Increased Mucin Expression in Oral Mucosal Epithelial Cells *in vitro*: A Potential New Role of Mycophenolate Mofetil

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Objective: Autologous cultured explants of human oral mucosal epithelial cells (OMEC) are a potential therapeutic modality in patients of bilateral ocular surface disease (OSD) with incapacitating dry eye. Mycophenolate mofetil (MMF) has been found to upregulate the mucin production in conjunctival goblet cells *in vitro*. The aim of this study was to evaluate the effects of MMF on mucin expression in primary cultures of OMEC.

Methods: With informed consent, oral mucosal epithelial tissue samples were obtained from patients undergoing oral surgery for non-malignant conditions. OMEC were cultured on human amniotic membrane (HAM) scaffold for 2 weeks. Mucin expression was quantified using RT-PCR and qPCR before and after treating cultured OMEC with MMF.

Results: Morphological studies revealed a confluent sheet of proliferating, stratified oral mucosal epithelial cells. Mucin mRNAs were elucidated by RT-PCR. Compared to untreated controls, MUC1, MUC15 and MUC16 mRNAs and MUC1 protein expression were found to be upregulated in MMF treated primary cultures of OMEC, as assessed by qPCR and immunocytochemistry respectively.

Conclusion: Our findings demonstrate that MMF can act as a novel enhancer of mucin production in OMEC *in vitro*. It has the potential to improve dry eye in patients undergoing OMEC transplantation for bilateral OSD.

Key words: Mucin, Mycophenolate mofetil, limbal stem cell deficiency

INTRODUCTION

Cornea is the avascular and transparent outermost part of the eyeball which facilitates clear vision by focusing light on the retina and protects the eyeball from external environment. Physiologically shed corneal epithelial cells are renewed by limbal stem cells. Apart from serving as a reservoir for corneal epithelial cells, limbal epithelium forms a barrier between corneal and conjunctival epithelium and prevents encroachment of conjunctival epithelium over cornea [1]. Injury or inflammation of the ocular surface in the form of burns, chemicals, Stevens Johnson syndrome or ocular cicatricial pemphigoid can lead to destruction and deficiency of limbal stem cells [2]. Limbal stem cell deficiency (LSCD) manifests in the form of severe ocular surface disease (OSD) characterized by persistent and recurrent epithelial defects, conjuntivalization of the corneal surface along with superficial neovascularization, scarring and ultimately opacity and loss of vision. Most of the cases of OSD are associated with severe dry eye pertaining to diminished mucin and aqueous secretion [3].

Limbal tissue transplantation from the contralateral normal eye, live related donors or cadaveric donors has been conventionally practiced for management of LSCD. *Ex vivo* expansion and transplantation of limbal stem cells has also been in use [4]. Major drawbacks of these practices include risk of iatrogenic LSCD in the donor eye [4–6] and complications associated with graft rejection and prolonged immunosuppressant drug usage in case of allografts [7]. Moreover, maintaining an autologous source for stem cells is a challenge in bilaterally affected LSCD patients. For this purpose, oral mucosal epithelial stem cells have been used in some studies where, a small buccal mucosal biopsy is taken and cultivated *ex vivo* on a substrate, known as cultured oral mucosal epithelial transplantation (COMET). Post 2–3 weeks of culture, the multi-layered confluent sheet formed, is transplanted in the affected eye [8–11]. Molecular characterization of OMEC has been done and it shows expression of cytokeratin K3 but not cytokeratin K12 which indicates oral mucosal epithelial cells (OMEC) have the ability to form a corneal epithelium like sheet but they do not transdifferentiate into corneal epithelium [9, 11–14]. COMET overcomes the prior mentioned complications of graft rejection and iatrogenic LSCD [12,13].

Long term follow-up studies of patients who had undergone COMET reported significant improvement

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in vision and stabilization of ocular surface. Most of these patients suffered from dry eye conditions, which was noted to be a major setback of COMET [15].

Mucins play important role in preventing dry eye as they are heavily glycosylated proteins with a tendency to attract and hold water molecules. These proteins keep the ocular surface wet and remove pathogens, particulate matter and debris, thereby protecting it from environmental insults [16]. Mucins have been shown to be expressed in both the ocular surface as well as oral mucosal epithelial cells (OMEC) [11,17]. He *et al*. in 2010 [18] reported that Mycophenolate mofetil (MMF) could potentially upregulate mucin expression in human conjunctival goblet cells *in vitro*. MMF, a pro-drug, is used as an immunosuppressant in the management of graft rejection and autoimmune diseases and has been proven to be safe and effective with improved tolerability and side effect profile [19–22]. The active ingredient of MMF, mycophenolic acid, acts by inhibiting inosine monophosphate dehydrogenase (IMPDH) in the de novo pathway of purine synthesis [23].

