

Safety and toxicity of human limbus-derived stromal/mesenchymal stem cells with and without alginate encapsulation for clinical application

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Abstract

Background

The purpose of this study was to assess the ocular and systemic toxicity of topically applied human limbus-derived stromal/mesenchymal stem cells (hLMSCs) with and without alginate encapsulation as per Indian regulatory guidelines for stem cell therapy.

Methods

The hLMSCs were obtained from cadaveric corneoscleral rims and expanded in a current good manufacturing practice compliant laboratory. The hLMSCs were checked for viability, chromosomal stability, growth kinetics, contamination, and endotoxin levels. Cells with (En^+ hLMSCs) or without (En^- hLMSCs) alginate encapsulation were used for the animal experiments. The study involved 3 groups of 6 New Zealand white rabbits each, which underwent corneal wounding followed by treatment with sham (G1), En^- hLMSCs (G2), and En^+ hLMSCs cells (G3). Ophthalmic assessment including intraocular pressure (IOP), blood investigations and inflammatory marker (IL-6, TNF- α , IgE) expression in serum and tears were assessed on days 1, 7, 14, 21, and day 28. At the end of 28 days, the animals were sacrificed, and the organs were subjected to histopathological examination.

Results

The hLMSCs had $88.33 \pm 2.37\%$ viability at the end of 6 hours and $78.21 \pm 1.47\%$ at the end of 24 hours. The cells showed positive expression for the stem-cell biomarkers (p63 α , Pax6, and ABCG2), extracellular matrix marker (Col-III) and mesenchymal biomarkers (VIM, CD73, CD90 and CD105). No contamination by the Mycoplasma species was found in either of the En^-/En^+ hLMSCs and the levels of bacterial endotoxins in the En^- hLMSCs and En^+ hLMSCs cell suspension was found to be within the permissible levels (≤ 0.12 EU/mL). Ophthalmic examination showed no significant difference in IOP, corneal clarity and conjunctival congestion between the three groups at every time point. Haematological parameters were comparable between the three groups. The inflammatory markers in tear and serum (TNF- α and IL-6) were not significantly elevated in the groups receiving En^+/En^- hLMSCs. Histological examination did not show any abnormality in the ocular or corneal tissue, and the viscera.

Conclusions

The results of the study show that hLMSCs do not cause any local or systemic toxicity in recipients, implying that these cells are safe for clinical use and their efficacy can be assessed in human clinical trials.

1. Introduction

Cornea is the outermost, transparent part of the eye. Any damage to the cornea can lead to partial or complete visual impairment, and ultimately blindness. Anatomically, the cornea consists of three major layers, namely the epithelium, stroma and endothelium. Corneal transparency is mainly maintained by the special alignment of collagen fibrils in the stromal layer (1–2), which once disturbed either due to infection, inflammation or trauma, results in loss of corneal transparency (3–4). One of the major causes of corneal opacification is scarring or fibrosis, which occurs due to the misalignment and deposition of irregular, unorganized collagen fibrils, abnormal proteoglycans and differentiation of corneal keratocytes into myofibroblasts (4–8). This occurs following corneal wounding and as a part of the natural healing mechanism.

Medications to reverse corneal opacification due to scarring are currently not available and corneal transplantation is the definitive standard of care for severe cases with advanced visual impairment or blindness. Corneal transplantation has its limitations and necessitates clinical monitoring throughout the transplant survival period (9–11). The vast gap between the demand and supply of the donor corneas globally further complicates the situation. Recent research has focused on developing alternate therapies for the prevention and treatment of corneal opacification due to scarring, one of which is cell-based therapy.

Human limbus-derived stromal/mesenchymal stem cells (hLMSCs) have been shown to prevent corneal scarring, *in vitro* (4, 12–16) and *in vivo* in various rabbit and mice models. These cells were previously proven to have immune-modulatory properties (17) and cause no xenogeneic reaction to the mice models (4, 18), rendering them to be safe. The hLMSCs and other mesenchymal stem cells are now being evaluated in various clinical trials to assess their safety and efficacy (19–24). The hLMSCs could potentially lower the need for corneal transplantation, therefore reducing the need for donor corneas. It has also been shown that encapsulation of hLMSCs in sodium alginate, retains their phenotype and maintains the viability, while being stored or transported at varied temperatures and for prolonged durations (3–5 days) (25). This easy-to-use technology, which obviates the need for an expensive cold chain, could increase the accessibility of hLMSC-therapy, particularly in remote geographical locations, without requiring the patient to travel hundreds of miles, particularly in developing countries. However, before these innovative approaches can be translated from the bench to the bedside, the safety and toxicity profile of these cells (with or without encapsulation) needs to be established.

In the present study, we examined our current good manufacturing practice (CGMP) compliant clinical grade hLMSCs for their safety and toxicity after topical application in animals, as per the Indian regulatory guidelines. The data also includes the characterization of the hLMSCs in terms of viability and stability during culturing and passaging, and the various quality checks for CGMP-grade hLMSCs that need to be performed before using these cells in a clinical trial.

2. Materials And Methods

2.1. Study protocol

This study was conducted in collaboration with an accredited contract research organization, Sipra Labs Limited (Compliance certificate number: GLP/C-107/2017; Accreditation certificate number: TC-5417), Hyderabad, adhering to the guidelines of Schedule – Y (26), Drugs and Cosmetics Rules act, 2019, Government of India (27). This study was carried out in compliance with the OECD (Organization for Economic Cooperation and Development) principles of Good Laboratory Practice, 1997 (28) and the guidelines of the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) M3 (R2) (29).

This study protocol was approved by the Institutional Review Board (LEC 05-18-081), Institutional Committee for Stem Cell Research (08-18-002), LV Prasad Eye Institute, Hyderabad and the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Sipra Labs Limited, Hyderabad (SLL/PCT/IAEC/110 – 19). The procedures used in this study were designed to conform to the accepted practices and minimize or avoid the risk of causing pain, distress or discomfort to the animals.

2.2. Experimental design

New Zealand White strain rabbits, aged 12 to 16 weeks, $n = 18$ (9 male and 9 female); were acclimatized at least 5 days before the experimentation and randomized to three groups using stratified randomization method. A veterinary inspection was performed to ensure the normal health and suitability of the animals to the study, before conducting the experiments. Animals were distributed to three groups with $n = 6$ each (3 male and 3 female), viz. control or sham-treated group (G1); G2 (En^- hLMSCs), treated with hLMSCs that were not encapsulated and G3 (En^+ hLMSCs) group treated with hLMSCs which were encapsulated in sodium alginate and then transported at room temperature.

On the day of the experiment, the rabbits were anaesthetized by Ketamine (35mg/Kg body weight) and Xylazine (10mg/Kg body weight) mixture through intramuscular route, followed by 1–2 drops of topical anaesthesia in the left eye (0.5% proparacaine hydrochloride). The eyes were then cleansed using a cotton swab dipped in 0.5% povidone-iodine eye drops and then gently scraped with a sterile needle. The test eyes of groups G2 and G3 were then administered with 5×10^5 each of En^- hLMSCs and En^+ hLMSCs respectively dissolved in 100 μ L of the commercially available fibrin glue composition (TISSEEL LYO, Baxter International Inc., Illinois, USA) respectively. Whereas the control or sham-treated group received only vehicle i.e. fibrin glue composition. Post administration of the analyte, the eyelids were gently held together for about 3–5 seconds to prevent loss of the test item. Treated eyes were applied with a sterile dressing pad until the rabbits' recovery from anaesthesia. Further investigations of ophthalmic and blood parameters and the collection of blood and tear fluid were performed at their respective time points. The gross pathological examinations were performed on day 29 after sacrificing the animals.

2.3. Isolation of hLMSCs and their encapsulation for transport at room temperature

The hLMSCs were obtained from the limbus of donor corneas, as previously described (25). Briefly, after washing the donor cornea with 2X Antibiotic-Antimycotic (15240062, Thermo Fisher Scientific, Massachusetts, USA) fortified PBS (14190250, Thermo Fisher Scientific, Massachusetts, USA), limbal rims were dissected, fragmented and subjected to gentle mincing. The minced limbal tissue fragments were digested with Collagenase-IV enzyme (17104019, Thermo Fisher Scientific, Massachusetts, USA). Digested tissue is washed and then cultured in DMEM/F12 medium (BE04-687F/U1, Lonza, Basel, Switzerland) fortified with 2% serum (SH30084.03, Cytiva Life Sciences, Massachusetts, USA) and other growth factors. The primary cultures (P0) were split after attaining 80–90% confluence and subcultured for 3 generations/passages. A pure population of the hLMSCs was obtained at passage 3 (P3), which post-viability checks using 0.4% Trypan Blue (15250061, Thermo Fisher Scientific, Massachusetts, USA), were encapsulated using sodium alginate.

