC-derived exosomes on eye clinical parameters. Heatmap displaying the indications present in individuals within clusters

disorders due to its immunomodulatory and tissue-repairing effects [32].

It has been found that intravenous infusion of MSCs, derived from bone marrow or umbilical cord, can alleviate the symptoms of SS [33, 34]. Prior animal trials showed that allogenic MSCs improved salivary gland histopathology and saliva flow rate in rodents with primary SS [35]. A recent study by Zhou et al. [36] demonstrated that graft-versus-host disease (GVHD)-associated DES could be alleviated by administering MSC-derived exosomes in both human and animal models. Similar to our study, the researchers observed significant improvements in OSDI scores, tear secretion, TFBUT, and corneal fluorescein staining when MSC exosomes were applied as eye drops. Notably, the authors proposed that the immunosuppressive effects of MSC exosomes were mediated by the reprogramming of pro-inflammatory M1 macrophages into an immunosuppressive M2 phenotype, which was facilitated by the targeting of the IL-6/IL-6R/Stat3 pathway by miR-204. The crucial role of miR-204 was further substantiated by observing the abolition of MSC exosomes effects upon blocking miR-204 while

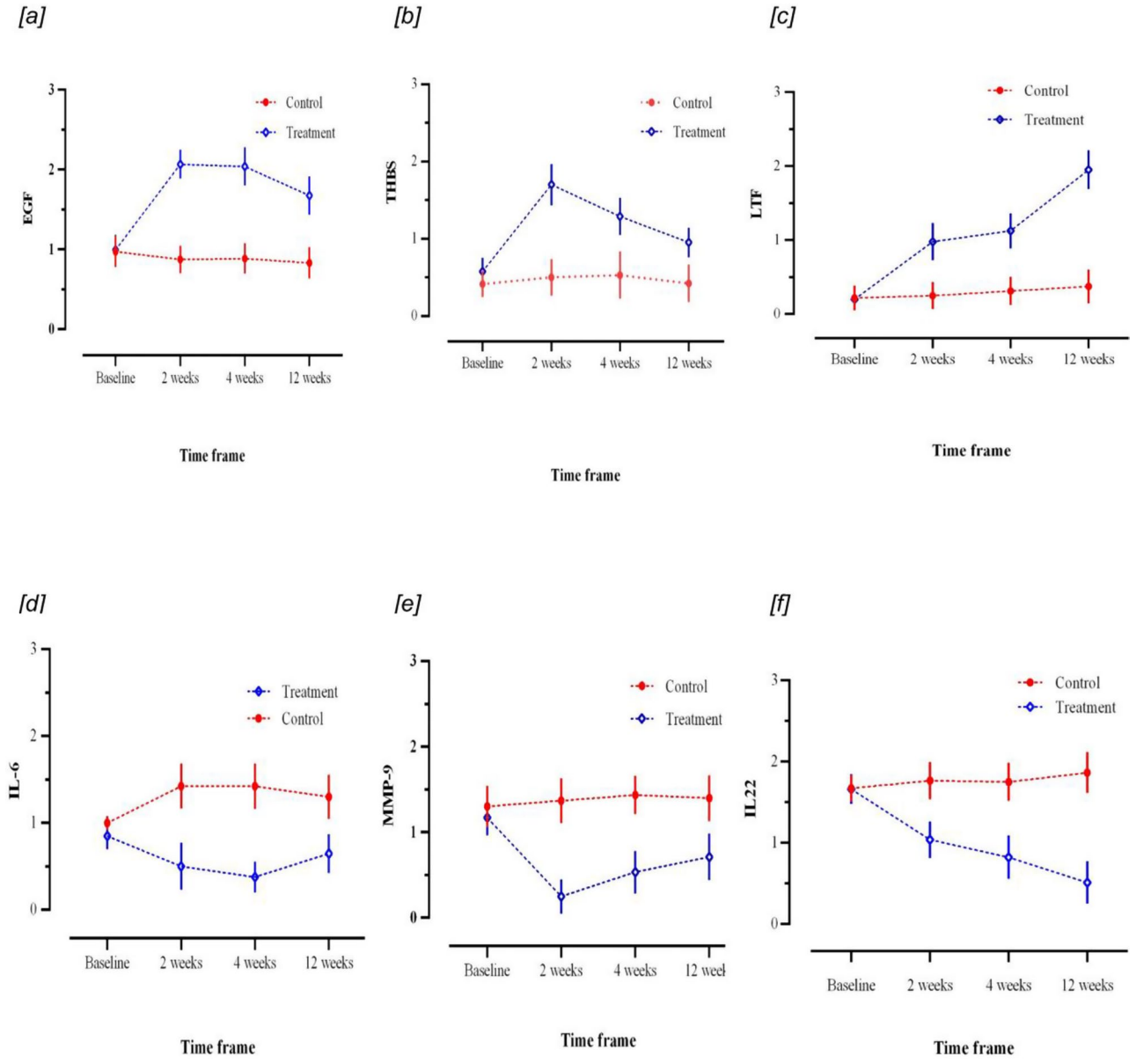

