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Levels of oxidative DNA damage are low in *ex vivo* engineered human limbal epithelial tissue

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ABSTRACT.
Purpose: **To examine levels of oxidative DNA base damage and expression of selected genes and proteins related to DNA damage repair in human limbal epithelium engineered *ex vivo*.**
Methods: **Cells were expanded from limbal tissue on cell culture-treated inserts in medium containing fetal bovine serum, recombinant growth factors, hormones and cholera toxin (COM) and in medium with human serum as the single growth-promoting additive (HS). Cells were analysed after two, three and four weeks in culture for DNA strand breaks and oxidized purine bases (Comet assay using the enzyme formamidopyrimidine DNA glycosylase, Fpg) and for expression of DNA repair enzymes APE1, OGG1 and Polβ by *in situ* hybridization (ISH) and by immunohistochemistry (IHC).**
Results: **Levels of strand breaks were substantial while levels of net Fpg-sensitive sites (8-oxoguanine and ring-opened FaPy bases) were relatively low in cells engineered in COM and in HS. Both types of medium were found to support expression of base excision repair (BER) enzymes APE1, OGG1 and Polβ at the gene level. At the protein level, expression of APE1 and OGG1 was noticeable in both conditions while expression of Polβ was low.**
Conclusion: **Our findings indicate low levels of oxidative stress and/or efficient DNA purine base damage repair in human limbal epithelium engineered in a medium with human serum as the single growth-promoting additive as well as in traditional medium with xenobiotics.**

Key words: cornea – DNA damage – epithelium – limbus – oxidative damage – tissue engineering

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Introduction

Continuous regeneration of corneal epithelium depends critically on an intact pool of limbal stem cells (Davanger & Evensen 1971; Cotsarelis et al. 1989; Tseng 1996; Dua et al. 2000; Bath et al. 2013). Limbal stem cell deficiency may cause recurrent corneal epithelial erosions, inflammatory reactions and ingrowth of fibro-vascular tissue onto the corneal surface. Clinical symptoms include ocular pain and loss of vision (Tseng 1996; Dua et al. 2000). Removal of the diseased tissue and transplantation of *ex vivo* engineered epithelium may restore the integrity of the corneal surface (Pellegrini et al. 1997; Nakamura et al. 2004; Sangwan et al. 2011; Pathak et al. 2013; Romero et al. 2014; Zakaria et al. 2014; Ljubimov & Saghizadeh 2015; Szabó et al. 2015; Etxebarria et al. 2017; Liu et al. 2017). For such engineering, a sample containing limbal epithelium is removed from the donor eye, and cultures may be initiated by the suspension culture technique or by the explant culture technique. In the former, dissociation of epithelial cells is achieved by incubation of the tissue in one or more enzyme solutions, and cells suspended in medium are subsequently seeded on a substrate and incubated (Pellegrini et al. 1997; Meyer-Blazejewska et al. 2010; Rama et al. 2010; Lorenzo et al. 2013). Animal feeder cells may be used in cultures to support *ex vivo* engineering of tissues for transplant purposes (Pellegrini et al. 1997; Rama et al. 2010). However, production of tissues destined for transplantation to human patients should preferably be performed in an environment without cells derived from other species, that is, in xenobiotic-free systems. The use of animal feeder cells represents a safety issue wherefore transplantation of tissues generated in such environments is not allowed by health regulatory authorities in some countries.

In the explant culture technique, an intact or minced sample obtained from the donor eye is placed on a substrate and incubated in medium (Shahdadfar et al.

2012; Pathak et al. 2013; Zakaria et al. 2014). Epithelial tissue is generated by outward epithelial migration and proliferative activity on the substrate surrounding the original samples.