The encapsulation of hLMSCs using sodium-alginate was performed using the commercially available BeadReady™ kit (Atelerix Ltd, UK), as described previously (25). Briefly, the alginate-cell suspension (2.5×10^6) formulation was dropped into a calcium chloride-based gelating buffer, using a sterile needle, which then polymerises to form bead-like structures. These beads containing hLMSCs, suspended in DMEM/F12–2% serum medium, were transported in a specialized container that maintains room temperature, for 3–5 days. After which, the cells were released from beads using a Trisodium citrate-based buffer and then sedimented for further use/analysis.

2.4. Assessment of characteristic phenotype and viability of the hLMSCs

Before the administration to the rabbit eyes, the hLMSCs in both groups (En^+/En^- hLMSCs) were subjected to phenotypic assessment of their characteristic biomarker expression using immunofluorescence. Cells were cultured on 18mm diameter coverslips in 12-well culture plates at a density of 20,000 cells/cm² at 37°C with 5% CO₂ in a humidified incubator until confluence. These cells were assessed for the expression of characteristic biomarkers of the mesenchymal stem cell (MSC) phenotype, as described previously (25). The antibody panel was composed of (a) ABCG2, Pax6, p63-α and Col-III as positive markers of the human limbal stem cell phenotype; (b) VIM, CD73, CD90, and CD105 as positive markers of the mesenchymal phenotype, and CD45 as a negative marker for mesenchymal origin.

This antibody panel was selected in accordance to the International Society for Cellular Therapy's guidelines of minimal criteria for defining multipotent mesenchymal stromal cells (30). The panel of secondary antibodies included anti-mouse Alexa Fluor 594 (Thermo Fisher Scientific, Massachusetts, USA) and anti-rabbit Alexa Fluor 594 (Thermo Fisher Scientific, Massachusetts, USA). Cells were mounted using Fluoroshield mounting medium with DAPI (ab104139, Abcam, UK) and imaging was done using a fluorescent microscope (Axio Scope A1, Carl Zeiss AG, Germany) with 20x or 40x objective. This experiment was performed on biological triplicates.

The viability of the cells in both the experimental groups was quantified using the dye -exclusion method that utilizes 0.4% Trypan Blue solution and viable cells were counted using the Neubauer chamber. The viability is expressed in (percentage \pm SD) format and the minimum acceptance criterion was $\geq 70\%$.

2.5. Evaluation of the stability of hLMSCs

2.5.1. Assessment of the viability hLMSCs stored as a pellet

Before applying on the corneal surface, the En^-/En^+ hLMSCs cell suspension (post-harvest or post-release from encapsulation respectively) was centrifuged at 1000rpm for 3 minutes to remove the supernatant. These cells, in the form of a pellet, were stored at ice-cold conditions ($2-4^{\circ}\text{C}$) because there is usually a delay in applying the cell to the patient's eyes and/or during their transit from the GMP laboratory to the operating suite. It is recommended to assess the stability as a pellet to determine the ideal time duration within which the cells should be transplanted on to the corneal surface. It was determined by quantifying the viability of these cells in the form of a pellet, from 0th hour to the end of 24hours. The cells suspension, after initial assessment for the viability, was then equally distributed to 6 individual vials (0.5×10^6 cells/vial/time point) and stored in ice-cold conditions. The percentage of viable cells at 0.5 hours, 1 hour, 3 hours, 6 hours, 12 hours and 24 hours, was calculated using the dye-exclusion method and plotted as a graph.

2.5.2. Chromosomal stability of the hLMSCs

The hLMSCs were checked for chromatin aberrations and mutations, via karyotyping, by an accredited third-party laboratory. The basic steps involved in this process are as follows. Three to four-day-old culture of hLMSCs (without encapsulation and post-encapsulation) were arrested, for the spindle formation during metaphase using colcemid. The cells were given a hypnotic treatment to release the chromosomes outside of the cell. Slides are then prepared using the G-banding method and observed under a bright-field microscope. The analysis was performed using Cytovision software.

2.5.3. Determining the kinetics of growth

In addition to the above, the kinetics of the cell growth of the hLMSC population were determined. This was performed by quantifying the number of viable cells via both MTT assay and dye-exclusion method from the 0th hour to the end of Day 6, in the culture. The data was plotted as a graph to obtain the growth curve and determine the doubling time of the hLMSCs.

2.6. Mycoplasma assessment

The absence or presence of any contamination in the hLMSCs culture was assessed using a kit method as per the manufacturer's instructions (LT07-318, MycoAlert™ Mycoplasma Detection Kit, Lonza, Basel, Switzerland). The spent media of the cells at every passage and the end of passage 3 were checked for mycoplasma presence, and the emitted light signal was read using a Luminometer (GloMax® 20/20 Illuminometer, E5321, Promega, Madison, USA).

2.6.1. Determining the endotoxin levels

The levels of bacterial endotoxins (BET) in the cell suspension were determined using a gel-clot based kinetic method (N283-125, PYROGENT™ plus Gel Clot LAL Assay, Lonza, Basel, Switzerland) as per the manufacturer's instructions. The maximum allowed levels of endotoxins are ≤ 0.2 EU/mL, as per the FDA guidelines (31).

2.7. Body weights and mortality

All animals were observed for morbidity and mortality twice a day. Individual body weights were recorded on the day of treatment and at weekly intervals thereafter. The body weights were measured in kilograms (Kg).

2.8. Ophthalmic observations and Intraocular pressure (IOP)

Slit-lamp examinations (PSLAIA-11, Appasamy Associates, India) were performed to detect the changes in cornea, conjunctiva, iris and aqueous humour. Fluorescein sodium ophthalmic strips were used for ophthalmic examinations of cornea and conjunctiva. The ophthalmic observations were assessed according to the numerical scoring system listed in the OECD Guidelines for the Testing of Chemicals, Test No. 405 "Grading of Ocular Lesions" (28) and as per schedule Y (26). Both slit lamp and IOP observations were performed before dosing and on the 3rd, 6th, 12th, 24th hours of day1, on days 7, 14, 21 and 28 of post-dosing. The criteria of scoring are given in Supplementary Table 1.

2.9. Assessment of inflammatory markers and immunogenicity in tear fluids and serum

Blood samples (3–4 mL) of all the animals were collected in plain vacutainers at 1, 6, 12 and 24 hours and on days 7, 14, 21 and 28 after administration. Sera was isolated from the blood samples and stored at -80°C . Tear fluid samples were collected using tear strips at 1, 3, 6, 12 and 24 hours and on days 7, 14, 21 and 28. The samples collected were stored at -80°C for the assessment of IL-6, TNF- α and IgE marker expression.

2.9.1. Tear fluid extraction from Schirmer's strips

The tear fluids were extracted from the frozen Schirmer's strip (Tear Strips, Care Group, Gujarat, India) using the protocol previously described by Posa, Andreas *et al.*, 2013 (32). Briefly, the frozen strips were inserted with the help of forceps, close to the base of a sterile 0.5mL microcentrifuge tube. These 0.5mL microcentrifuge tubes were punctured with a sterile 22 ½ gauge needle. This entire arrangement was inserted into a 1.5mL microcentrifuge tube. Around 10-50uL of 1x PBS was added to the strip, based on the length (mm) of the strip to the extent the tear fluids were absorbed and then incubated at $2-4^{\circ}\text{C}$ for 30minutes. The setup was then centrifuged at 13000rpm for 5minutes at 4°C . One microliter each of the tear fluid extracted was used for protein quantification, while the rest was stored immediately at -80°C for future analysis purposes.

2.9.2. Protein quantification using Bicinchoninic Acid (BCA) Assay.

Quantification of protein in the Tear fluid was performed using the BCA assay, a colorimetric assay (786 – 570, G-Biosciences, Geno Technology Inc., Missouri, USA), according to the manufacturer's protocol. The unknown samples' concentration was calculated against the standard graph obtained. The standards ranged from 2000 µg/mL to 0 µg/mL and the absorbance was read at 562nm, using SpectraMax M3 microplate reader system (Molecular Devices, California, USA).

2.9.3. Assessing through Immunoassay

The levels of rabbits' inflammatory markers were assessed using the sandwich ELISA methods. The quantification was done using commercially available antibody-coated kits procured from KinesisDx, Krishgen Biosystems, USA (IgE, K09-0071; IL-6, Ref: KLX0003, TNF- α , KLX0065). In brief, 40uL of each of the samples (sera/tear) was added to respective wells, followed by 10uL each of respective biotinylated antibodies. The standards were devoid of any biotinylated antibodies. Wells were then added with 50uL each of Streptavidin-HRP conjugate solution and incubated in dark for 1 hour at 37°C. Wells were then washed with 1x wash buffer 4 times, using an automated washer system (Erba Lisa Wash II, Erba Mannheim, London, UK) and firmly tapped onto an absorbent paper to remove the residual buffer. Wells were then added with 50uL each of substrate A followed by substrate B and incubated for 10 minutes. The reaction was stopped by adding 50uL each of stop solution and the resultant colour formed was read at 450nm, using the SpectraMax M3 microplate reader system, Molecular devices, USA.