Fig. 6 Gene expression of pro- and anti-inflammatory modulators in the treatment and control groups, including (**a**) IL-22, (**b**) LTF, (**c**) THBS1, (**d**) MMP-9, (**e**) IL-6, and (**f**) EGF. In the statistical analysis, each eye was considered separately. The data are presented as Median [IQR] ($n = 8$ for each group). The symbol * represents P value < 0.05, and the symbol ** P value < 0.01. IL: Interleukin; LTF: lactoferrin; THBS1: thrombospondin-1; MMP-9: metalloproteinase-9; EGF: Epidermal Growth Factor

enhancing L929 exosomes with miR-204 significantly mitigated DES [37–39].

In this study, we analyzed tear samples obtained from participants at various time points before and after treatment to assess the gene expression levels of key pro-inflammatory cytokines namely IL-6, IL-22, and MMP-9 and multifunctional peptides accelerating healing process including, EGF, LTF, and THBS1.

Notably, we observed a significant reduction in MMP-9 expression in tear samples collected from SS-related DES participants one month following treatment. As previously stated, inflammation can augment MMP-9 levels via multiple pathways, including activating MAPK signaling in ocular epithelia and releasing pro-inflammatory cytokines such as IL-6 from immune cells. A decrease in MMP-9 levels has been observed to align with improvements in dry eye tests, including tear secretion tests, TFBUT, OSDI, eye redness, and fluorescent staining. Given that MMP-9 has been implicated in both ocular surface damage and inflammatory signaling, its diminished presence in tears after treatment indicates a potential therapeutic effect and supports its viability as a