The purpose of this study was to check if MMF treatment of *ex vivo* cultured OMEC could increase mucin expression. This could suggest a role of MMF in management of patients with LSCD having dry eye after COMET.

MATERIALS AND METHODS

Collection and preparation of Human amniotic membrane (HAM)

HAM has anti-inflammatory, anti-scarring, non-immunogenic and remarkable cell adhesive properties, which make it a suitable scaffold for expansion of epithelial cells [24–26]. HAM was obtained from sero-negative donors (HIV, HBV, HCV) undergoing elective Caesarean section for obstetric indications in the Department of Obstetrics & Gynecology, AIIMS, New Delhi, after taking Institutional Ethical Committee (IEC) approval and written informed consent in accordance with the principles outlined in the Declaration of Helsinki. HAM was processed and cryopreserved in sterile vials containing DMEM and glycerol (1:1) at -70°C after cutting into small pieces (4cm x 4cm) as described by Sen *et al*., 2011 [11]. Cryopreserved HAM was thawed before using it by keeping the vials at 37°C for 30 minutes. The epithelial lining of HAM was denuded by treating it with 0.25% Trypsin-EDTA for 30 minutes at 37°C.

Oral mucosal epithelial tissue collection

Thirty oral mucosal tissue samples were obtained from patients undergoing oral reconstructive surgery for non-malignant conditions in the Department of Oral & Maxillofacial Surgery, Centre for Dental Education & Research, AIIMS, New Delhi; after taking Institutional Ethical Committee (IEC) approval and written informed consent in accordance with the principles outlined in the Declaration of Helsinki. Patients who were long term tobacco users and/or with malignant lesions of oral cavity were excluded from the study. After appropriate antisepsis and pre-operative assessment by an oral surgeon, 4mm x 4mm mucosal biopsies were obtained under local anesthesia from the inner side of cheek of donors. Specimens were transported to the culture laboratory in sterile vials containing DMEM with antibiotics.

Explant culture of oral mucosal epithelial cells

The biopsy specimens were washed thoroughly with normal saline containing antibiotics. Mucosal layer was mechanically sliced off the adjoining connective tissue by a sterile surgical blade and cut into small $1mm^2$ pieces (explants). Mucosal explants were transferred to de-epithelialized HAM kept in sterile 35mm petri plate. Petri plates containing HAM and mucosal explants were transferred to a CO_2 incubator (95% air & 5% CO_2) at 37°C. Explants were allowed to adhere to HAM surface for 1 hour and growth media containing DMEM & Ham's F12 in ratio 1:1 with FBS (10%v/v), EGF (10ng/ml), insulin (5µg/ml) and antibiotics were added after that. Samples were divided into four parts and grown in duplicates to ensure availability of proper controls. Growth media was changed every alternate day and OMEC growth on HAM surface was monitored under a phase contrast inverted microscope.

MTT assay

In order to determine the toxic potential and appropriate *in vitro* dosage of MMF, MTT assay was performed on OMEC as described previously [27]. Briefly, the cells were grown in a 96 well microtiter plate and treated with increasing concentrations of MMF (Sigma Aldrich, USA) in triplicates. Twenty-four hours later growth media containing MMF was removed and 100µl of MTT solution (5mg/ml) was added to each well. At the end of incubation period of 4 hours at 37°C, dark blue colored formazan crystals were formed which were dissolved in DMSO followed by measurement of absorbance at 570nm. Appropriate vehicle controls were used. The experiment was repeated three times. Based on the results of MTT assay, maximum tolerable non-toxic dosage of MMF was used for treating OMEC cultures.

MMF treatment of cultivated OMEC

After allowing OMEC to grow in duplicates for two weeks, cells were treated with 100ng/ml dose of MMF for 24 hours duration, keeping an untreated control of the same biological sample. DMSO (Duchefa, Netherland) was used as a diluent for MMF.

