Lacrispheres from human Lacrimal Gland: Stemness and Secretory Potential

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Abstract

Purpose: Lacrimal gland (LG) dysfunction leading to dry eye syndrome (DES) is an important cause of ocular morbidity. One of the promising long-term management therapies for restoration of LG function could be transplantation of autologous *ex-vivo* expanded stem cells. The present study was aimed at exploring the 2D and 3D cultures of human LG, identifying inherent stem cells and evaluating their secretory potential.

Methods: Fresh HuLG (n=5) from patients undergoing exenteration were harvested after IRB approval. The gland was enzymatically digested and the isolated cells plated in HepatoStim media supplemented with l-glutamine, epidermal growth factor, fibroblast growth factor and N2. The native HuLG and the *DIV*14-16 spheres were evaluated for presence of stem cells (CD117) expression, quiescence, BrdU label retention, cell cycle and colony forming efficiency and differentiation (secretion of tear proteins).

Results: Under the established culture conditions, suspension cultures of human 'lacrispheres' could be maintained and propagated for 3-4 weeks. The spheres consist of both acinar as well as ductal cells with evidence of stem cells. This was supported by expression of CD117 (0.8%±0.05%), BrDU label retention (9.31±0.41%), co-localization of CD117 in the G0/G1 phase of cell cycle (76.9%) and colony forming units (3.1%). The lacrispheres also secreted quantifiable levels of tear proteins (lysozyme, lactoferrin, scIgA) into the conditioned media.

Conclusion: The study provides promising, first-of-its-kind evidence for the generation of 'lacrispheres' from fresh HuLG, with enriched population of stem cells and secretory

competent differentiated cells. The dual properties of these spheres make them highly suitable source of transplantable cells for restoring the structure and function of damaged lacrimal gland.

Introduction

The human lacrimal gland is a tubulo-acinar exocrine gland responsible for the secretion of the aqueous component of the tear film, the stability and integrity of which is essential for the health of the ocular surface epithelium. In conditions of lacrimal gland dysfunction or destruction, this stability is lost with subsequent damage to the ocular surface and development of chronic aqueous deficient dry eye.

Development of aqueous deficient dry eye (ADDE) is multifactorial and could be sequelae of many factors that affect the lacrimal gland and its function. Some of the causes of lacrimal gland dysfunction/destruction include: bone marrow transplantation and cancer management therapies like radiotherapy and chemotherapy, ocular spectrum of graft versus host disease, post-menopausal hormonal therapy, low androgen pool, low dietary intake of omega-3-fatty acids, anti-histaminic drugs, and refractive surgeries like Laser Assisted In-situ Keratomileusis (LASIK)(1). It has been reported in animal studies that the gland is able to withstand small destructive influences and can regenerate itself (2). However, when the damage becomes extensive, this natural course of tissue regeneration fails and the signs and symptoms of dry eye develop causing significant discomfort to the patient. The current line of therapeutics available for the management of dry eye unfortunately, does not halt the progress of this morbid condition, with the patient having to significantly compromise quality of vision and life.

One of the promising avenues for the management of this condition could be replacement of the gland and restoration of its function through cell therapy. Prerequisites for cell therapy would be to explore the presence of stem cells that could be harvested and cultured. The cultured cells containing stem cells would self-renew as well as differentiate towards functionally competent cells both of which could contribute to regeneration of the injured tissue. There is increasing evidence for the presence of stem-like cells in the lacrimal gland of mice (2) and rat (3) that contribute towards regeneration of the injured gland. Studies published by You *et al* and Shatos *et.al.,* have shown the presence of BrdU label retaining quiescent cells in the gland, which have the potential to heal the gland following an insult. The study by You *et.al.* (2) showed that post-interleukin injection into the murine lacrimal gland, which destroys areas in the gland, stem-like cells migrate towards the site of injury and heal the wound. At the same time, the number of label retaining cells (LRC) increased by nearly 7-fold in these mice during the healing phase, providing evidence that the injured murine lacrimal gland contains LRCs that are recruited to salvage the damaged gland. These cells could be harvested and cultured under *in-vitro* conditions, unlike the uninjured gland, which showed minimum growth *in-vitro*. In contrast, the study by Shatos *et.al.* on rat lacrimal gland also showed stem-like cells to be present in the native, uninjured gland which could be maintained under appropriate *in-vitro* conditions(3).