The composition of the medium used for engineering of transplantable epithelial tissue differs between clinics. A medium containing fetal bovine serum, recombinant growth factors, hormones and cholera toxin was used in the first clinical treatments of limbal stem cell deficiency. This protocol has been shown to support engineering of grafts with a high density of stem cells (Pellegrini et al. 1997; Rama et al. 2010; Yu et al. 2016). Concern regarding the content of xenobiotics has spurred development of alternative protocols wherein some or all of these supplements have been omitted (Nakamura et al. 2004; Di Girolamo et al. 2007; Kolli et al. 2010; Albert et al. 2012; Shahdadfar et al. 2012; Pathak et al. 2013, 2016; Shortt et al. 2014; Zakaria et al. 2014; Bobba et al. 2015). Long-term renewal of the epithelium after transplant surgery depends critically on the presence of a pool of well-functioning stem cells. The *ex vivo* systems represent a foreign microenvironment; wherein, oxidative reactions and other stressors may alter cell function temporarily or permanently by distortion of the integrity of cellular molecules, and microarray analysis could indicate that the levels of reactive oxygen species are higher in tissues generated in medium with human serum versus in the traditional complex medium with xenobiotics (Pathak et al. 2016). Previous studies indicate that levels of DNA strand breaks and, in particular, oxidative damage to DNA purine bases may provide information regarding the quality of a culture system (Lorenzo et al. 2009; Haug et al. 2013; Osnes-Ringen et al. 2013). *In vivo*, oxidative damage to DNA bases is generally efficiently repaired by the base excision repair pathway (BER) involving the action of a number of enzymes including APE1, OGG1 and Polβ (Collins et al. 2003). Maintained integrity of the DNA is a prerequisite for long-term proper cellular functioning, replication and viability of cells in the generated tissue.

Procedures for *ex vivo* engineering are still relatively new, and scant knowledge is available on types and levels of DNA damage in *ex vivo* engineered epithelial tissue. We here aimed to examine levels of DNA strand breaks and levels of oxidized purine bases in human limbal epithelium expanded *ex vivo* in the traditional complex medium and in medium with human serum as the single growth-promoting additive. Cultures were initiated by the explant culture technique. Due to variations in culture time in current protocols and to possible differences in susceptibility towards such damage depending on spatial location of cells in the generated tissues, cells in central as well as in peripheral parts of the tissues were examined after two, three and four weeks in culture. The levels of oxidative DNA damage depend on types and magnitude of oxidative stress and also on expression of repair enzymes. To examine whether culture conditions supported expression of such enzymes, gene and protein expressions of APE1, OGG1 and Polβ in cultivated cells were visualized at the different time intervals using *in situ* hybridization and immunohistochemistry.

Materials and Methods

All experiments were conducted in accordance with the Declaration of Helsinki, and all tissue harvesting was approved by the Local Committees for Medical Research Ethics.

Tissue

Human corneoscleral tissue was obtained from rings available after Descemet's stripping automated endothelial keratoplasty (DSAEK) and preserved in organ culture prior to use. For organ culture, donor corneas were incubated as described previously (Slettedal et al. 2007). Briefly, the corneas were incubated in minimal essential medium with Earle's Salts and L-glutamate, buffers, antibiotics, antifungals and 8% heat-inactivated fetal bovine serum and incubated at 32°C. For age, sex, post-mortem time and time in organ culture, see Table 1.

Each corneoscleral ring was divided into 12 equal segments (approx. 2 mm^2) and cultured in parallel in medium with human serum as the single growth-promoting supplement or in complex culture medium.

Culture media

Culture medium with human serum (HS)
Culture medium with HS was prepared using DMEM/F12 (31331028; Invitrogen, Carlsbad, CA, USA), penicillin/streptomycin (100 U/ml, P4333), amphotericin B (2.5 μg/ml), A2942 and 10% HS, pooled normal human AB serum (Novakemi AB, Sollentuna, Sweden).

Culture medium with complex medium (COM)
Culture medium COM consisted of DMEM/F12 (31331028; Invitrogen), penicillin/streptomycin (100 U/ml, P4333), amphotericin B (2.5 μg/ml, A2942), 5% FBS(F2442), epidermal growth factor (2 ng/ml, E9644), ITS (insulin (5 μg/ml), transferrin (5 μg/ml) and sodium selenite (5 ng/ml), I1884), cholera toxin A (30 ng/ml, G120, Biomol International, LP, Plymouth Meeting, PA, USA), dimethyl sulfoxide (0.5% 34869) and hydrocortisone (15 μM, H0888).