RT-PCR and qPCR

After 24 hours of MMF treatment, treated and corresponding untreated OMEC were mechanically detached from the HAM scaffold using sterile cell scrapers. Total RNA was extracted from these cells using One Step RNA reagent (Bio-Basic, Canada) according to manufacturer's protocol. Quality and yield of extracted RNA was quantified by NanoDrop spectrophotometry and RT-PCR was set up to synthesize cDNA with 1µg total RNA using MMLV-RT (Thermo Scientific, USA) and anchored oligodT primer. PCR was performed with cDNA of MMF treated and corresponding untreated samples using gene specific primers (IDT, USA, as shown in Table 1). PCR products were electrophoresed on 1.5% agarose gel and analyzed on gel documentation system (DS2400, Alpha Innotech, USA). Based on the results of PCR, qPCR was performed on Bio-rad CFX96 Touch™ Real-Time PCR Detection System

Table 1 Primers used for RT-PCR and qPCR

Serial Number	Gene	Primer sequence	Product Size (bp)
1.	Beta Actin	F 5' ACTGGAACGGTGAAGGTGAC 3' R 5' AGAGAAGTGGGGTGGCTTTT 3'	169
2.	MUC1	F 5' AGACGTCAGCGTGAGTGATG 3' R 5' CAGCTGCCCGTAGTTCTTTC 3'	172
3.	MUC5B	F 5' CACCTCCTTCAACACCACCT 3' R 5' GAACGTAGCTGCAGTCACCA 3'	161
4.	MUC6	F 5' AGCGAAGCCATCATCTCAGT 3' R 5' CTCCACCAGAACCATGAGGT 3'	171
5.	MUC13	F 5' TAAACACAGCCACCAACCAA 3' R 5' GGGAGCAGGTGAAGTAGCTG 3'	167
6.	MUC15	F 5' GTGCCTTGGAATGCACCTAT 3' R 5' TGTTTGTGGTAAGCCATCCA 3'	226
7.	MUC16	F 5' AGCATCCTGGACGTAACCAC 3' R 5' CAGGTGGAAGGGTGTTCTGT 3'	173

F–forward
R–reverse

using DyNAmo Flash SYBR Green qPCR kit (Thermo Scientific, USA) and gene specific primers (Table 1). Beta actin was used as an internal reference gene for normalization. Relative fold change in gene expression was calculated using $2^{-\Delta\Delta CT}$ method.

Immunocytochemistry

Based on the results of RT-PCR and qPCR, immunocytochemistry was done to validate protein expression in two representative samples. Cultured OMEC were fixed on APES (Aminopropyltriethoxysilane) coated glass slides using acetic acid and methanol (1:3) as fixative and stored at 4°C. At the time of processing, slides were taken out of 4°C and incubated at room temperature for one hour before washing them with 0.1M PBS (pH = 7.4). Endogenous peroxidases were blocked with 4% hydrogen peroxide in PBS and non-specific binding was blocked using protein blocking solution provided with CRF Anti-Polyvalent HRP Polymer (DAB) Immunocytochemistry kit (ScyTek Laboratories, USA). The slides were then incubated with MUC1 primary antibody (Santa Cruz Biotechnology, USA) in 1:100 dilution in PBS at 4°C for 12–16 hours. Immunodetection was achieved by avidin-biotin horse radish peroxidase based colorimetric method according to manufacturer's protocol with 3,3'-diaminobenzidine tetrahydrochloride (DAB) as a chromogen and H_2O_2 as the substrate, followed by light counterstaining with hematoxylin and examination under a microscope.

Statistical analysis

All data are expressed as mean ± standard error of mean (SEM). Statistical comparisons of qPCR results were made using Wilcoxon signed rank test (Graph Pad Prism, version 5). A p- value of less than 0.05 was considered statistically significant.

RESULTS

Morphology of cultivated OMEC

Initially, within 3 to 4 days after setting up the explant culture, small, oval shaped OMEC started growing and migrating from the edges of the explant. After one to two weeks, a multi-layered confluent sheet of OMEC was formed on the surface of HAM. A heterogeneous population of cells was observed in which some cells were differentiated into large and irregularly shaped polygonal epithelial cells (Fig. 1A–1D). Out of 30 samples collected, a total of 15 primary cultures could be established successfully. There was no appreciable difference in the morphology of MMF treated and untreated control OMEC cultures.