We have earlier reported the establishment of human lacrimal gland cultures under *in-vitro* adherent conditions with the potential to secrete quantifiable levels of major secretory tear proteins(4).The study also provided preliminary evidence of

formation of spheres and duct-like structures in adherent cultures and expression of stem cell markers ABCG2 and CD117 in some cells of the native tissue and *in-vitro* cultures. Encouraged with this progress and that observed in other exocrine tissues like breast, prostate and salivary gland, we explored the potential of the human lacrimal gland to form 3D lacrispheres. In the present study, we attempted to establish cultures of human lacrispheres and evaluate the stem cell component by immunophenotyping, clonal assays, label retaining studies, cell cycle analysis and quantification by flow cytometry. We also aimed to evaluate the secretory efficiency of the lacrispheres by estimating the secreted tear proteins in the conditioned media. We believe that the results of this study would be helpful in choosing appropriate and suitable cells for potential clinical transplantation in patients with chronic ADDE.

Materials and Methods

Chemicals: Hank's balanced salt solution (HBSS), HepatoSTIM (BD Biosciences), N2 supplement (Invitrogen), epidermal growth factor (Sigma Aldrich), basic fibroblast growth factor (bFGF)(Sigma Aldrich), Antibiotic-antimycotic (Thermo Fisher Scientific) ultra-low attachment plates (Nunc), anti c-kit (Millipore, Temecula, CA; Dako, Glostrup, Denmark, Alexa Fluor 488 (Invitrogen, Carlsbad, CA, USA), Phycoerytherin (eBioscience), polymer horse radish peroxidase (HRP), DAB substrate, agarose (Sigma Aldrich, St Louis, MO, USA), Propidium iodide.

Human Tissue Source

The study was conducted at the L V Prasad Eye Institute (LVPEI), Hyderabad and School of Medical Sciences, University of Hyderabad. The use of human tissue was approved by the Ethics Committee of the LV Prasad Eye Institute (ECR/ 468/Inst./AP 2013) and is in accordance with the tenets of the Declaration of Helsinki.

Human lacrimal gland tissues (n=5) harvested from orbital tissues of patients undergoing therapeutic exenteration at LV Prasad Eye Institute were included in the study after written informed consent. After histologic confirmation of normal, disease free lacrimal gland, the tissues were subjected to further processing. The fresh gland was collected in DMEM-Ham's F-12 media supplemented with antibiotics and transported to the lab where it was immediately taken for processing.

Establishing 3-D lacrispheres under serum free conditions

Isolation of cells from human lacrimal gland was done as published in our previous study(4).Briefly, fresh human lacrimal gland was washed with HBBS to remove red blood cells. The gland was chopped into small bits using a scalpel blade (#21). The tissue mince was then incubated with the enzyme cocktail of collagenase (130 units per ml) and hyaluronidase (300 units per ml) for 90 minutes at 37ºC with intermittent shaking. At the end of the incubation period, the suspension was filtered through a 75μm cell sieve and the cell pellet obtained by centrifugation at 1500 rpm for 20 min. The single cell suspension was seeded on ultra-low attachment plates in HepatoSTIM media supplemented with N2, 25ng/ml EGF, 10ng/ml bFGF, 2mM l-glutamine, 10μg/ml

insulin, and antibiotics. The cultures were allowed to proliferate with media change every third day. In contrast to the previous protocol, the media was devoid of serum to propagate the 3D spheres, which were subjected to further analysis. On day 14, the spheres were collected for phenotypic and functional characterization and compared with native lacrimal gland. Cryopreservation of the cultured spheres were carried out on day 14-16 using a cryoprotectant medium and stored at -196^{0}C. Revival efficiency of the spheres was calculated following cryopreservation.

Evaluating stem-like cells in lacrispheres

The lacrispheres of 14-18 days and normal human lacrimal gland were evaluated for the presence of stem-like cells by expression and immunolocalization studies (immunohistochemistry/immunocytochemistry and flow cytometry), quiescence studies (label retaining & cell cycle analysis) and colony forming assay.

CD117 expression and immunolocalization studies: Immunohistochemistry and immunocytochemistry: Expression of CD117 (c-kit) was evaluated in formalin-fixed paraffin-embedded sections of fresh human lacrimal gland by immunohistochemistry and in fixed D14- lacrispheres.

Thin 3 µm sections were taken on silane coated glass slides and used for immunostaining according to the previously published standard protocol of the laboratory (4).