2.10. Blood investigations

The haematological parameters were determined using a Haematology cell counter (SYSMEX-XP 100, Japan). Blood smears were prepared from the haematology sample and stained with Leishman stain. The differential leukocyte count for these smears was performed by conventional microscopy. Sera isolated from the blood samples were subjected to clinical chemistry analysis. The clinical chemistry parameters were determined using a fully automated Random Access Biochemical Analyser (EM-360, Erba Mannheim, London UK).

2.11 Tissue investigations

2.11.1. Necropsy and gross observations

On day 29, all the animals of sham, En^{-} hLMSCs and En^{+} hLMSCs groups were euthanized and subjected to detailed necropsy. External observations suggesting any abnormalities were recorded. An *in situ* examination of organs was carried out and the individual organs were examined for gross morphological changes.

2.11.2. Organ weight and Histopathology

On completion of gross pathology examination, the organs were collected and specified organs were weighed. The organ weight ratios as a percentage of body weight were determined. The collected organs were preserved in a 10% buffered formalin solution for histopathological examination.

2.12. Statistical Analysis

All the data were expressed as Mean \pm SD. All the data was subjected to statistical analysis at a significance level of 0.05, using GraphPad software. The data was analysed using student's t-test and non-parametric one-way ANOVA tests (Kruskal-Wallis).

3. Results

3.1. Phenotypic assessment

No deviations were observed in the phenotypic expression of the biomarkers by the hLMSCs (Fig. 2). The cells showed positive expression for the stem-cell biomarkers p63 α , Pax6, ABCG2 and the extracellular matrix marker Col-III. Mesenchymal biomarkers such as VIM, CD73, CD90 and CD105 showed positive expression while CD45 did not shown any expression, as expected.

3.2 Assessment of the stability and viability of hLMSCs

Both the cell populations of En^{-}/En^{+} hLMSCs assessed through karyotyping, did not show any numerical or chromatic aberrations (Fig. 2B-2C). The hLMSCs stored as a pellet in the ice-cold conditions had $88.33 \pm 2.37\%$ of viable cells at the end of 6 hours and $78.21 \pm 1.47\%$ of cells viable at the end of 24hours (Supplementary Fig. 1C). The growth kinetics studies revealed that the hLMSCs have a doubling time of ~ 61 hours. No contamination of the Mycoplasma species was found in either of the En^{-}/En^{+} hLMSCs given to the test animals in the study. The levels of bacterial endotoxins in the En^{-} hLMSCs and En^{+} hLMSCs cell suspension was found be within the permissible levels (≤ 0.12 EU/mL).

3.3 Clinical signs, body weights and mortality

All the sham and test (En^{+}/En^{-} hLMSCs) group animals were found normal for clinical signs. No mortality was observed in vehicle control and test groups. In all the test groups, the body weight gain was found normal, when compared to the control group (Supplementary Fig. 2).

3.4. Ophthalmic observations and IOP

All the ophthalmic observations were found to be normal. However, Grade 1 ocular inflammation was observed in the conjunctiva of the left eye in all three groups at the 3-hour time-point. The same was observed during the 6-hour time-point, in one animal of the sham group and all the animals of En^{-} hLMSCs group. No ocular inflammation was noticed from the time-point of the 12th hour onwards. Intraocular pressure was found to be normal and comparable in all three groups. No significant

differences were found in the IOP of both the test groups compared to the sham or control group (Table 1 and Fig. 3).

Table 1

Serial evaluation of intra-ocular pressure after treatment with En^+/En^- hLMSCs: Median levels of the IOPs of the treated eyes at different time points (n = 6 per group). Statistical analysis done with Kruskal Wallis test (non-parametric one-way ANOVA). **G1** – Sham treated group; **G2** – Treated with cells without encapsulation/transit (En^- hLMSCs); **G3** – Treated with cells post-encapsulation and transit (En^+ hLMSCs).

Intraocular Pressure									
Group	Pre-dose	3 hours	6 hours	12 hours	24 hours	Day 7	Day 14	Day 21	Day 28
G1	10.5	10.0	11.0	11.0	13.0	13.0	12.0	13.0	12.5
G2	12.5	9.0	10.0	11.5	13.0	11.0	12.0	15.0	12.0
G3	12.0	12.0	9.0	11.0	12.5	13.0	13.0	11.0	12.5
p-value	0.185	0.063	0.268	0.855	0.953	0.154	0.718	0.069	0.349

Table 2

Serial evaluation of immunological and inflammatory markers in serum and tears post En+/En- hLMSCs treatment. Median levels are provided; Statistical analysis done with Kruskal Wallis test (non-parametric one-way ANOVA). *3-hour time-point blood samples were not collected; however, tear samples were collected. **G1** – Sham treated group; **G2** – Treated with cells without encapsulation/transit (*En*⁻ hLMSCs); **G3** – Treated with cells post-encapsulation and transit (*En*⁺ hLMSCs)

Group	1 hours	3 hours	6 hours	12 hours	24 hours	Day 7	Day 14	Day 21	Day 28
IgE Sera (µg/mL)									
G1	0.961	NA*	0.684	0.327	0.592	1.205	0.789	0.84	0.626
G2	0.787	NA*	0.659	0.736	0.685	1.555	1.085	1.655	0.537
G3	1.845	NA*	1.588	2.28	2.145	1.875	2.165	1.865	1.95
p-value	<i>0.048</i>	NA*	0.067	<i>0.016</i>	<i>0.022</i>	0.549	<i>0.024</i>	0.206	<i>0.017</i>
IL-6 Sera (pg/mL)									
G1	1025	NA*	294.1	345.2	462.2	162.1	251.8	199.9	363.6
G2	735.5	NA*	291.6	209.6	189.8	148.3	212.5	207.9	211.2
G3	373.6	NA*	209.2	176.3	195.6	180.4	171.2	215.6	174.6
p-value	<i>0.019</i>	NA*	0.737	0.504	0.258	0.222	0.331	0.549	0.296
TNF-α Sera (pg/ml)									
G1	171.4	NA*	26	44.2	37.1	36.6	31.2	49.5	41.4
G2	165.7	NA*	28.9	23.7	37.3	33.9	25	34.3	27.1
G3	110.9	NA*	18.2	20.1	15.9	26.9	24.1	31.7	23.9
p-value	0.386	NA*	0.199	0.544	0.089	0.354	0.471	0.271	0.187
IgE Tears (µg/mL)									
G1	32.5	29.5	30.2	31.8	25.5	17.77	25.2	9.7	23.2
G2	165.7	98.5	26.4	17.5	27.1	22.73	17.9	18.5	22.1
G3	30.7	26.4	26.1	26.9	21.1	5.87	18.9	7.4	18
p-value	<i>0.023</i>	<i>0.026</i>	0.471	0.341	0.737	0.116	0.528	0.344	0.603
IL-6 Tears (pg/mL)									
G1	284.3	274.5	345.2	325.5	275.7	316.2	345.2	454.9	357.7

Group	1 hours	3 hours	6 hours	12 hours	24 hours	Day 7	Day 14	Day 21	Day 28
G2	177.9	188.7	192.7	154.5	191.8	198.6	166.2	176.3	195.1
G3	282.2	269	257.8	279.7	114.9	98.3	91.4	100.8	110.6
p-value	<i>0.021</i>	<i>0.008</i>	<i>0.012</i>	<i>0.012</i>	<i>0.006</i>	<i>0.005</i>	<i>0.004</i>	<i>0.004</i>	<i>0.014</i>
TNF- α Tears (pg/mL)									
G1	67.2	51.9	67.9	71.1	56.4	50.5	59.6	61.1	54.5
G2	28.7	27.6	27.9	30.2	31.9	30.7	31.4	30.8	32.9
G3	31.1	43.1	42.6	47.9	34.7	33.1	27.3	25.6	42.6
p-value	0.091	0.108	<i>0.005</i>	<i>0.004</i>	<i>0.253</i>	<i>0.109</i>	<i>0.071</i>	<i>0.071</i>	<i>0.028</i>

Table 3

Haematological observations of rabbits after treatment with hLMSCs: Table of the blood parameters of the rabbits of groups G1, G2 and G3. No significant changes were observed in test item administered animals when compared with vehicle control group animals. Values are expressed as mean \pm SD; $p > 0.05$. **G1** – Sham treated group; **G2** – Treated with cells without encapsulation/transit (En^- hLMSCs); **G3** – Treated with cells post-encapsulation and transit (En^+ hLMSCs).