MTT assay and MMF dosage determination

On treating OMEC with MMF doses ranging from 5ng/ml to 100ng/ml for 24 hours, the cell viability was found to be in the range of 86.38% to 93.83% and was comparable to DMSO vehicle control (96.09%, Fig. 2). The maximum tolerated non-toxic drug dose of 100ng/ml was fixed for treating confluent cultures of OMEC. The duration of treatment was finalized to be 24 hours.

Effect of MMF treatment on mucin expression

MUC1, MUC15 and MUC16 genes were found to be expressed in OMEC by RT-PCR. Gene expression levels of MUC1, MUC15 and MUC16 were observed to be higher in MMF treated OMEC compared to untreated controls as per gel documentation analysis of PCR products after gel electrophoresis (Fig. 3A–3D). qPCR data confirmed the PCR results as the expression of MUC1 gene was increased by 2.69 folds (p value = 0.03), MUC15 gene by 2.71 folds (p value = 0.05) and MUC16 gene by 2.29 folds (p value = 0.20) in MMF treated OMEC compared to untreated controls after normalization with beta actin (Fig. 4). MUC5B, MUC6 and MUC13 were not found to be expressed uniformly in all the samples. MUC1 protein expression was found to be higher in OMEC treated with MMF as compared to untreated controls which further validates the results of PCR and qPCR experiments (Fig. 5A–5B).

DISCUSSION

Mucins are high molecular weight glycoproteins which attract and hold water molecules due to their hydrophilic nature, making them indispensable for the protection of epithelial surfaces including cornea. Mucins provide protection from pathogens, particulate