Lacrispheres of Day 14 were collected and processed for immunocytochemistry. Briefly, the cells were fixed with 4% fresh paraformaldehyde (PFA) for 10 minutes, followed by blocking with 5% BSA for 2 hours at room temperature. The cells were then incubated with 1:100 dilution of primary antibody (CD117 and ABCG2) for 2h at room temperature followed by washes and incubation with 1:200 dilution of the secondary antibody (Phycoerythrin) for 45 min. The coverslips were mounted in 50% glycerol and the images acquired using Carl Zeiss Laser Scanning Microscope LSM 510.

Flow cytometry: The D14 lacrispheres were mechanically dissociated and processed for flow cytometry. Briefly, $1x10^6$ cells were fixed with 4% fresh PFA for 10 minutes, blocked with 5% BSA and incubated with 1:100 dilution of directly conjugated CD117-PE antibody for 1 hour at room temperature. After incubation, the pellet was washed thrice with PBS, resuspended in 500µl of FACS buffer and acquired on BD FACS ARIA™ Special Order System. Appropriate controls were used for the experiment. Gates were set to exclude dead cells and doublets from the analysis. A total of 20000 to 50000 events were acquired and the analysis was done using BD FACSDiva™ software.

Quiescence Studies:

BrdU Label retaining studies: (I) BrdU pulsing: On Day 7, the media of human lacrispheres cultures was discarded and replaced with fresh media containing 10µM BrdU reagent. This BrdU pulsing was done for 24 hrs after which the media was replaced with fresh media without BrdU. In order to determine the number of

proliferating cells, the culture was processed for anti-BrdU staining immediately. To evaluate the number of cells that retain the label after a chase period of 14 days (i.e. quiescent cells), another set of similarly pulsed culture was allowed to grow for a further period of 14 days without BrdU and then processed similarly for anti-BrdU staining.

II) Immunostaining for anti-BrdU: Briefly, the cells were fixed with 70% ice-cold ethanol for 30 minutes at 4°C followed by washes with 1X PBS. DNA was denatured with 2N HCl (denaturing solution) for 30 mins at 37°C and the conditions neutralized immediately with 0.2M borate buffer (neutralizing solution) for 10 minutes at room temperature. The cells were then blocked with 10% serum for 60 minutes at room temperature followed by incubation with 1:50 dilution of anti-BrdU antibody for 60 minutes at room temperature. Secondary antibody (FITC or PE) at 1:200 dilution was used against the primary BrdU antibody. Lastly the cells were washed, mounted in 50% glycerol and images acquired using Carl Zeiss Laser Scanning Microscope LSM 510.

Cell Cycle Analysis: Cell cycle analysis was done to determine the fraction of cells present in various phases of cell cycle. The cells were also co-stained with CD117 (c-kit) to determine the co-localization profile. The 14-16 DIV cells were fixed with 4% PFA and incubated for 1 hour with anti-human CD117-PE. At the end of the incubation period the cells were washed thrice with 1X PBS and processed for cell cycle analysis by permeabilization with 70% ice-cold ethanol followed by incubation with 50 µg/ml Propidium iodide on ice for 30 min. The cells were then treated with 0.25 mg/ml RNase A for 45mins at 37^0C to remove double stranded RNA. Cells were analyzed by flow cytometry at an excitation wavelength of 488nm.

Colony forming efficacy: The lacrispheres were grown in agarose as single cells to assess their colony forming potential. The spheres from Day 14 growing under serum free conditions were collected and mechanically dissociated. A cell pellet was obtained after centrifugation. The pellet was resuspended in 1 ml of DMEM medium with 10% FCS and 2mM l-glutamine. Two percent agarose was coated onto a well of six-well plate as a base coat, and further overlaid with cell suspension (1,000 cells in 1% agarose). Plates were incubated in humidified tissue culture incubator at 37°C, 5% CO_2, for two weeks. Visible colonies with cell number >50 were counted under a phase-contrast microscope and the CFE was determined.

Secretory potential evaluation

The day 14 lacrispheres were evaluated by reverse transcriptase polymerase chain reaction (RT-PCR) to determine the mRNA expression of water channel aquaporin 5, mucin and tear protein lysozyme in comparison to the native gland and adherent monolayer culture. Total RNA was extracted from the freshly isolated tissue as well as lacrispheres using the TRIzol reagent according to the manufacturer's instructions. The quality of the RNA isolated was checked by visualization on agarose gel and by Nanodrop spectrophotometry. 2μg of RNA was used for cDNA synthesis per 25μl of the reaction volume using the Superscript™ First Strand Synthesis System for RT-PCR (Life Technologies, USA) according to the manufacturer's instructions. The primer sequences used for RT-PCR are summarized in Table 1.