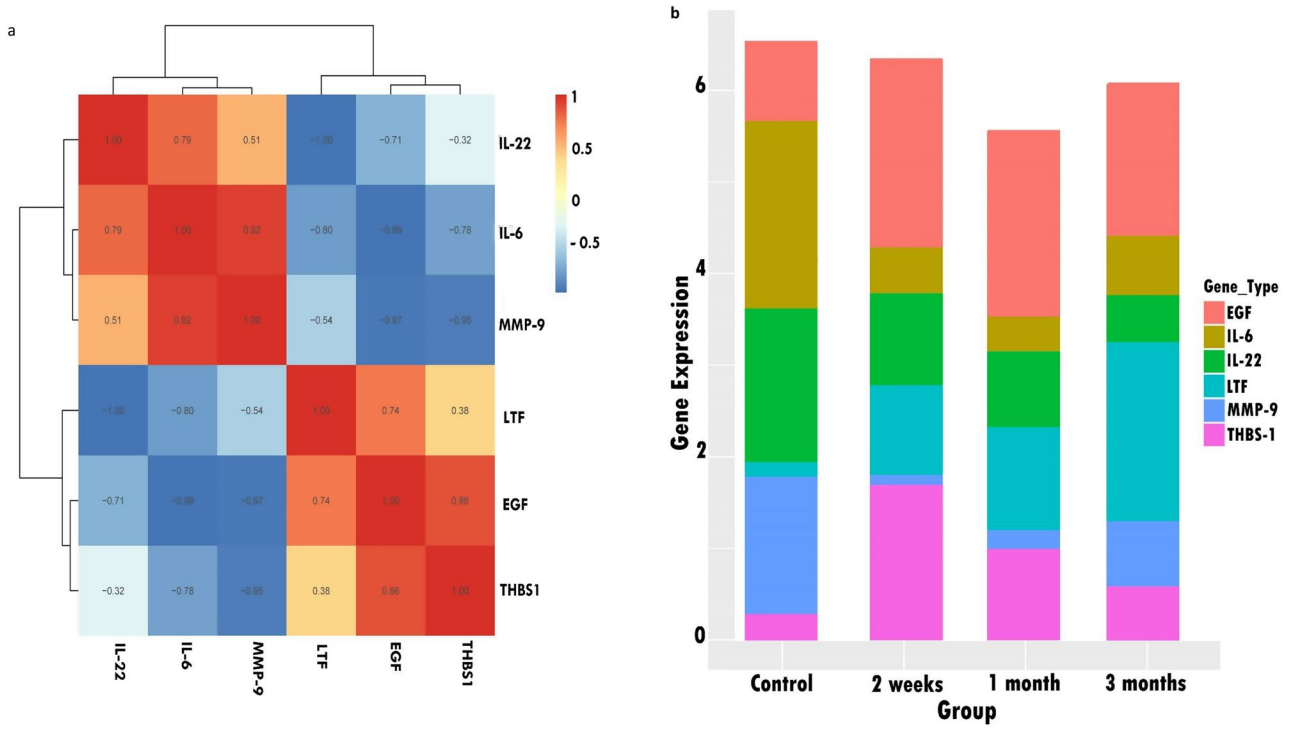

Fig. 7 **a** Heatmap of a pairwise correlation for the gene expression profiles in tear samples. (red: high correlation; blue: low correlation). **b** Gene expression of the log2 fold changes mean during different time points

biomarker for disease monitoring. Based on the Hierarchical cluster analysis, eye clinical parameters including TFBUT, tear secretion, OSDI, TMT and redness scores demonstrated more correlation between 2 weeks and 1 month. Also, reduction in the expression of pro-inflammatory cytokines such as IL-6, IL-22, and MMP-9 genes was observed during treatment. In contrast, LTF, EGF and THBS genes showed higher expression compared to the control group at 2 weeks and 1 month after treatment. Addition to the similar alterations in the up and down regulation of IL-6, IL-22, and MMP-9 genes, LTF, EGF and THBS genes revealed the same expression in terms of reduction or increment.

Following treatment, the expression levels of IL-6 were decreased in the tears of participants with DES. This correlation between IL-6 concentration and ocular surface parameters has been established in participants with SS who also suffer from DES [40]. IL-6, an effector cytokine of Th-17 cells, along with other pro-inflammatory cytokines (IL-1b, TNF-a), have been reported to reduce tear production by the lacrimal gland through neuronal and hormonal effects [41, 42]. Furthermore, IL-6 may be implicated in goblet cell apoptosis via various signaling pathways, which is associated with dry eyes [43]. The reduced presence of IL-6 in tears post-treatment suggests a potential therapeutic effect and supports its use as a biomarker for disease monitoring.

Conversely, we found an upregulation of EGF expression in tears of SS-associated DES participants one-month post-treatment. A decrease in EGF has been observed in participants with SS compared to healthy subjects. EGF, a vital component of both tear film and serum, belongs to the EGF family of growth factors and serves as a potent polypeptide mitogen. It is secreted by the lacrimal gland and plays a critical role in preserving the integrity of the corneal epithelium and promoting wound healing [44]. As evident in the results, there has been a significant reduction in the number of dots in the corneal fluorescein staining. This decrease has been substantial up until three months following treatment. One of the potential explanations for this restoration could be the increase in EGF levels and its associated healing effects.

MSC-derived exosomes are considered a promising cell-free therapeutic approach due to their biological similarity to source cells. In contrast to cell-based therapies, MSC exosomes offer advantages such as ease of storage and direct transportation to affected areas through targeted modifications or local administration, effectively bypassing some limitations. Although additional investigation is necessary, the application of MSC exosome therapy holds great promise in addressing tissue damage associated with DES. However, the clinical application of MSC-exosome therapy encounters certain challenges that necessitate careful consideration. These challenges

include, the absence of standardized techniques for isolating and purifying exosomes, and ensuring the stability of MSC exosomes during storage and transport [45, 46]. Furthermore, the exosomes obtained from cell cultures manifest noteworthy variations and irregular characteristics, despite originating from identical donor cells [47]. These issues highlight the limitations of existing cell culture and exosome purification methodologies, thereby impeding the establishment of standardized and large-scale exosome manufacturing processes.