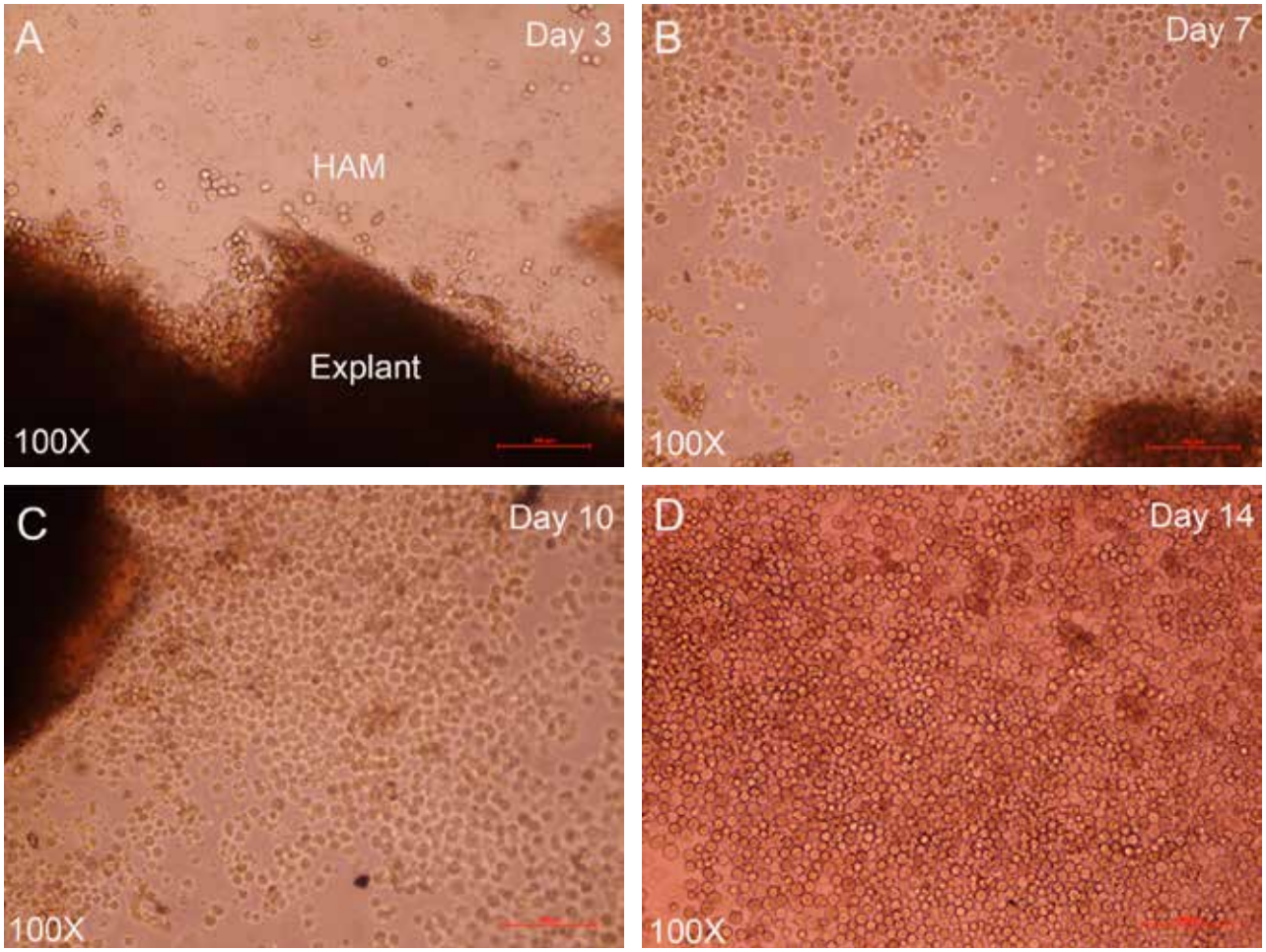

Fig. 1 Morphology of primary cultures of OMEC grown on surface of human amniotic membrane (HAM) scaffold. **A**: Initiation of growth of OMEC from the explant edge on day 3 of setting up the culture. **B**: OMEC growing outwards from the explant edge as shown on day 7. **C**: Continuous growth of OMEC as shown on day 10. **D**: Formation of a multilayered confluent sheet of OMEC as shown on day 14 after setting up the culture. Magnification, X100 (scale bar 100 μm).

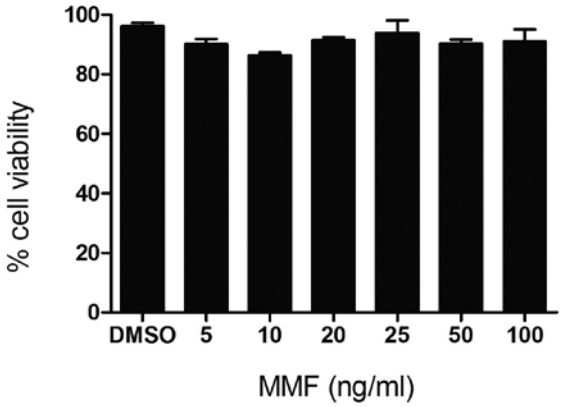

Fig. 2 MTT assay to estimate cytotoxic potential of MMF. Percent cell viability was calculated using MTT assay on OMEC treated with increasing doses of mycophenolate mofetil (MMF) and vehicle control (DMSO). The bar graph represents mean ± standard error of mean (SEM) of three experiments (n = 3).

matter and other environmental toxins by flushing them off from epithelial surfaces. They play an important part in keeping the corneal epithelial surface wet and decreased mucin production from corneal epithelium can lead to dry eye syndromes. Limbal stem cell deficiency (LSCD) caused by destruction of limbal stem cells results in failure to replenish physiologically shed corneal epithelial cells which ultimately manifests in the form of ocular surface disease (OSD) characterized by recurrent epithelial defects, conjunctivalization and neovascularization of cornea, decreased mucin production and severe dry eye symptoms [3]. Limbal stem cell transplantation (LSCT) and *ex vivo* cultured limbal epithelial transplantation (CLET) provide an alternative source of corneal epithelial cells renewal but are associated with risk of inflicting iatrogenic LSCD in donor eye in addition to complications of graft rejection and prolonged immunosuppressant use in case of allografts. Cultivated oral mucosal epithelial transplantation (COMET) gained remarkable success especially in bilateral LSCD as it is free from complications associated with LSCT and CLET. Long term