Table 1: Primer Sequences

S. No.	Name	Sequence	Product Size (bp)
1.	Lysozyme - F	5'-CTCTCATTGTTCTGGGGC-3'	350
	Lysozyme - R	5'-ACGGACAACCCTCTTTGC5-3'	
2.	Mucin-5AC- F	5'-TCCACCATATACCGCCACAGA-3'	103
	Mucin-5AC- R	5'-TGGACGGACAGTCACTGTCAAC-3'	
3.	Aquaporin-5- F	5'-CCTGTCCATTGGCCTGTCTGTCAC-3'	249
	Aquaporin-5- R	5'-GGCTCATACGTGCCTTTGATGATG-3'	
4.	GAPDH - F	5'-CAGAACATCATCCCTGCATCCACT-3'	250

The potential of the lacrispheres to secrete tear proteins like scIgA, lactoferrin and lysozyme were detected in the culture supernatant of day 6–7 and day 14 cultures using sandwich ELISA kits according to the manufacturer's instructions. All the reagents used were supplied as a part of the kit. The optical density (OD) was measured at 450 nm on ELISA microplate reader (BioRadiMarkTM Microplate Reader). A standard curve was generated for each experiment performed.

Statistical Analysis

Values are expressed as mean of triplicate readings±SEM unless otherwise indicated. The statistical test used was two-way ANOVA with post-hoc Tukey test. Statistical

package SPSS Version 19 was used for analysis and graphs plotted using Microsoft excel. The results were statistically compared with fresh media (as a negative control) and also with each group and were considered as statistically significant if $p \leq 0.05$.

Image Analysis: Images were analyzed using the ImageJ software.

Results

Generating Lacrispheres from human lacrimal tissue

Under serum free conditions, lacrisphere could be generated from 4 out of 5 human lacrimal tissues processed for this study (Figure 1). They grew in size over a period of time in culture from 60.38µm on day 7 to about 200µm by day 14-16. These lacrispheres were passaged for 3-4 passages by mechanical dissociation to generate secondary spheres and could be maintained in culture for more than 30-35 days. These spheres could be cryopreserved and revived with a revival efficiency of about 50-60%.

Figure 1: Lacrispheres from human lacrimal gland. *The figure documents the increase in size of lacrispheres from 60.38µm on day 7 to about 200µm by day 14-16 in-vitro.*

CD117 expression and immunolocalization studies: CD117 expression was seen in the day 14 cultured lacrispheres. These CD117 positive cells did not seem to have any specific location within the sphere- they were seen both at the center and the peripheral region of the lacrisphere (Figure 2 Panel B).

In the normal human lacrimal gland, CD117 localization was noted within the cell membrane of acinar (44%) and a few ductal cells (10%) (Figure 2 Panel A). These percentages were calculated using ImageJ software. Staining also appears to cluster near the branching point of the ducts. The staining was intercellular and basal in a few cells while the others showed uniform staining all over the cell membrane. None of the myoepithelial cells (which envelope the acinar and ductal cells) showed CD117 positivity.

Flow cytometric evaluation of freshly isolated human lacrimal gland cells and day 14 lacrisphere cultures showed the expression of CD117 in about 6.7 ± 2.0% of the cells at t=0 (4)and in 0.8% of the lacrispheres (Figure 2 Panel C).

Figure 2: Expression of stem cell marker CD117. A) Normal human lacrimal gland: CD117 localization was seen in the cell membrane of some acinar and ductal cells (arrow). **B) Cultured human lacrispheres:** CD117 expression is seen in some cells of the Lacrisphere. Fluorescent tag is PE and DAPI is the nuclear counter stain. Magnification 10X. **C) Flow cytometric evaluation of CD117 expression in lacrispheres at 14DIV:** Data shows 0.8±0.05 % of the cells to be positive for CD117 (P3 gate)

Quiescence studies:

BrdU Label retention: When the pulsed lacrispheres were immediately fixed and immunostained with anti-BrdU antibody post-treatment, a majority of cells (51.6±3.8%)

in the sphere stained positive for BrdU indicating presence of proliferative cells. The number of LRC reduced to 9.31±0.41%(Figure3) after a chase period of 14 days.