Epithelial cell culture

Limbal biopsies were cultured on Netwells® tissue culture-coated plastic polyester membrane inserts (CLS3450), in HS or in COM (Pathak et al. 2016). The cultures were incubated at 37°C, 5% CO_2 and 95% air without feeder cells and without air-lifting. The culture medium (HS or COM) was changed every 2–3 days. Samples were cultured for 2, 3 or 4 weeks.

Fixation and processing of expanded cells

After culturing for 2, 3 or 4 weeks, the expanded tissue in each insert was divided into one central and one

Table 1. Characteristics of the donor rings obtained for the experiments.

	Ring				
	1	2	3	4	5
Age (years)	61	88	71	82	75
Sex	Male	Male	Female	Male	Female
Post-mortem time (hr)	20	9	12	10	19
Time of storage in organ culture (days)	15	18	34	30	27

peripheral part for investigative purposes. One part was fixed in 4% formalin overnight at 4°C, dehydrated through a graded series of ethanol up to 100% and then embedded in paraffin for immunohistochemistry and RNA *in situ* hybridization assay. Another part was divided into central and peripheral area and processed for cell dissociation for use in the Comet assay. Duplicated samples were incubated with 250 μl of 0.05% trypsin–EDTA for 30 min using 96-well plates (Nunclon Surface, Nunc, Denmark) at 37°C in a humid atmosphere containing 5% CO_2. At the end of the incubation, enzyme activity was terminated by adding an equal amount of medium. Cells were dispersed by gentle pipetting ×20 using a 1000 μl micropipette. The dissociated cells were transferred to tubes on ice and processed for DNA damage analyses.

Comet assay for evaluation of DNA damage

Using a standard comet assay protocol, the basal levels of strand breaks (SBs) were measured as described (Collins 2004). As an experimental control, human lymphocytes were used. Cells were suspended in PBS at 0.25×10^6 cells/ml. Twenty microlitres of cell suspension was mixed with 94 μl of 1% low-melting point agarose, and 5 μl drops was placed onto agarose-precoated slides following a format of 12 minigels/slide (Shaposhnikov et al. 2010). Cells were lysed in 2.5 M NaCl, 0.1 M EDTA, 0.01 M Tris and 1% Triton X-100 (pH 10) at 4°C for at least 1 hr. Nucleoids were incubated with or without the bacterial repair enzyme formamidopyrimidine DNA glycosylase (Fpg), which breaks DNA at oxidized purines (notably 8-oxoguanine, a distinctive product of oxidative damage to DNA). After that, slides were immersed in electrophoresis solution (0.3 M NaOH and 1 mM EDTA) for 20 min. Electrophoresis was carried out at 1.3 V/cm for 20 min in the same solution. Slides were washed for 10 min in PBS, 10 min in water, fixed in ethanol 70% for 15 min and in absolute ethanol for further 15 min. Comets were stained with SYBR Gold at the dilution recommended by the manufacturer in a large dish at 4°C with agitation. Using a fluorescence microscope, 100 comets (50 on each gel) were visually classified into five categories (0–4), representing increasing relative tail intensities. Summing the scores (0–4) of 100 comets therefore gives an overall score between 0 and 400 arbitrary units (2).

Immunohistochemistry (IHC)