S.No.	Parameter	G1	G2	G3	Units
1	Haematocrit	44.8 \pm 3.31	42.5 \pm 4.87	40.73 \pm 2.35	%
2	Haemoglobin	13.83 \pm 1.06	13.10 \pm 1.61	12.93 \pm 0.72	gm/dL
3	Mean Corpuscular Volume	72.35 \pm 2.59	70.33 \pm 3.23	70.90 \pm 1.97	fL
4	Platelets	384 \pm 152.69	330.5 \pm 69.85	461.17 \pm 215.31	10 ³ / μ L
5	Red Blood Corpuscles	6.19 \pm 0.4	6.06 \pm 0.77	5.75 \pm 0.24	10 ⁶ / μ L
6	White blood Corpuscles	9.97 \pm 3.04	7.93 \pm 1.54	8.63 \pm 3.01	10 ³ / μ L
7	Differential Count				%
	Neutrophils	40.67 \pm 8.90	32.0 \pm 5.29	43.83 \pm 15.68	%
	Lymphocytes	52.50 \pm 10.08	62.67 \pm 5.76	50.17 \pm 15.42	%
	Monocytes	4.5 \pm 1.38	3.67 \pm 0.75	4.00 \pm 0.58	%
	Eosinophils	2.33 \pm 0.75	1.67 \pm 0.47	2.0 \pm 0.58	%
	Basophils	0.00 \pm 0.00	0.00 \pm 0.00	0.00 \pm 0.00	%
8	Reticulocyte Count	1.83 \pm 0.08	1.86 \pm 0.06	1.87 \pm 0.05	%
9	Bleeding Time	3.06 \pm 0.29	3.25 \pm 0.14	3.08 \pm 0.30	Minutes
10	Coagulation Time	6.98 \pm 0.33	7.22 \pm 0.37	6.98 \pm 0.33	Minutes
11	Prothrombin Time	16.17 \pm 1.07	16.33 \pm 0.94	16.67 \pm 1.11	Seconds
12	Activated Partial Thromboplastin Time	39.67 \pm 4.03	41.00 \pm 2.08	40.33 \pm 1.70	Seconds
13	Erythrocyte Sedimentation Rate	4.0 \pm 3.21	5.67 \pm 3.35	5.33 \pm 1.89	mm/1st hour

3.5. Immunoassay based assessment for inflammatory markers and immunogenicity

A decreasing trend of the inflammatory markers TNF- α and IL-6 was observed from day 0 to day 28 of the study, in the rabbit sera. The mean levels of these analytes in both the test groups (En^+/En^- hLMSCs) were observed to follow a decreasing trend similar to that of the control group (G1) (Fig. 5B-5C). In the tear samples as well, the levels of the inflammatory molecules TNF- α and IL-6 were found to be significantly low and had a decreasing trend during the study (Fig. 5E-5F), except for few initial time points (levels of TNF- α in tears at 1st and 3rd hours, post-treatment) (Fig. 5F). The levels of IgE in serum were found to be high in En^+ hLMSCs group compared to the other two groups at 5 of 8 time points (Fig. 5A). Whereas in the tear samples, the levels of IgE have shown to follow a decreasing trend except for the initial hours of treatment viz 1st and 3rd hour of En^- hLMSCs group (Fig. 5D). Overall, the levels of IgE in tears remained comparable in all three groups.

3.6. Haematology

All the haematological parameters of the control (sham) and test item administered groups (En^+/En^- hLMSCs) were found to be normal (Tables 4). Bone marrow analysis revealed no significant changes in the haematopoietic system. No significant evidence of erythropoiesis, granulopoiesis and lymphopoiesis was observed in the precursors of cells of erythroid, myeloid and lymphoid cells in all test groups when compared to the control group. None of the animals from the G1, G2 and G3 groups showed any hypocellularity or hypercellularity, hypochromatism or hyperchromatism of the cellular population (Tables 4).

Table 4

Clinical chemistry observations of the rabbits. Table of the biochemical parameters of the rabbits of groups G1, G2 and G3. Changes observed in the levels of the clinical parameters did not show any impact on the systemic organs. Values are expressed as mean \pm SD. * $p \leq 0.05$. **G1** – Sham treated group; **G2** – Treated with cells without encapsulation/transit (En^- hLMSCs); **G3** – Treated with cells post-encapsulation and transit (En^+ hLMSCs).

S. No.	Parameter	G1	G2	G3	Units
1	Serum Glucose	112.83 \pm 17.49	117.67 \pm 12.76	115.33 \pm 6.39	mg/dL
2	Blood Urea Nitrogen	21.50 \pm 2.99	21.67 \pm 3.35	22.50 \pm 3.64	mg/dL
3	Serum Creatinine	0.95 \pm 0.13	0.85 \pm 0.08	0.98 \pm 0.29	mg/dL
4	Serum Total Bilirubin	0.05 \pm 0.00	0.05 \pm 0.01	0.05 \pm 0.00	mg/dL
5	Alanine Aminotransferase	61.83 \pm 12.56	59.50 \pm 26.91	81.67 \pm 32.03	IU/L
6	Aspartate Aminotransferase	62.17 \pm 13.03	74.17 \pm 67.73*	61.00 \pm 11.65	IU/L
7	Serum Alkaline phosphatase	86.67 \pm 39.77	134.83 \pm 48.47	107.83 \pm 28.37	IU/L
8	Serum Total protein	6.0 \pm 0.18	5.63 \pm 0.38	5.82 \pm 0.25	g/dL
9	Serum Albumin	2.40 \pm 0.07	2.36 \pm 0.20	2.46 \pm 0.10	g/dL
10	Globulin	3.60 \pm 0.21	3.27 \pm 0.21*	3.38 \pm 0.23	g/dL
11	Serum Total Cholesterol	56.33 \pm 29.03	59.00 \pm 13.76	74.83 \pm 37.75	mg/dL
12	High density lipoprotein	23.50 \pm 11.91	29.17 \pm 9.15	30.83 \pm 10.35	mg/dL
13	Low density lipoprotein	27.00 \pm 16.90	25.67 \pm 7.36	36.50 \pm 23.68	mg/dL
14	Serum Phosphorous	5.83 \pm 0.39	6.22 \pm 0.46	7.35 \pm 1.11	mg/dL
15	Serum Calcium	12.98 \pm 0.19	12.80 \pm 0.46	12.67 \pm 0.40	mg/dL
16	Serum Sodium	158.40 \pm 3.19	153.26 \pm 5.01*	152.47 \pm 1.86	mmol/L
17	Serum Potassium	4.84 \pm 0.21	4.73 \pm 0.36	5.50 \pm 0.91	mmol/L
18	Gamma glutamyl transferase	8.42 \pm 3.30	10.65 \pm 3.46	9.48 \pm 4.33	IU/L

However, mild variations in the production of granulopoietic cells were observed in one animal from G1 [1 of 6], and one animal from the G2 group [1 of 6]. The G3 (En^+ hLMSCs) group did not show any such changes in granulopoietic cellular populations. There were no significant changes in variation in granulopoietic activity in test item administered animals when compared with sham group. The changes

of granulopoietic activity were observed in few animals of control and test item administered groups, indicates the occurrence of spontaneous immune changes in the animals.

Bone marrow smears of all animals of groups G1, G2 and G3 did not show any dose-dependent variation or toxicity were observed in the production of erythroid, myeloid and lymphoid precursor cells in G2 and G3 groups compared to the control group, where different doses of test item were administered.

3.7. Clinical Chemistry

All the clinical chemistry parameters were found normal except for the following observations. An increase in the levels of Phosphorous (7.70 ± 0.85) and GGT levels of the G3 group (13.47 ± 2.30) was observed when compared with sham group (5.53 ± 0.40 ; 7.03 ± 1.47). In the G2 group, decreased levels of AST (40.33 ± 3.06), total proteins (5.40 ± 0.36), globulin (3.17 ± 0.25), sodium (151.37 ± 3.75) were observed and in animals of G3 group, and a decreased level of sodium (152.35 ± 1.20) was observed when compared with the sham group animals (59.67 ± 5.13 , 6.17 ± 0.06 , 3.80 ± 0.00 , 160.27 ± 4.08 respectively) (Tables 5). The changes observed did not show any impact on the systemic organs of the tested animals.

3.8. Necropsy, gross observations and organ weights

External and *in situ* examinations of organs were found normal in all the groups of animals. (Supplementary Table 2). The organ weights were found normal in all the test item administered groups (G2 and G3). No significant changes were observed in test item administered animals when compared with control group animals.

3.9. Histopathology

Histopathological examination was carried out for all the groups. The organs of spleen, heart, aorta, adrenal, trachea, thyroid, parathyroid, oesophagus, duodenum, Jejunum, colon, rectum, lymph node, thymus, pancreas, urinary bladder, muscle, skin, testes, epididymis, ovary, uterus, spinal cord, mammary gland and middle ear did not show any abnormal changes in the test groups when compared to control (Supplementary Table 3).