Figure 3: BrdU label retaining studies: *One day post pulse 51.6±3.8% of cells in the lacrisphere stained positive for BrdU. After a chase period of 14 days 9.3±0.41% cells in the lacrispheres retained the BrdU label.*

Cell Cycle Analysis: Cell cycle analysis was done on cells at t=0 (immediately after isolation) as well as t=14 (DIV 14) lacrispheres. At t=0, 79.9% of the cells of the native human lacrimal glad were in G0/G1 phase and 18.9% in the G2/S/M phase (Figure 4).

In the t=14 lacrispheres, 76.9% of the cells were in the G0/G1 phase and 22.9% in the G2/S/M phase. Of these 76.9% cells in the G0/G1 phase about 0.3% were positive for stem cell marker CD117 (Figure 4).

Figure 4: Cell cycle analysis A) Fresh human lacrimal tissue: *Immediately at isolation, 79.9%of the cells were in G0/G1 phase and 18.9% in the G2/S/M phase*
B) Lacrispheres at t=14:*At day 14 76.9% of the cells were in the G0/G1 phase and 22.9% in the G2/S/M phase*

Colony forming efficiency of human lacrispheres

Cells, growing as lacrispheres, were plated on agarose and their colony forming efficiency evaluated. At the end of 14 days the number of colonies (Figure 5) was

manually counted under phase contrast microscope. From the 1000 cells of the lacrisphere initially plated, an average of 31 colonies was generated, with a colony forming unit (CFU) efficiency of 3.1%.

CFU = (No of colonies/ No. of cells plated)*100 = (31/1000)*100=3.1%

Figure 5: Colony formation assay on agarose: From the 1000 cells of the lacrisphere initially plated on agarose, an average of 31 colonies was generated, with a colony forming unit (CFU) efficiency of 3.1%.

Secretory potential of human lacrispheres

The secretory function of the lacrispheres was evaluated by assessing the expression of water channel aquaporin 5, mucin and tear protein lysozyme in day 7 lacrispheres as well as by detecting and quantifying the levels of scIgA, lactoferrin and lysozyme secreted by the day 7 and day 14 lacrispheres in the culture media by sandwich ELISA.

The RT-PCR study showed the expression of mRNA for water channel aquaporin 5, mucin and tear protein lysozyme in day 7 lacrispheres (Figure 6).

Figure 6:A)Secretory potential: RT-PCR analysis for GAPDH, AQP5, MUC5AC and LYZ *in native (N) lacimal gland,day 14 in-vitro adherent cultures (M) and lacrispheres (L).*

The ELISA results show that D7 lacrispheres secrete 135.28±2.9ng/ml of scIgA, 35.29±0.46 ng/ml of lactoferrin and 411.18±36.08 ng/ml of lysozyme. The day 14 lacrispheres secrete 153.69 ng/ml of IgA, 33.60±0.80 ng/ml of lactoferrin and 551.69±6.66ng/ml of lysozyme. (Table 2; Figure 7).

With increasing duration of culture, there was no significant increase in total protein or individual protein secretion (p=0.7988), however the quantity of lysozyme in both the D7 lacrispheres as well as D14 lacrispheres is much higher as compared to the other two tear proteins.

Figure 7: **Quantification of secreted proteins by sandwich ELISA.** The day 7 lacrispheres secrete 135.28±2.9ng/ml of scIgA, 35.29±0.46 ng/ml of lactoferrin and 411.18±36.08 ng/ml of lysozyme. The protein secretion is seen to increase in day 14 lacrispheres, which secrete 153.69 ng/ml of scIgA, 33.60±0.80 ng/ml of lactoferrin and 551.69±6.66ng/ml of lysozyme.

Table 2:Quantification of Protein Secreted by Lacrispheres

Samples	**ELISA VALUES (ng/ml** ± SEM**)**		
	IgA	**LTF**	**LYZ**
Lacrisphere D7	135.28±2.9	35.29±0.46	411.18±36.08
LacrisphereD14	153.69±6.4	33.60±0.80	551.69±6.66

Discussion

Dry eye syndrome is a chronically debilitating condition that affects about 11-22% of people worldwide with nearly one sixth of them developing aqueous deficient dry eye (5-7). The current therapeutic modality does little to arrest the progression of the disease and does not provide adequate long-term relief to the patients. The avenue of cell therapy remains a very lucrative option for the long-term management of chronic dry eye patients. The present work is an important step forward towards a cell therapy for this syndrome.