After fixation, 3.5 μm sections were stained for APE1 (13B8E5C2, 1:800; Enzo Life Sciences, New York, NY, USA), OGG1 (Rabbit Polyclonal, 1:500; Novus Biologicals, Littleton, CO, USA) and Polβ (Goat Polyclonal, 1:50; Santa Cruz, Santa Cruz, CA, USA) for expression of DNA repair enzymes. An Autostainer 360 (Lab Vision Corporation, Fermont, CA, USA) was used, and positive immunoreactions were detected by a secondary antibody conjugated with peroxidase-labelled polymer with diaminobenzidine (DAB). Positive control was used as recommended by distributor. Three trained observers evaluated semiquantitatively the expression of APE1, OGG1 and Polβ at the protein levels and classified it on a scale from 0 to 3+, where 0 indicates a negative reaction; 1+ indicates a slight reaction, with nuclear details clearly visible; 2+ indicates a moderate reaction, where nuclear details are partially obscured; and 3+ represents a strong reaction, where nuclear details can no longer be distinguished. Images were captured with ZEISS Imager M1 microscope (ZEISS, Oberkochen, Germany) using 100× magnification. *Ex vivo* expanded cultures were assayed for the presence of CK3 (clone AE5-Mono, 1:100, Abcam, Cambridge, UK). Epithelium expanded for 2, 3 and 4 weeks in medium with HS and COM where all found positive for this marker.

RNA *in situ* hybridization assay (ISH)

The assay was performed using RNAscope 2.5 HD detection kit brown for formalin-fixed, paraffin-embedded (FFPE) tissue (ACD, Hayward, CA, USA) according to the manufacturer's protocols.

Formalin-fixed, paraffin-embedded tissues were sectioned at 3.5 μm, mounted on SuperFrost Ultra Plus slides (Thermo Fisher Scientific, Waltham, MA, USA) and deparaffinized as recommended by manufacturer plus, incubated for 30 min at 60°C to avoid detachment of the sections, incubated with pretreat one reagent at room temperature for 10 min, boiled with pretreat two reagent for 15 min and washed with water. The sections were incubated for 30 min at 60°C and then pretreated with reagent 3 for 15 min at 40°C. Samples were hybridized with RNAscope Negative Control Probe 310043, RNAscope Positive Control Probe-Hs-PPIB 313901, RNAscope Probe-Hs-APEX 1 480481, RNAscope Probe-Hs OGG1 480391 and RNAscope Probe-Hs-POLB 480491 for 2 hr at 40°C in a humidity chamber in a HybEzTM oven. Following hybridization, slides were washed for 2 min twice in wash buffer, and the signal was amplified using a specific set of amplifiers (Amp) 1–6: Amp1 for 30 min at 40°C; Amp2 for 15 min at 40°C; Amp 3 for 30 min at 40°C; Amp 4 for 15 min at 40°C; Amp 5 for 60 min at room temperature; Amp6 for 15 min at room temperature. Washing steps with wash buffer were performed twice between reagent steps indicated above. The signal was detected using DAB for 10 min at room temperature. Slides were counterstained with 50% haematoxylin (Sigma-Aldrich, Saint Louis, MO, USA/GHS116) for 20 seconds, and bluing was performed with 0.02% ammonia water for 10 seconds. Slides were washed 3–5 times with water, for 2 min in 70% ethanol, for 2 min in 96% ethanol and for 5 min in xylene. Coverslips were mounted with mounting medium. Images were captured with ZEISS Imager M1 microscope (ZEISS) using 63× magnification.

Results

The comet assay

Some levels of DNA strand breaks were observed in epithelial cells expanded in COM and also in HS after 2, 3 and 4 weeks of cultivation. Any noticeable time-dependent increase or any difference between cells obtained from the central versus peripheral areas could not be detected (Fig. 1A). Levels of net Fpg-sensitive sites were low at all time-points in both types of medium in cells in the central as well as in the peripheral parts of the tissues (Fig. 1B; Table 2).