Sinusoidal haemorrhages in the liver were noticed in two rabbits from all the groups G1, G2 and G3 group [2 of 6]. Foci of necrosis and infiltration of inflammatory cells were observed in one animal from the G1 group [1 of 6], whereas other groups did not show any such changes in the liver. Alveolar wall thickening or alveolar inflammation was noticed in the lungs of five animals from the G1 group [5 of 6] and five animals from the G2 group [5 of 6]; four animals from the G3 group [4 of 6].

Tubular degeneration was observed in the kidneys of one male animal from the G2 group [1 of 6] and one female animal from the G3 group [1 of 6]. Foci of tubular inflammation or interstitial inflammation noticed in two males and one female from the G1 group [3 of 6] and one male animal of the G2 group [1 of 6] and one female animal from the G3 group [1 of 6]. Foci of necrosis noticed in the brain, in the cerebral hemisphere and Perivascular cuffing noticed in two male animals from the G1 group [2 of 6] and

one male animal from the G2 group [1 of 6], whereas the G3 group did not show such changes in the brain. Submucosal lymphoid tissue hyperplasia noticed in ileum mucosa in one male animal from the G1 group [1/6] and one male animal from the G2 group [1/6] and one female animal from the G3 group [1/6].

However, there were no dose-related toxic changes in lung, liver, kidney, eye and ileum in G2 and G3 groups when compared with the G1 group. The lesions discussed in these organs might be spontaneous as they appeared in both vehicle control and test item group. Also, there is no consistency or significance of lesion in these organs in test item administered animals when compared with vehicle control animals. In summary, there were no major reactive and toxic changes in all the systemic organs (Supplementary Table 3).

4. Discussion

Many potential alternatives to corneal transplantation for the treatment of corneal opacification and scarring have emerged in the recent past. These include cell-based approaches, biomimetic hydrogels, and molecular approaches. Various studies have shown the promise of different hydrogels (with and without cells), as a viable alternative to stromal replacement with donor tissue (33–36). Others have shown the importance of various secretory molecules like exosomes (37), anti-TGF- β (7, 38–39), anti-PDFG (7, 40–41), and HGF (42–43) in preventing or reverting the corneal scars. Studies have shown that corneal scars can be healed either by inhibiting the TGF- β / SMAD signalling or by reversing myofibroblasts to fibroblast during wound healing (4, 44–47). The hLMSCs, in recent years, have shown promising potential in scarless wound healing of the damaged cornea due to various pathologies (4). These cells have also been shown to retain their characteristic properties and have enhanced shelf life over prolonged durations at varied temperature conditions when encapsulated in alginate (25). Alginate encapsulation can facilitate these cells being transported across long distances without needing expensive cold-chain systems. Since the burden of corneal blindness due to stromal scarring or opacification is most acute in the developing world, simpler and cheaper transportation will increase accessibility to patients in remote areas, at lower costs. The current study was aimed at evaluating the ocular and systemic toxicity of the LMSCs after being topically applied to rabbit corneas. The cells were treated with or without encapsulation in sodium alginate followed by transit at room temperature.

The LMSCs isolated from the limbus of the donor corneas were cultured in a CGMP-grade, certified cell culture facility as reported in the earlier studies (25). The LMSCs that were encapsulated in the sodium alginate and under transit for 3 days and the non-encapsulated cells which were not under any transit, were assessed for their toxicity after topical application on the rabbit eyes with the corneal wound. The control group did not receive any cells but received the vehicle as a sham control. Thorough evaluations for systemic and ocular toxicity were performed through ophthalmic, haematological and tissue investigations. Zero mortality was observed during the study. All the rabbits were sacrificed at end of the study and all major organs and tissues including the eyes were harvested and subjected to a detailed histopathological evaluation. The ophthalmic investigations revealed normal observations with no significant changes in the intraocular pressure of the treated rabbit eyes (left eye) (Fig. 4). The

parameters of haematological examination were comparable in all three groups. Corneal tissues of the rabbit eyes were found to have no abnormalities, after histopathological assessment (Fig. 6). Both the experimental groups did not show any signs of a significant inflammatory response with respect to the sham or control group, in tears or sera (TNF- α and IL-6) (Fig. 5A-5F). This study provides additional evidence for the safety of the hLMSCs implying that these cells may be assessed for their clinical applications in human clinical trials.

Recent advances in regenerative medicine have opened the doors for a variety of treatment modalities for various disease conditions and disorders. Mesenchymal stem cells are one of the major therapies that are being assessed for their efficacy in the treatment of various diseases related to the heart, ear, bone, and eye (48–49) in clinical trials across the world. However, ensuring the safety of the patient is non-negotiable and forms the most essential crux and primary priority of any clinical trial or drug-development process. This necessitates compliance with various regulatory requirements and preclinical testing to establish the toxicity or safety profile of the drug or cell product of interest. The Central Drugs Standards Control Organization (CDSCO), the Indian regulatory body, an equivalent organization to the FDA in the USA; and the Drug Controller General of India, the body within CDSCO, together govern the pharmaceutical regulations in India. These bodies mandate the assessment of safety of each drug or any form of surgical intervention, as mentioned in the Schedule Y of the Drugs and Cosmetic Rules act 2018, Government of India (26–27).

The hLMSCs assessed in this study were evaluated abiding by the guidelines of Schedule Y and the OECD guidelines for Good Laboratory Practice (GLP). Studies by *Wright et al* (2016) (50) and *Damala et al* (2019) (25) have shown that encapsulation of the corneal epithelial cells and hLMSCs in sodium alginate could enhance the shelf life of the cells allowing them to be transported at room temperature conditions while retaining their characteristic phenotype and viability. This technology greatly favours the economics of this emerging cell-based therapy by removing the expensive and laborious cold-chain transport, thereby potentially reducing the costs involved to a greater extent.

The assessment of inflammatory molecules revealed that the hLMSCs did not cause any ocular toxicity to the recipient as evident from the relatively low levels of the cytokines TNF- α and IL-6 in tears (Fig. 5E-5F). Similar observations found with regards to the systemic toxicity of these cells as well, from the levels of these analytes TNF- α (Fig. 5C) and IL-6 (Fig. 5B) in the blood serum of the rabbits. Whereas the levels of the IgE molecules, which indicates any possible allergic reactions to the organism, in the group treated with encapsulated cells were found to be significantly different to the groups of control/sham and the group treated with non-encapsulated cells at certain time points. However, there was no definite trend of the varying levels of IgE (Fig. 5A), and the same was not observed in the levels of IgE in the tears of the test animals (Fig. 5D). Both experimental arms showed a significantly reduced expression of TNF- α and IL-6 in the tear samples (Fig. 5E-5F). In addition, the ophthalmic investigations revealed no significant changes in the levels of IOP and corneal clarity with no ocular lesions observed post 12th hour of the treatment, till the end of the study (Fig. 3). The variations found in the haematological and clinical chemistry parameters did not show any effect on the systemic organs as evident from the

histopathological investigations (Fig. 6 and Supplementary Table 3). Apart from the above, the data supporting the stability, sterility of the cells and the absence of any chromosomal aberrations (Fig. 2B-2C) provide additional evidence regarding the safety of these cells towards their use in human trials.

This study finds its strength in being carried out at a NABL-accredited (National Accreditation Board for Testing and Calibration Laboratories), and GLP-certified animal facility. All the observation-making personnel (veterinarians/ biochemists/ pathologists) were completely masked to the intervention being studied. The study has involved only one time-point duration (3-days) of transit after alginate-encapsulation of the hLMSCs, compared to the previous study, which may be a plausible limitation. However, this duration was chosen considering that a 3-day duration would be sufficient for the cells to reach any remote location of the country from the site of distribution. The evaluation of the tears from the untreated or normal eye as well may have provided better picture of the ocular toxicity. The application of the LSMCs assessed in this study was limited to the surface of corneas only. However, injecting these cells into the subconjunctival space could have provided an opportunity to evaluate not only the safety of these cells but also exploring the different mechanism of the cell delivery as well. This shall be explored in further studies.

5. Conclusion

In conclusion, our study aimed at assessing the toxicity of the hLMSCs with or without alginate encapsulation and transit, in mildly wounded rabbit corneas. The findings of our study suggest that the hLMSCs are non-toxic to the recipient, not inducing any inflammatory response, rendering them safe. This ascertains the usage of these cells for human application to assess their efficacy in treating corneal wound healing, and eventually making them available at the remotest geographical locations, at significantly lower costs, obviating the need for long-distance travel.