The results of the present study document the generation of 3D cultures of human lacrimal gland under serum-free non-adherent conditions. These free-floating spheres, which we term as "***lacrispheres***", appear to be more enriched in stem cells with higher CD117 expression, higher LRC as compared to previously reported adherent cultures, exhibiting greater degree of quiescence, increased clone forming ability with preserved secretory potential (as evident from the estimation of secreted proteins in the conditioned media). This study not only reiterates the previously established capacity of human lacrimal gland cells to grow in 2D cultures but also provides evidence for its capacity to grow in 3D cultures with retained secretory potential, which we believe, is a significant step towards exploring the avenue of cell therapy for chronic debilitating lacrimal gland disorders.

The various techniques for maintaining and enriching stemness *in-vitro* include use of feeder cells, simulating niche conditions using bioreactors or co-culture systems

or by maintaining stem-like cells as 3D spheres under serum-free conditions(8-10). The method that we explored in this study was stem cell enrichment by generating 3D spheres under serum free condition. In our study, by altering the EGF, bFGF concentration and serum withdrawal, lacrispheres could be generated *in-vitro*. These lacrispheres were capable of surviving for greater than 35 days in culture and generating secondary spheres for 3-4 passages. Schrader et al. had earlier attempted sphere generation from rabbit lacrimal gland, under microgravity conditions(11). Contrary to their evidence that spheres could only be maintained in culture for about 3 weeks after which they degenerate due to spreading central necrosis, our study provides evidence that the spheres survive and proliferate for greater than 35 days in-vitro.

The study of stem cells has been plagued with the fact that there is no single marker that can be counted upon as the true stem cell marker. In order to compensate for this shortcoming, a wider range of strategies, like evaluating the expression of development and pluripotency markers, functional assays like clone formation, sorting studies and gene expression signature profiling are usually employed. In the present study, the presence of stem like cells was explored by evaluating the expression of stem cell marker (CD117), quiescence studies (BrdU label retention & cell cycle analysis), and evaluation of clonogenic and differentiation potential.

CD117 has been shown to identify stem-like cells in exocrine tissues like salivary gland, breast, prostate (8, 9, 12). The possible presence of CD117 positive cells in the normal human lacrimal gland was explored by immunohistochemistry and flow cytometry.

CD117 localization studies show a basal and baso-lateral staining pattern on cells of acinar (44%) and ductal (9.8%) compartment. This observation contrasts what has been reported in the salivary gland and breast wherein, CD117 positive stem-like cells have been shown to reside in the terminal ductioles of the gland(9). While CD117 staining has not been previously attempted in normal lacrimal gland, You et al., had attempted to study the stem cell compartment in the murine lacrimal gland using BrdU label retaining studies. They reported BrdU label retaining cells to localize in the murine acinar (58.2±3.6%), myoepithelial (26.4±4.1%), ductal (0.4±0.4%) and stromal (15.0±3%) compartments (2). Their findings support our observation of presence of stem-like cells in both the acinar and ductal compartments. In our study however we did not find evidence for presence of stem cell markers in the myoepithelial cells of normal human lacrimal gland.

Flow cytometry data of freshly isolated cells from the human lacrimal gland, published previously by our group(4), showed that 3.1±0.61% of the cells were $ABCG2^{+}$ and 6.7±2.0% were $CD117^{+.}$ The adherent cultured cells also showed the presence of stem cells. However, they tend to undergo differentiation and their percentages fall to 0.3±0.15% ABCG2 and 0.2±0.05% CD117 by day 14 in-vitro. These values further reduce to 0.2±0.13% ABCG2 and 0.13±0.03% CD117 respectively by DIV 21.Presence of stem cells as show by both qualitative and quantitative parameters along with retained secretory potential was a promising evidence in our previous study. However the observation of reduction in stem-like cells over a period of time, with a relative increase in proportion of differentiated secretory cells (as evidenced by the increase in

secretion of tear protein over a two week period) prompted us to consider that the limitation could be in our culture system which probably does not support the proliferation of both stem cells and differentiated cells beyond 2- 3 weeks. We hypothesized that optimizing conditions that would sustain both these compartments would enable us to mimic the lacrimal gland function *in-vitro*. The present study provides evidence in support of our hypothesis. The number of stem cells (CD117 positive cells) showed a four-fold increase in lacrisphere cultures as compared to adherent cultures (0.8% vs0.2% cells in D14 cultures). It is important to note that these findings are also comparable to the salispheres from salivary glands, which have shown to have enriched stem cells ($CD117^{+}$cells) (13).