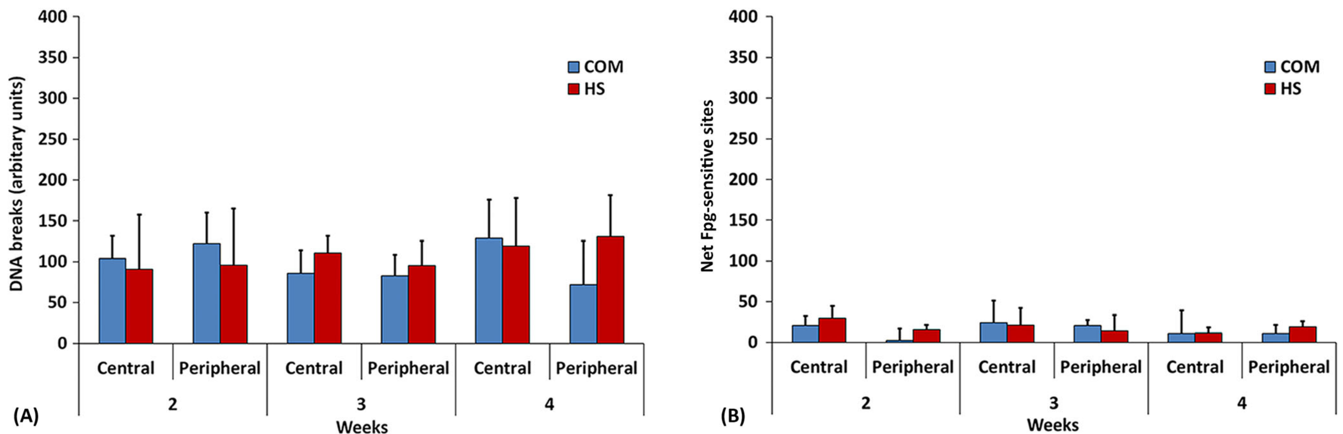

Fig. 1. DNA strand breaks (A) and net Fpg-sensitive sites (B) detected in central and in peripheral parts of human limbal epithelial tissues expanded *ex vivo* for 2, 3 and 4 weeks in complex medium (COM) and in human serum (HS). The results are expressed in arbitrary units in a range between 0 and 400 where increasing values represent increasing levels of DNA damage. Significant differences were not detected between levels of damage (strand breaks and net Fpg-sensitive sites) in tissue sample after 2, 3 and 4 weeks in culture or between COM and HS (p-value < 0.05).

Table 2. Measured levels of DNA strand breaks and of net Fpg-sensitive sites in central and peripheral parts of human limbal epithelial tissues expanded *ex vivo* for 2, 3 and 4 weeks in complex medium (COM) and in human serum (HS).

	DNA strand breaks				Net Fpg-sensitive sites			
	COM	STDEV	HS	STDEV	COM	STDEV	HS	STDEV
2								
Central	104	27.49	90.75	66.8	20.5	11.81	30	14.5
Peripheral	122	38.03	96	69.13	2.5	14.21	15.5	6.11
3								
Central	85.5	28.42	110.75	20.66	24	27.15	21.25	20.75
Peripheral	82.5	25.65	95.42	29.87	20.5	6.79	14	19.56
4								
Central	129	46.61	119	59.01	11	28.25	11.5	6.95
Peripheral	72	53.71	131	50.52	11	10.55	19	6.83

ISH and IHC

By ISH, expression of APE1, OGG1 and Polβ at gene level was observed in tissues engineered in both types of culture medium and at all time-points and in superficial as well as in basal cells (Fig. 2A). Using IHC for detection of these enzymes at the protein level, the expression of APE1 and of OGG1 was classified as moderate/strong in cells in superficial as well as in basal layers in tissues engineered in both types of medium and at all time-points. Reaction for APE1 was most intense at the level of the nuclei while the reaction for OGG1 was more evenly dispersed. For both proteins, a positive reaction was visualized in superficial as well as in basal cells. At all time-points and in both types of medium, reaction for Polβ at the protein level was considerably less pronounced, and in some sections below the level for detection. Representative images of tissues expanded for 3 weeks are shown in Fig. 2B, and semiquantitative evaluation of expression is illustrated in Table 3.

Discussion

The main purpose of our study was to examine levels of DNA strand breaks and oxidative DNA purine base damage in human limbal epithelium expanded *ex vivo* in medium commonly used for engineering of transplantable tissues. This is the first time that specific types of damage to DNA have been measured in such expanded tissues. Our main finding was that levels of strand breaks were substantial while levels of net Fpg-sensitive sites (8-oxoguanine and ring-opened FaPy bases) were relatively low in cells expanded in COM and in HS. Both types of medium were found to support expression of BER enzymes APE1, OGG1 and Polβ at the gene level. At the protein level, expression of APE1 and OGG1 was noticeable in both conditions while expression of Polβ was low.