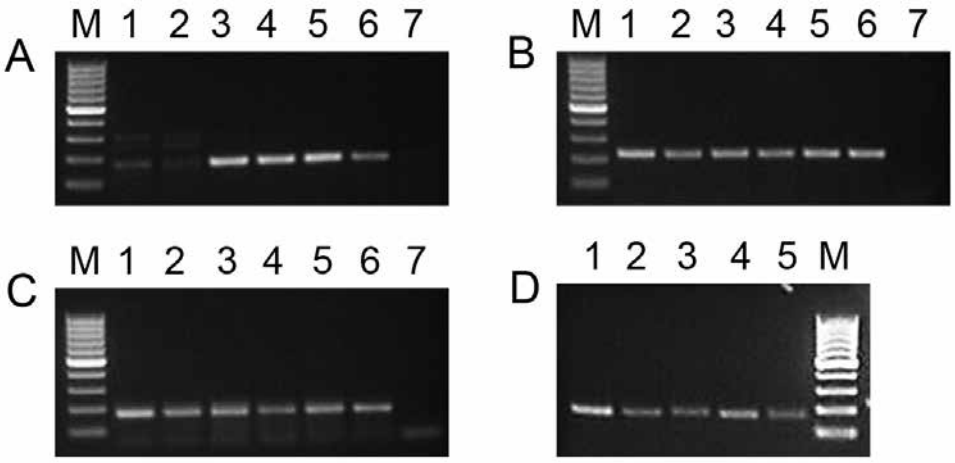

Fig. 3 RT-PCR analyses of different genes in representative samples of untreated and treated (with 100ng/ml of MMF for 24 hours) OMEC isolated after 2 weeks culture on HAM. **A**: MUC1 mRNA expression; M, marker (100bp ladder); 1, sample 1 MMF treated; 2, sample 1 untreated control; 3, sample 2 MMF treated; 4, sample 2 untreated control; 5, sample 3 MMF treated; 6, sample 3 untreated control; 7, NTC (No template control). **B**: MUC15 mRNA expression; M, marker (100bp ladder); 1, sample 1 MMF treated; 2, sample 1 untreated control; 3, sample 2 MMF treated; 4, sample 2 untreated control; 5, sample 3 MMF treated; 6, sample 3 untreated control; 7, NTC. **C**: MUC16 mRNA expression; M, marker (100bp ladder); 1, sample 1 MMF treated; 2, sample 1 untreated control; 3, sample 2 MMF treated; 4, sample 2 untreated control; 5, sample 3 MMF treated; 6, sample 3 untreated control; 7, NTC. **D**: Beta actin (housekeeping gene) mRNA expression; 1, sample 1 MMF treated; 2, sample 1 untreated control; 3, sample 2 MMF treated; 4, sample 2 untreated control; 5, sample 3 MMF treated; M, marker (100bp ladder).

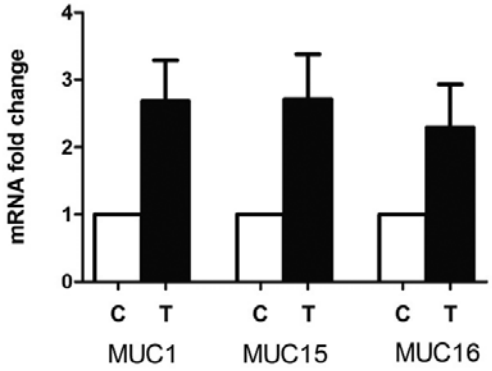

Fig. 4 Relative fold mRNA expression of MUC1, MUC15 and MUC16 genes in OMEC isolated after 2 weeks culture on HAM and treated with 100ng/ml of MMF for 24 hours as determined by qPCR and normalized to beta actin. MUC1 mRNA expression: mean is 2.69 folds compared to untreated control (p value = 0.03, n = 12); MUC15 mRNA expression: mean is 2.71 folds (p value = 0.05, n = 9); MUC16 mRNA expression: mean is 2.29 folds (p value = 0.20, n = 9). The bar graph represents mean ± standard error of mean (SEM). C, control; T, treated.

follow-up studies conducted on patients undergone COMET, reported significantly improved vision and relief from other symptoms of OSD. However, these patients complained of variable degrees of dryness in the operated eyes [15].

In the present study we simulated an *in vitro* model of COMET by cultivating OMEC on de-epithelialized HAM scaffold as described previously [11]. OMEC formed a multi-layered confluent sheet at the end of about 2 weeks period and their growth pattern was in concordance with the previous studies [8–11]. For the first time in our study we have analyzed the effect of MMF on mucin expression in cultivated OMEC. A previous paper reporting MMF induced increase in MUC5AC expression in cultivated conjunctival goblet cells [18] prompted us to check the effects of MMF on mucin production in cultivated OMEC. MMF is widely used in clinical practice as an immunosuppressant and its cytostatic effect is highly selective for T and B lymphocytes as its active ingredient mycophenolic acid is five times more potent inhibitor of type II isoform of IMPDH expressed in T and B lymphocytes as compared to type I isoform expressed by other cells of body [23]. Despite MMF being used clinically, MTT assays were performed on OMEC to determine the maximum tolerable non-toxic *in vitro* dose of MMF for epithelial cells. Repeated MTT assays with different concentrations of MMF led us to define non-cytotoxic nature of MMF for epithelial cells and we used 100ng/ml concentration to treat primary cultures of OMEC for 24 hours.