Declarations

Ethics approval and consent to participate: This study protocol was approved by the Institutional Review Board (LEC 05-18-081), Institutional Committee for Stem Cell Research (08-18-002), LV Prasad Eye Institute, Hyderabad and the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Sipra Labs Limited, Hyderabad (SLL/PCT/IAEC/110-19).

Consent for publication: Not applicable.

Availability of data and material: Data available upon request.

Competing interests: There are no potential conflicts of interest to declare. Two clinical trials have been approved by the Indian regulatory agencies (CDSCO/DCGI) based on the data provided in this study (<http://ctri.nic.in/Clinicaltrials/advancesearchmain.php>; [CTRI/2020/07/026891](#) and [CTRI/2021/07/035034](#)). These trials have not started recruiting patients yet.

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Authors' contributions:

Mukesh Damala: Conception and design, provision of study material, collection and/or assembly of data, data analysis and interpretation, manuscript writing.

Naveen Pakalapati: Collection and/or assembly of data, data analysis and interpretation.

Sayan Basu: Conception and design, financial support, administrative support, provision of study material, data analysis and interpretation, manuscript writing, final approval of manuscript.

Vivek Singh: Conception and design, financial support, administrative support, provision of study material, data analysis and interpretation, manuscript writing, final approval of manuscript.

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Figures

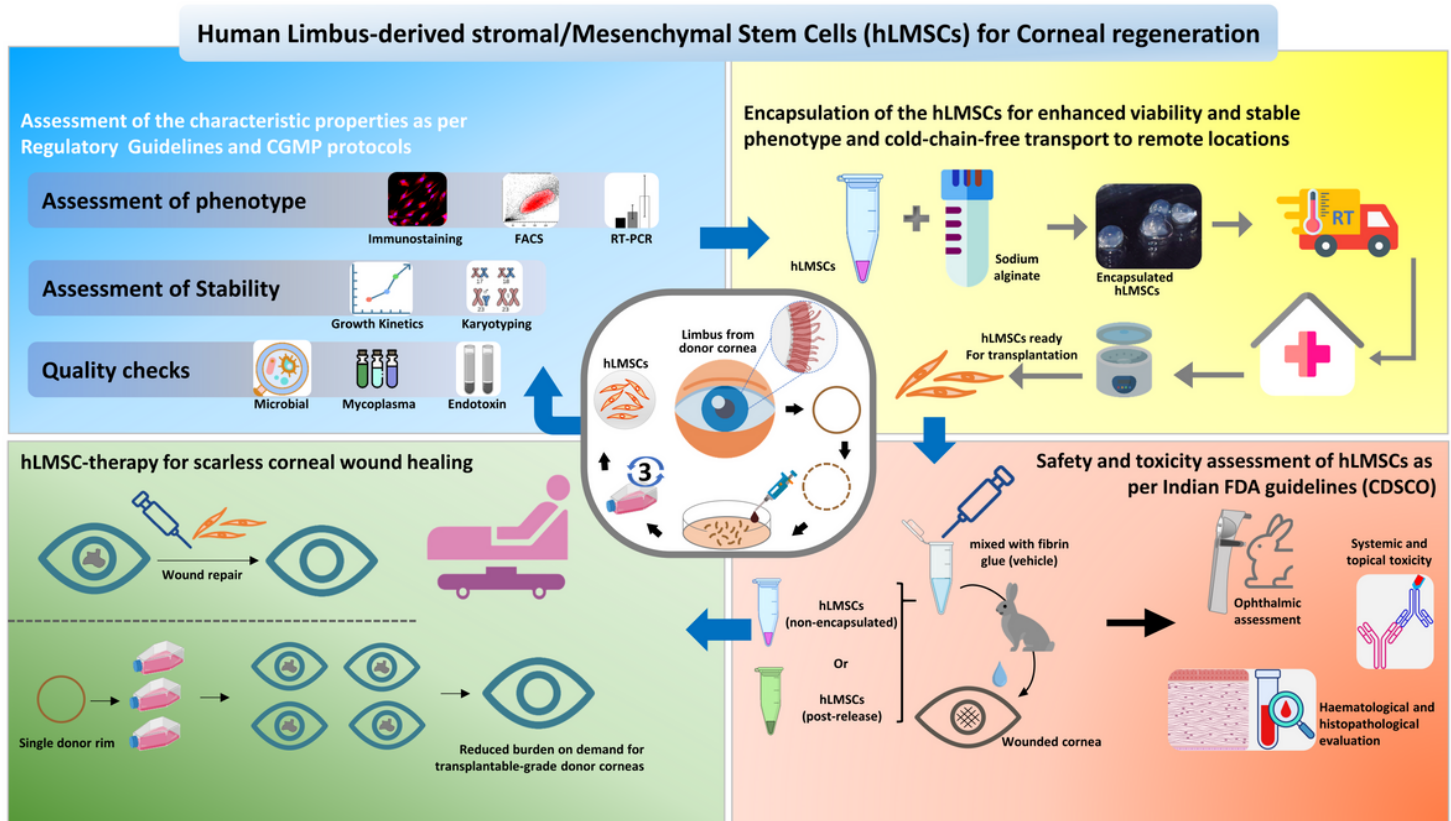


Figure 1

Schematic diagram of the study protocol. Descriptive flow chart showing the study design. Human limbus-derived stromal/mesenchymal stem cells (hLMSCs) cultured in a CGMP-grade facility were assessed for their phenotypic assessment. These cells with or without encapsulation in sodium alginate were assessed for toxicity in rabbit eye with corneal wound. The study has 3 groups of animals (n=6 each) which were treated with sham and hLMSCs with (En+ hLMSCs) or without encapsulation (En-hLMSCs). The animals were subjected to ophthalmic investigations during the study. Blood and tear samples were collected for assessment of immunogenicity. At the end of the study, the animals were sacrificed and subjected to histopathological evaluation.