Scant information is available regarding types and levels of DNA damage in the corneal epithelium in the human eye. A gradient of DNA damage has been described in the corneal epithelium in animal eyes where levels of DNA strand breaks were observed to decrease from the surface towards the basal layer (Choy et al. 2005). Levels of DNA strand breaks and levels of oxidized bases are generally very low in the epithelium on human donor corneas maintained in cold storage. Increases in levels of strand breaks and also in levels of oxidized bases in the epithelium may be observed after transfer of the donor corneas to organ culture (Haug et al. 2013). Environmental stressors such as UV-irradiation and airborne particulate matter have been shown to induce DNA strand

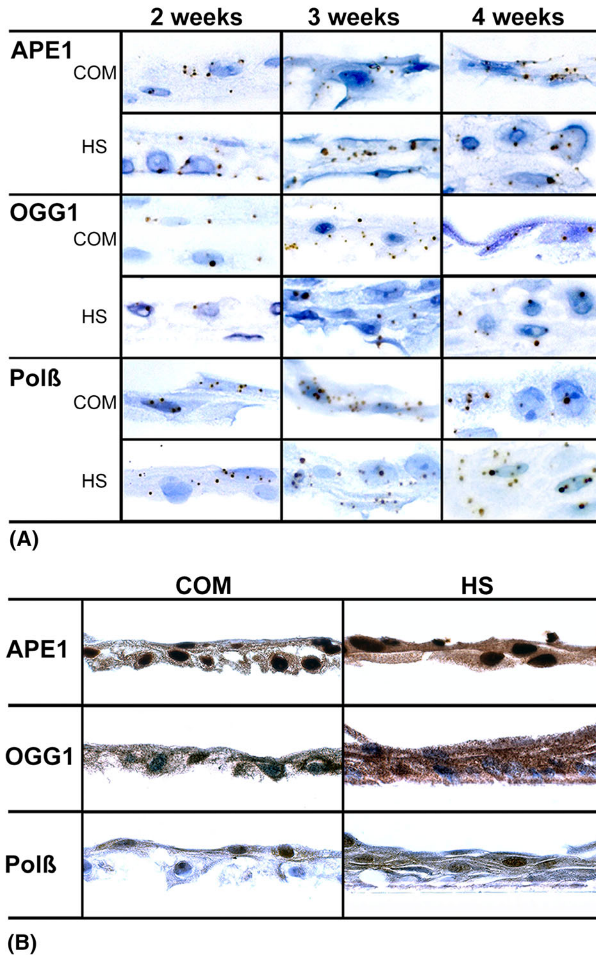

Fig. 2. Expression of APE1, OGG1 and Polβ at the gene level in tissues engineered *ex vivo* in complex medium (COM) and in human serum (HS) was visualized using ISH (A). Gene transcripts are observed as distinct chromogen precipitates and were detected at all time-points. In (B), the protein expression of these enzymes in tissues expanded for 3 weeks is visualized using IHC (Mag.: A: 63× and B: 100×).

Table 3. The sections from tissues expanded *ex vivo* in complex medium (COM) and in human serum (HS) were evaluated by three independent observers for expression of APE1, OGG1 and Polβ at the protein level and classified in a scale from 0 to 3+ where 0 indicates a negative reaction, and 3+ indicates a strong reaction.

	APE1	OGG1	Pol β
COM 2w	2+	2+	0
HS 2w	2+	2+	+
COM 3w	2+	2+	+
HS 3w	3+	2+	+
COM 4w	3+	3+	0
HS4w	2+	2+	2+

breaks in cultivated human and animal epithelial cells (Choy et al. 2005; Gao et al. 2016). Unrepaired, such damage may act to destabilize cell function through an increased level of transcriptional errors (Ishibashi et al. 2005).