This prospective clinical trial, aimed at evaluating the safety and efficacy of MSC exosomes eye drop therapy in participants with SS-associated DES, is accompanied by notable limitations. The first limitation pertains to the relatively small sample size and short-term follow-up, which may limit the generalizability and long-term assessment of the treatment outcomes. Based on the study findings, it appears that the therapeutic effects of exosome treatment have a limited duration following its completion. Furthermore, a majority of the underlying factors deteriorate three months' post-treatment. This highlights the need for future investigations to assess the potential benefits of sustained and prolonged exosome therapy. On the other hand, the prolonged administration of MSC exosomes, given its immunoregulatory nature, may potentially lead to adverse effects comparable to those observed with steroid or immunosuppressive medications. These effects include the risk of cataracts, glaucoma, or infection. Therefore, it is crucial to conduct additional research to assess the long-term effectiveness and safety of MSC exosomes eye drops.

Conclusion

Our research establishes the safety and notable therapeutic potential of exosomes derived from MSCs in the context of SS-associated DES. Specifically, the compelling anti-xerophthalmic effectiveness of MSC-derived exosomes observed in our clinical investigation opens up exciting prospects for the broader utilization of these exosomes in treating severe DES, encompassing GVHD and other connective tissue disorders. It is highly recommended to consider participant's recruitment encompassing a wider range of severe DES cases and conducting long-term follow-ups to derive more robust and conclusive outcomes .

Abbreviations

SS	Sjögren's syndrome
DES	Dry eye syndrome
MSC	Mesenchymal stem cell
MMP-9	Metalloproteinase-9
IL	Interleukin
EGF	Epidermal Growth Factor
LTF	Lactoferrin
THBS1	Thrombospondin-1
OSDI	Ocular Surface Disease Index
miRNA	microRNA
HWJMSC	Human Wharton's Jelly Mesenchymal Stem Cell
RCT	Randomized controlled trial
DMEM	Dulbecco's modified Eagle's medium
PBS	Phosphate-Buffered Saline
FBS	Fetal Bovine Serum
EDTA	Ethylene Diamine Tetra Acetic acid
AA2P	Ascorbic Acid-2 Phosphate
SDS	Sodium Dodecyl Sulphate
PVDF	Poly Vinylidene Fluoride
TBST	Tris-Buffered Saline with Tween
HIV	Human immunodeficiency virus
HCV	Hepatitis C virus
HBV	Hepatitis B virus
CMV	Cytomegalovirus
EBV	Epstein-Barr virus
cGMP	current Good Manufacturing Practice
TEM	Transmission electron microscopy
DLS	Dynamic Light Scattering
BCA	Bicinchoninic acid
TFBUT	Tear Film Break Up Time
FLC	Fluorescein Staining score
TMT	Tear Meniscus Thickness
RT-PCR	Reverse Transcription Polymerase Chain Reaction
GVHD	graft-versus-host disease

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12886-025-04078-9.

Supplementary Material 1

Acknowledgements

Not applicable.

Author contributions

Conceptualization: NA, AH Methodology: NA, MS, AK, and MN Investigation: AH, MS, MD and AK Supervision: NA and AK Writing: AH and NA.

Funding

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Data availability

All data generated or analyzed during this study are included in this published article [and its supplementary information files].

Declarations

Ethics approval and consent to participants

Before their inclusion in the study, written informed consent was obtained from the WJ tissue donors and participants with SS. The trial was conducted in accordance with the guidelines for conducting clinical trials on human participants and the Declaration of Helsinki. Shiraz University of Medical Sciences Ethics Committee, Shiraz, Iran, approved the study protocol (IR.SUMS.REC.1400.852). The study protocol was also registered in the Iranian Registry of Clinical Trials (IRCT20211102052948N1) at 2022-04-20.

Consent for publication

Before the participant enters the study, written informed consent was obtained from WJ tissue donors and SS participants for the publication of results that will be used in a completely confidential manner and solely for research purposes, and the participants' identity will remain confidential within the framework of the law.

Competing interests

The authors declare no competing interests.

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Received: 14 February 2025 / Accepted: 16 April 2025
Published online: 20 May 2025

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