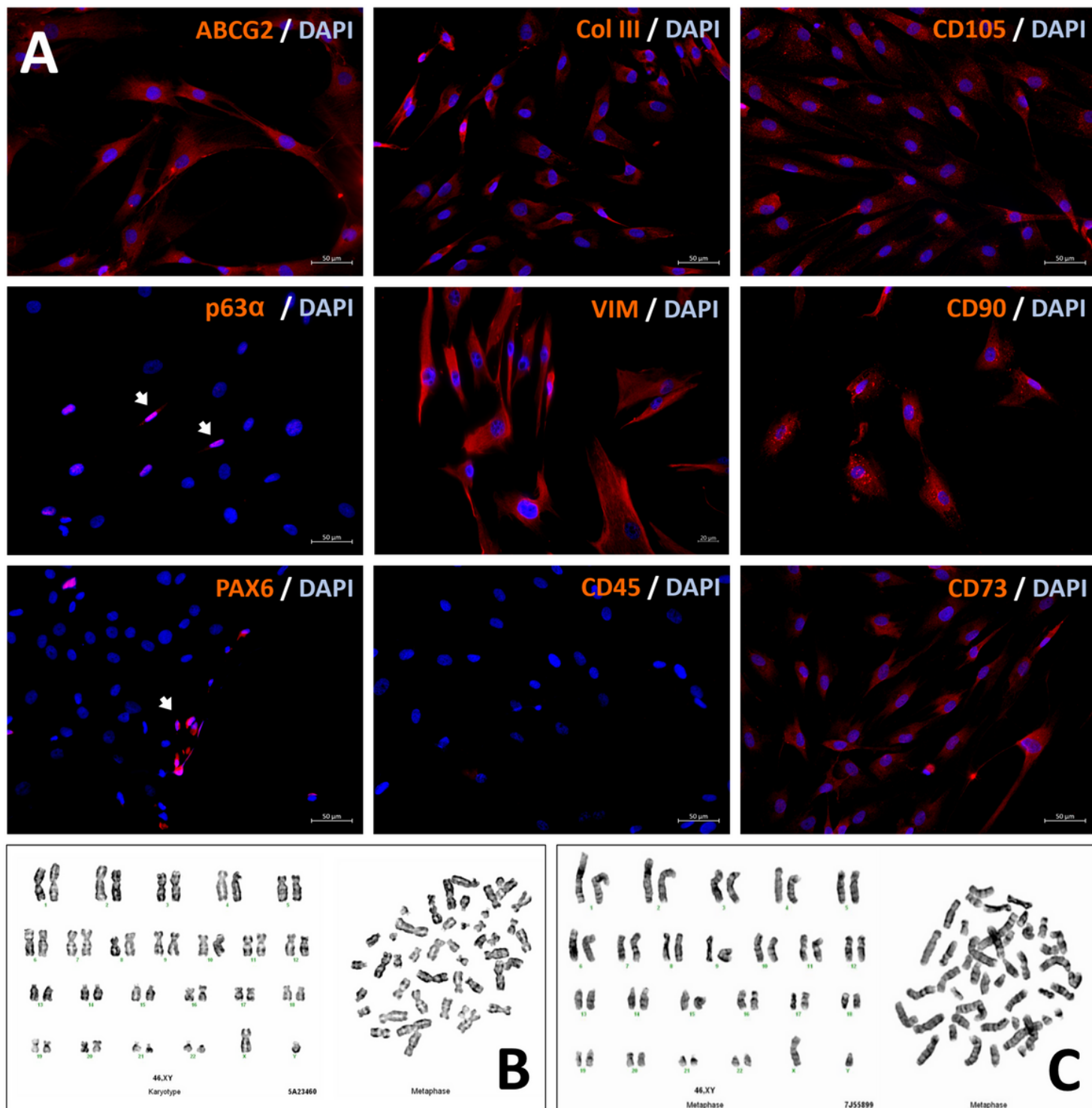


Figure 2

Assessment of characteristic phenotype and stability. (A) Phenotypic expression of the characteristic biomarkers: hLMSCs were assessed before their administration onto the rabbit corneas, for their characteristic phenotype, through immunostaining. Panel shows the expression of the stem-cell biomarkers (p63+, Pax6+ and ABCG2+) and mesenchymal biomarkers (VIM+, CD45-, CD73+, CD90+, and CD105+), stained in red against DAPI, nuclear stain (blue). Magnification: 40x; Scale: 50μM. (B) Karyotyping analysis of the hLMSCs, showing the stability of the cells with no numeric or structural

alterations detected (n=3). (C) Karyotyping analysis of the hLMSCs post-release from the encapsulation and transit (n=3). No numeric or structural alterations/ aberrations were observed.

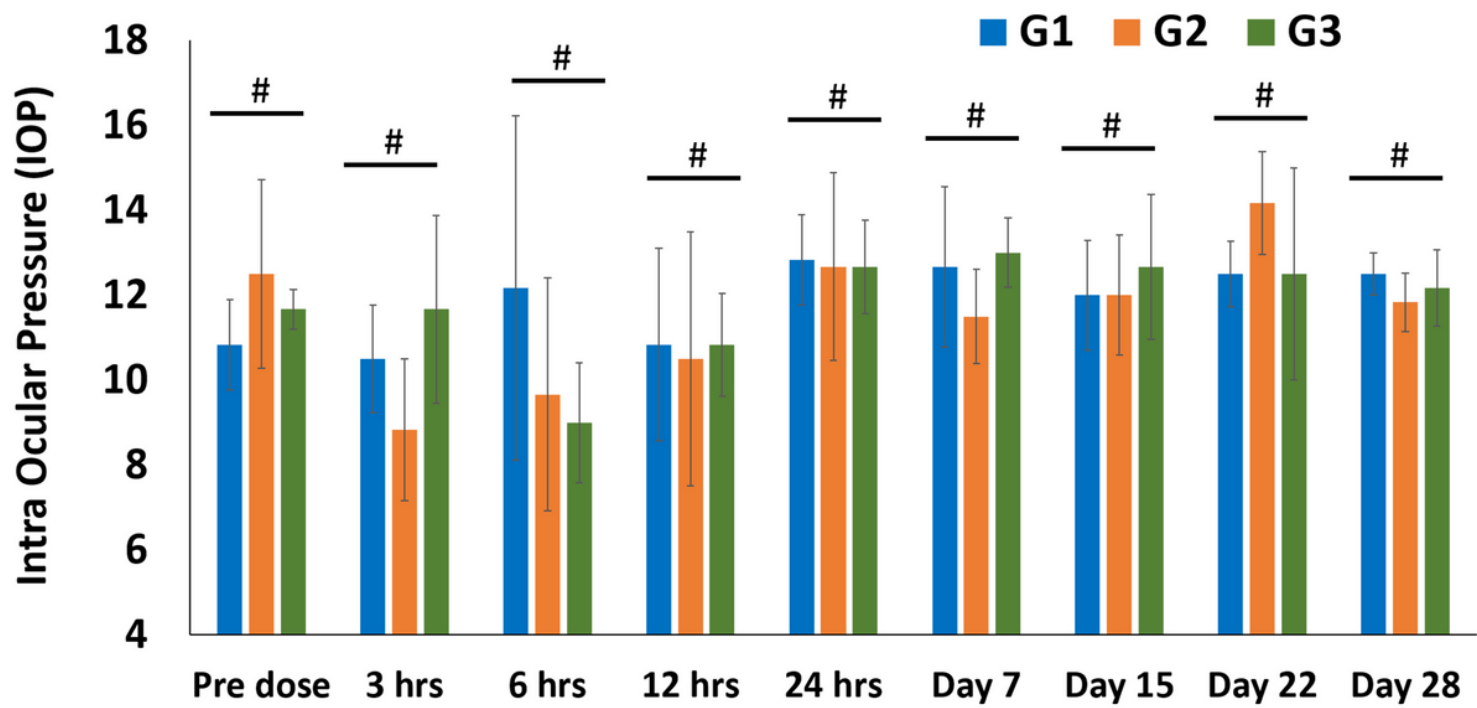


Figure 3

Change in Intraocular pressure of the rabbit eyes. Bar graph plot showing the changes in intraocular pressure (IOP) of the treated eyes. No significant changes in the levels of the IOP of the experimental groups (G2 and G3) were observed when compared to control group (G1). n=6; #p>0.05. G1 – Sham treated group; G2 – Treated with cells without encapsulation/transit (En- hLMSCs); G3 – Treated with cells post-encapsulation and transit (En+ hLMSCs).

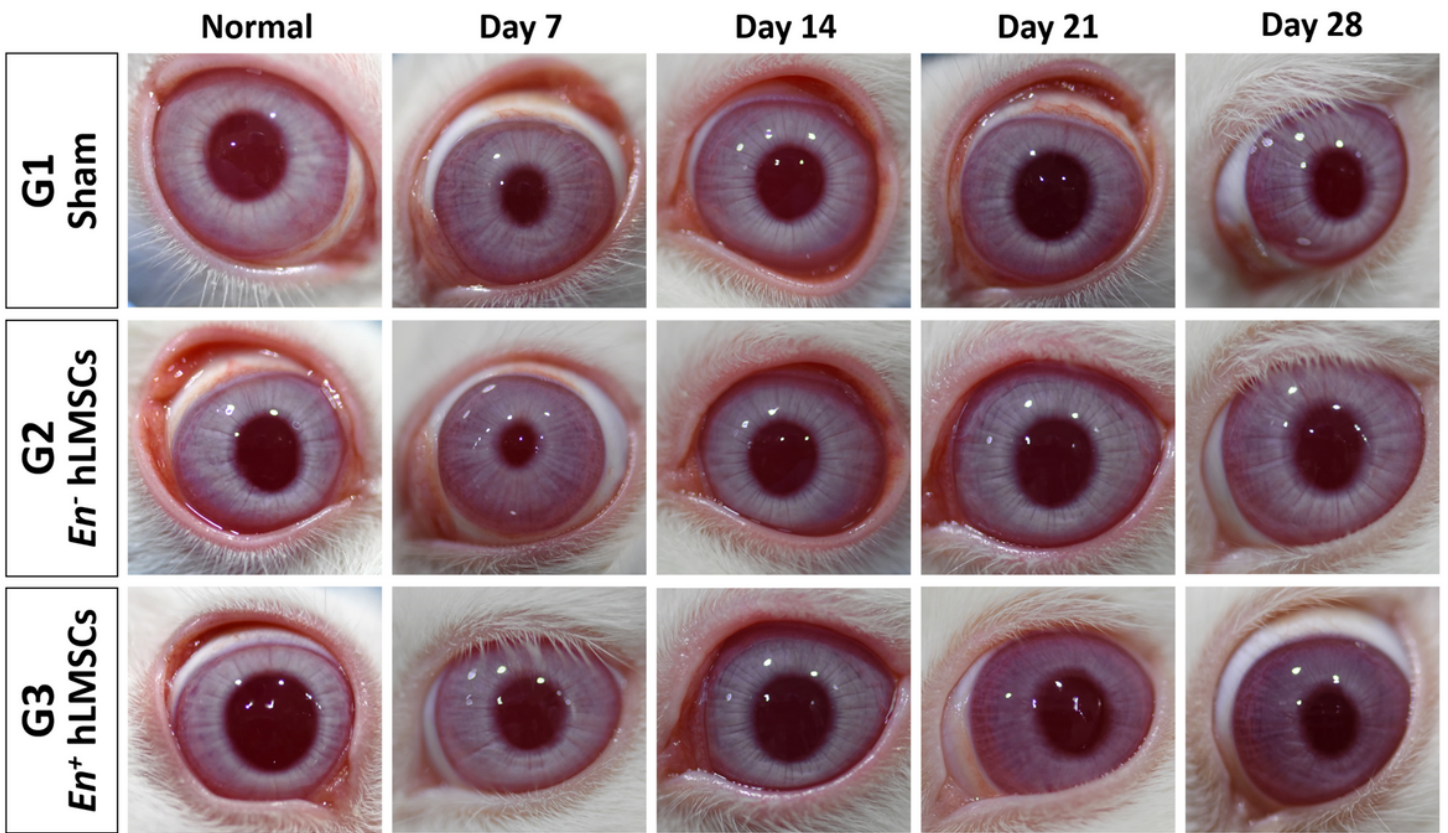


Figure 4

Representative photographs of the rabbit eyes after treating with En+/En- hLMSCs. Panel of representative clinical photographs of the rabbit eyes showing no signs of redness or irritability in the wounded eyes from days 7 to 28, with respect to the normal eyes. The photographs were captured using a DSLR camera (Nikon D7200) equipped with a Nikon AF-S VR Micro-NIKKOR 105mm f/2.8G IF-ED lens. G1 – Sham treated group; G2 – Treated with cells without encapsulation/transit (En- hLMSCs); G3 – Treated with cells post-encapsulation and transit (En+ hLMSCs).