We have also investigated the presence of quiescent cells in culture by BrdU label retention studies and cell cycle analysis. Our results indicate that over a chase period of 14 days, 9.3±0.4%of cells in the lacrispheres retain BrdU label. These are known as label retaining cells and are presumed to be potential stem-like cells. The other cells in culture lose the BrdU label indicating their proliferative nature. In the cell cycle analysis, we aimed at looking at the fraction of cells that would be in resting phase (G0/G1). In principle, this population would include quiescent cells (G0) as well as cells before entering into cell cycle (G1). In this study, we considered them together for logistic reasons. Cell cycle analysis showed that in the normal human lacrimal gland 79.9% of the cells are in G0/G1 or quiescent phase and 18.9% in the G2/S/M or active cell cycle phase. When huLG cells are cultured *in-vitro* as adherent monolayer, a higher fraction of the cells (33.9%) become active while the percentage of G0/G1 quiescent cells falls

to 66.9%. However, when maintained under serum-free conditions as lacrispheres, an increased *in-vivo* mimicry was seen with about 76.9% of the cells being in the G0/ G1 phase and 22.9% in the G2/S/M phase. Of the 76.9% of the cells in the quiescent phase, about 0.3% are CD117 positive.

Whether the lacrispheres have the potential to self-renew was investigated by evaluating their colony forming efficiency, from single cells, on agarose. The results of the assay showed that the cells of lacrisphere have clonogenic potential as they give rise to colonies on agarose with a colony forming efficiency of 3.1%; thereby indicating their potential to self-renew.

Another promising finding in this study was documentation of secretory products in the conditioned media. The presence of mRNA for aquaporin 5, mucin and lysozyme indicate that cells in the lacrisphere have the potential to be functionally competent. When the supernatants of day 7 and day 14 lacrisphere cultures were evaluated by sandwich ELISA, the results showed the presence of 135.28±2.92 ng/ml of scIgA, 35.29±0.46ng/ml of lactoferrin and 411±36.08ng/ml of lysozyme in day 7 cultures and 153.69 ±6.4 ng/ml of scIgA, 33.60±0.79ng/ml of lactoferrin and 551.69±6.66ng/ml of lysozyme in day 14 cultures. The increase in individual protein secretion was not found to be statistically significant (p=0.7988). It was observed that the level of lysozyme in both the D7 as well as D14 lacrispheres is much higher (411.18±36.08 ng/ml and 551.69±6.66ng/ml in D7 and D14 lacrispheres respectively) as compared to the other two tear proteins (IgA and lactoferrin). It is beyond the scope of this study to elaborate further on this; however these results provides an opportunity to extrapolate that the

secretion of various components could vary with different periods of time and culture conditions. This study provides the dual advantage of 3D lacrispheres that not only retain secretory function but are also enriched in stem cells. However, further *in-vivo* studies in appropriate models are warranted to investigate the regenerative potential of these lacrispheres to create a network of acinar and ductal structures that responds to external stimuli such as neurotransmitters and androgens.

The present study is not without its set of limitations. Though an attempt was made to determine the histoarchitecture of the lacrispheres by cryosectioning and electron microscopy, due to the fragile nature of the spheres it could not be executed. Another limitation of the study is an assumption of quiescence based on the cells in both G0/G1 phase (79.9% at t=0, 66.9% of adherent cultures and 76.9% of lacrispheres), which should ideally be restricted to G0 component. Hence the results are indicative of not just quiescent cells but also those preparing to re-enter the cell cycle leading to mitosis.

Conclusion: The present study provides a very promising, first of its kind evidence for the generation of 3D floating lacrispheres from native human lacrimal gland, which contain enriched population of stem cells as well as differentiated, secretory- competent cells. We strongly believe that further validation of the regenerating potential of these lacrispheres in an appropriate preclinical model would take us one step closer to potential clinical application.

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Conflict of Interest: None

References

1. The epidemiology of dry eye disease: report of the Epidemiology Subcommittee of the International Dry Eye WorkShop (2007). Ocul Surf. 2007;5(2):93-107.

2. You S, Kublin CL, Avidan O, Miyasaki D, Zoukhri D. Isolation and propagation of mesenchymal stem cells from the lacrimal gland. Invest Ophthalmol Vis Sci. 2011;52(5):2087-94.

3. Shatos MA, Haugaard-Kedstrom L, Hodges RR, Dartt DA. Isolation and characterization of progenitor cells in uninjured, adult rat lacrimal gland. Invest Ophthalmol Vis Sci. 2012;53(6):2749-59.