To the best of our knowledge this is the first study that evaluates a quantitative change in mucin expression in OMEC after the cells are treated with MMF. Through our RT-PCR and qPCR experiments, three mucin genes, viz. MUC1, MUC15 and MUC16 were shown to be expressed by cultivated OMEC in this study, which partly corroborates with findings of Sen *et al.*, 2011, as MUC5B, MUC6 and MUC13 genes expression was negligible to nil in our sample cohort which can possibly be explained by the concept of biological variability. Quantification of mRNA expression of mucin genes by qPCR led us to conclude that MMF increases MUC1, MUC15 and MUC16 expression in cultivated OMEC. We are reporting for the first time that MMF has the property to induce mucin production by cultivated OMEC on HAM scaffold. To validate the increased mucin production at protein level, we performed immunocytochemistry analysis for MUC1 protein on representative samples

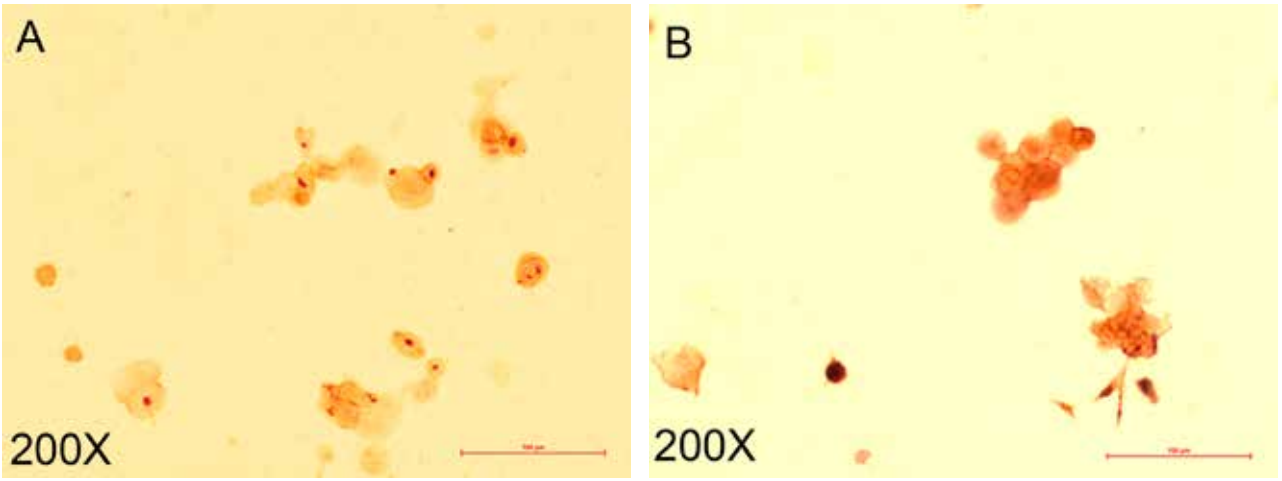

Fig. 5 MUC1 protein expression in representative OMEC samples, isolated after 2 weeks culture on HAM as assessed by immunocytochemistry. A, untreated and B, treated with 100ng/ml of MMF for 24 hours. Magnification, X200 (scale bar 100 μm).

of OMEC cultures before and after MMF treatment. Immunocytochemistry results also corroborated with our qPCR findings and MUC1 protein expression was observed to be higher in MMF treated OMEC when compared to untreated controls.

Our set of experiments and analyses proposes a novel role of MMF in induction of mucin production by cultivated OMEC. This finding has implications in improving the outcome of COMET as well as modifying the potential therapeutic protocols of dry eye management. Our study gives a novel idea that use of MMF in clinical settings along with OMEC transplantation can improve the dry eye symptoms in patients of LSCD and severe OSD. A well designed randomized control trial using MMF in patients undergoing COMET is required to prove the clinical efficacy of this drug in management of LSCD and dry eye syndromes.

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