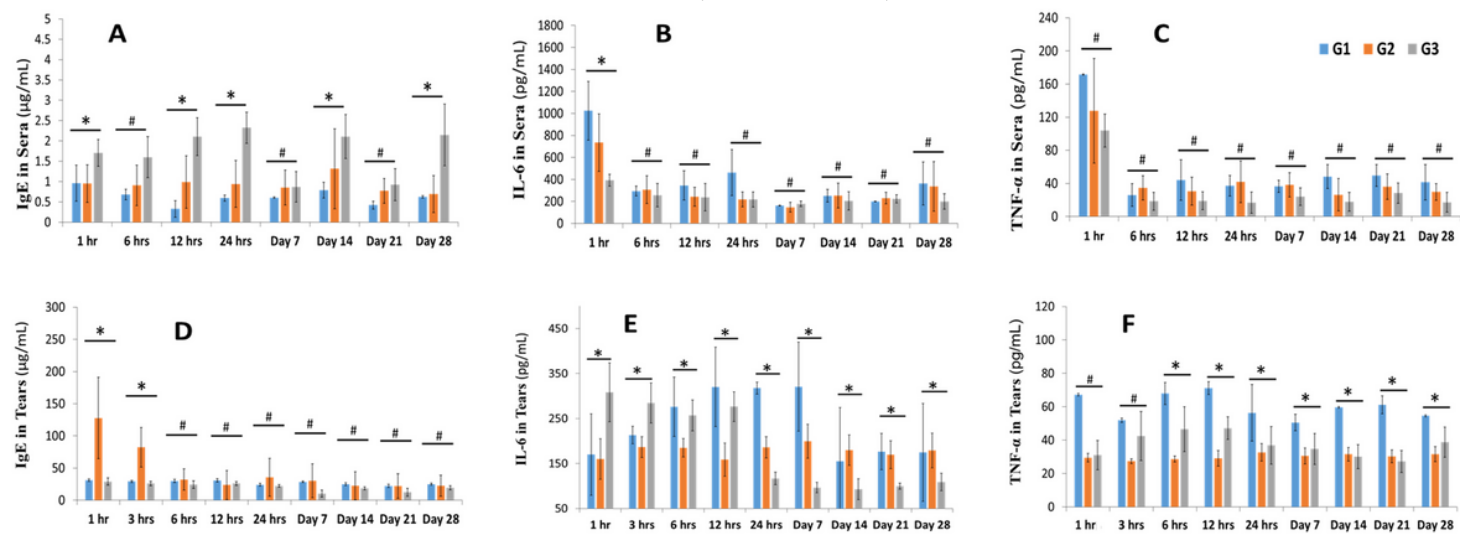


Figure 5

Graph plots showing the levels of cytokines IgE, IL-6, and TNF- α in serum and tears of rabbits post En+/En- hLMSCs treatment. (A-C) Bar graph plots showing the levels of the cytokines IgE, IL-6 and TNF- α in the sera of rabbits, assessed through ELISA. (D-F) Bar graph plots showing the levels of the cytokines IgE, IL-6 and TNF- α in the tear samples of rabbits. A decreasing trend of the cytokines was observed in the experimental and control groups, indicating no topical toxicity to the recipient eyes. * $p \leq 0.05$; # $p > 0.05$. G1 – Sham treated group; G2 – Treated with cells without encapsulation/transit (En- hLMSCs); G3 – Treated with cells post-encapsulation and transit (En+ hLMSCs)

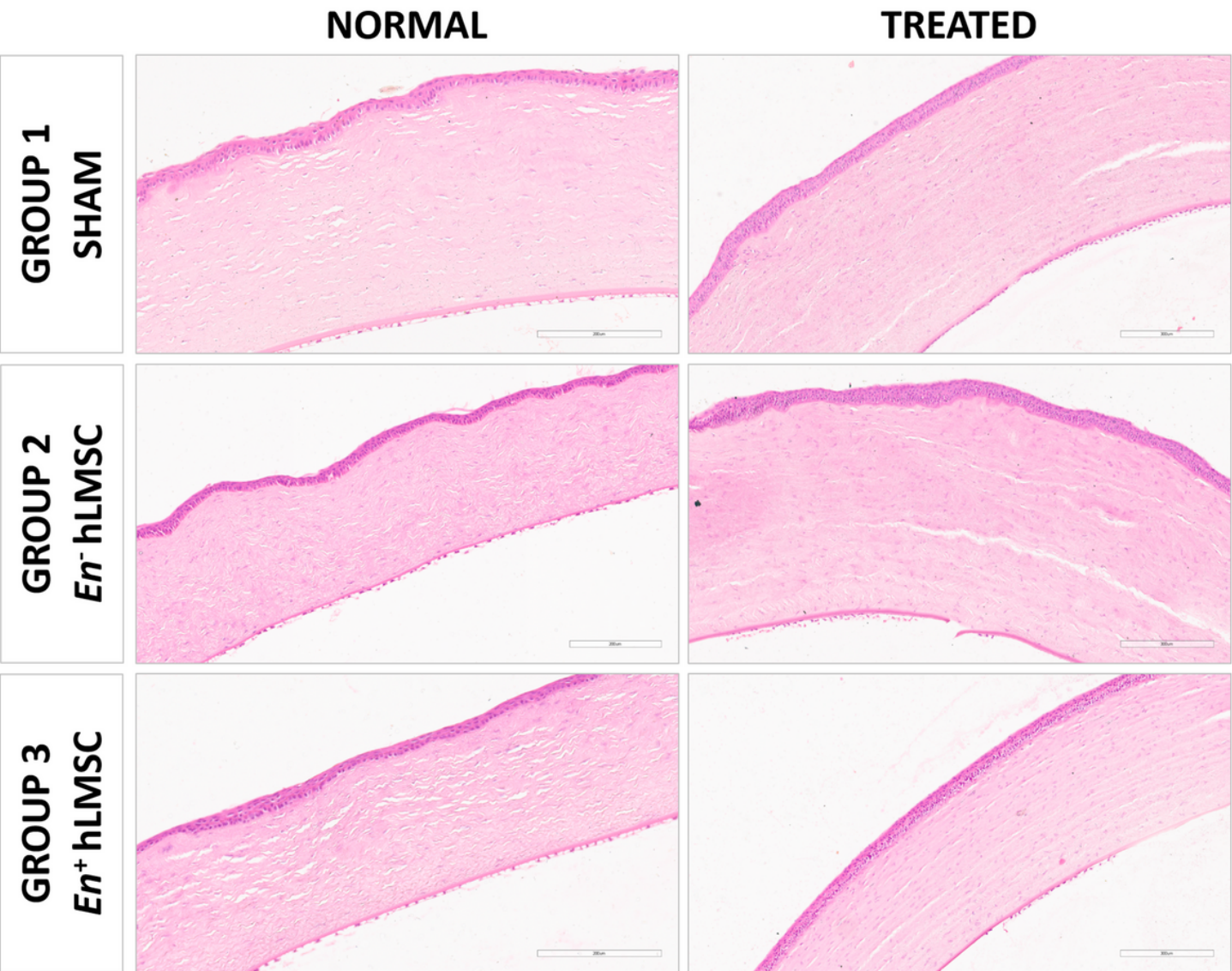


Figure 6

Histopathological sections of the corneas. Panel of the representative photomicrographs of the histopathological sections of the normal corneas versus treated corneas. Corneas were excised at the end of the study and stained with haematoxylin and eosin stain. Magnification: 40x; Scale: 200µM. G1 – Sham treated group; G2 – Treated with cells without encapsulation/transit (En- hLMSCs); G3 – Treated with cells post-encapsulation and transit (En+ hLMSCs).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Supplementarydata.docx](#)