4. Tiwari S, Ali MJ, Balla MM, Naik MN, Honavar SG, Reddy VA, et al. Establishing human lacrimal gland cultures with secretory function. PLoS One. 2012;7(1):e29458.

5. Abelson MB, Ousler GW, 3rd, Maffei C. Dry eye in 2008. Curr Opin Ophthalmol. 2009;20(4):282-6.

6. Lemp MA. Report of the National Eye Institute/Industry workshop on Clinical Trials in Dry Eyes. CLAO J. 1995;21(4):221-32.

7. Lemp MA, Crews LA, Bron AJ, Foulks GN, Sullivan BD. Distribution of aqueous-deficient and evaporative dry eye in a clinic-based patient cohort: a retrospective study. Cornea. 2012;31(5):472-8.

8. Leong KG, Wang BE, Johnson L, Gao WQ. Generation of a prostate from a single adult stem cell. Nature. 2008;456(7223):804-8.

9. Lombaert IM, Brunsting JF, Wierenga PK, Faber H, Stokman MA, Kok T, et al. Rescue of salivary gland function after stem cell transplantation in irradiated glands. PLoS One. 2008;3(4):e2063.

10. Woodward WA, Chen MS, Behbod F, Rosen JM. On mammary stem cells. J Cell Sci. 2005;118(Pt 16):3585-94.

11. Schrader S, Kremling C, Klinger M, Laqua H, Geerling G. Cultivation of lacrimal gland acinar cells in a microgravity environment. Br J Ophthalmol. 2009;93(8):1121-5.

12. Kondi-Pafiti A, Arkadopoulos N, Gennatas C, Michalaki V, Frangou-Plegmenou M, Chatzipantelis P. Expression of c-kit in common benign and malignant breast lesions. Tumori. 2010;96(6):978-84.

13. Nanduri LS, Lombaert IM, van der Zwaag M, Faber H, Brunsting JF, van Os RP, et al. Salisphere derived c-Kit+ cell transplantation restores tissue homeostasis in irradiated salivary gland. Radiother Oncol. 2013;108(3):458-63.

Figure Legends

Figure 1: Lacrispheres from human lacrimal gland. *The figure documents the increase in size of lacrispheres from 60.38µm on day 7 to about 200µm by day 14-16 in-vitro.*

Figure 2: Expression of stem cell marker CD117. A) Normal human lacrimal gland: CD117 localization was seen in the cell membrane of some acinar and ductal cells (arrow). **B) Cultured human lacrispheres:** CD117 expression is seen in some cells of the Lacrisphere. Fluorescent tag is PE and DAPI is the nuclear counter stain. Magnification 10X. **C) Flow cytometric evaluation of CD117 expression in lacrispheres at 14DIV:** Data shows 0.8±0.05 % of the cells to be positive for CD117 (P3 gate)

Figure 3: BrdU label retaining studies: *One day post pulse 51.6±3.8% of cells in the lacrisphere stained positive for BrdU. After a chase period of 14 days 9.3±0.41% cells in the lacrispheres retained the BrdU label.*

Figure 4: Cell cycle analysis A) Fresh human lacrimal tissue: *Immediately at isolation, 79.9%of the cells were in G0/G1 phase and 18.9% in the G2/S/M phase*

B) Lacrispheres at t=14:*At day 14 76.9% of the cells were in the G0/G1 phase and 22.9% in the G2/S/M phase*

Figure 5: Colony formation assay on agarose: From the 1000 cells of the lacrisphere initially plated on agarose, an average of 31 colonies was generated, with a colony forming unit (CFU) efficiency of 3.1%.

Figure 6:A)Secretory potential: RT-PCR analysis for GAPDH, AQP5, MUC5AC and LYZ *in native (N) lacimal gland,day 14 in-vitro adherent cultures (M) and lacrispheres (L).*

Figure 7: **Quantification of secreted proteins by sandwich ELISA.** The day 7 lacrispheres secrete 135.28±2.9ng/ml of scIgA, 35.29±0.46 ng/ml of lactoferrin and 411.18±36.08 ng/ml of lysozyme. The protein secretion is seen to increase in day 14 lacrispheres, which secrete 153.69 ng/ml of scIgA, 33.60±0.80 ng/ml of lactoferrin and 551.69±6.66ng/ml of lysozyme.

Table Legends

Table 1: Primer Sequences

Table 2: Quantification of Protein Secreted by Lacrispheres