The explant culture technique was used for initiation of cultures in the present study. Limbal tissue was obtained from human donor corneas maintained in organ culture. The donors differ in age as well as in gender, and post-mortem time and time in organ culture prior to initiation of the explant cultures differ considerably. For initiation by the suspension culture technique, limbal epithelial cells may be dissociated from organ cultured tissues or from fresh samples by incubation in 0.05% trypsin–EDTA at 37°C for 1 or 3 hr, respectively (Pellegrini et al. 1997; Zito-Abbad et al. 2006). This procedure has previously been shown to induce noticeable levels of strand breaks in the isolated cells (Lorenzo et al. 2013). Levels of strand breaks observed in the DNA in cells cultivated in the present experiment were found to be similar to those observed after dissociation of cells from organ cultured limbal epithelium. Although a considerably shorter incubation time was used for dissociation of cells from the *ex vivo* engineered tissues in the present study, to some extent, levels of DNA strand breaks may have been induced by this procedure. The culture system, be it organ culture or cell culture, represents a foreign microenvironment, where a number of constituents may contribute to such damage.

In culture, stability of the genome, levels of oxidative damage and patency of antioxidant defence and repair mechanisms depend on a number of variables including composition of medium and time in culture (Shahdadfar et al. 2005; Lorenzo et al. 2009; Johnsen-Soriano et al. 2012; Bath 2013; González & Deng 2013; Veréb et al. 2013; Hua et al. 2015; Szabó et al. 2015; Pathak et al. 2016; Jacobs et al. 2016). In particular, an upregulated gene expression of NOX4 in tissues engineered in HS (Pathak et al. 2016) could result in increased levels of reactive oxygen species (ROS) in tissues engineered in HS when compared to COM. Despite the lack of complexity, we did not observe any increase in DNA base oxidation damage in medium with human serum as the single supplement. Previous studies indicate that cultivated cells may show enhanced stability of the genome and ability to maintain an unmethylated state when expanded in medium with human serum compared with fetal bovine serum (Shahdadfar et al. 2005; Dahl et al. 2008). A possible increase in ROS in tissues expanded in HS may not necessarily be accompanied by an increase in the levels of oxidative damage; ROS serve as important signalling molecules and are involved in regulation of a number of cellular pathways and functions including in regulation of gene expression (Cortese-Krott et al. 2017).

Time in culture generally spans between 2 and 4 weeks, and phenotype may differ between cells in central versus peripheral areas of the generated tissue (Shahdadfar et al. 2012; Pathak et al. 2013, 2016; Zakaria et al. 2014; Lužnik et al. 2016). When cultivated in medium with an oxidative potential, a time-dependent increase in molecular oxidative damage and a decrease in antioxidant potential may be detected in epithelial cells (Johnsen-Soriano

et al. 2012). No noticeable increase in oxidative damage to the DNA was observed within the time frame of our study. Our findings indicate low levels of oxidative stress and/or efficient repair of DNA base damage in both types of culture medium. With time in culture, epithelial tissue is generated by outward migration and proliferation of cells from the limbal tissue. The compactness and phenotype of cells may differ between central and peripheral areas (Kolli et al. 2008), and the DNA damage response may vary during cell differentiation (Beckta et al. 2017). We did, however, not detect any spatial differences in DNA base oxidation damage in the engineered tissues.

Conclusion

Protocols for *ex vivo* engineering of epithelium for transplant purposes differ between clinics. Concern is linked to the content of, in particular, animal feeder cells, fetal bovine serum and cholera toxin in medium used for engineering of transplantable tissues. The use of medium containing xenobiotics represents a safety issue wherefore transplantation of tissues generated in such environment is not allowed by health regulatory authorities in some countries. For *ex vivo* generation of transplantable tissues, epithelium on freshly harvested autologous or allogeneic limbal tissue or on post-mortem donor tissue derived from Eye Banks may serve as origin. Our study, using post-mortem donor tissues, indicates that medium with human serum as a single supplement may support engineering of tissues without any increase in levels of DNA strand breaks and of DNA base oxidation damage when compared to the traditional complex medium containing xenobiotics.

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