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Research article

An investigation into corneal enzymatic resistance following epithelium-off and epithelium-on corneal cross-linking protocols

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ABSTRACT

The aim of this study was to investigate corneal enzymatic resistance following epithelium off and on riboflavin/UVA cross-linking (CXL). One hundred and fourteen porcine eyes were divided into four non-irradiated control groups and seven CXL groups. The latter comprised; (i) epithelium-off, 0.1% iso-osmolar riboflavin, 9 mW UVA irradiation for 10 min, (ii) disrupted epithelium, 0.1% hypo-osmolar riboflavin, 9 mW UVA for 10 min, (iii) epithelium-on, 0.25% hypo-osmolar riboflavin with 0.01% benzylalkonium chloride (BACS), 9 mW UVA for 10 min, (iv) epithelium-on, 5 min iontophoresis at 0.1 mA for 5 min with 0.1% riboflavin solution, 9 mW UVA for 10 min or (v) 12.5 min, (vi) epithelium-on, prolonged iontophoresis protocol of 25 min with 1.0 mA for 5 min and 0.5 mA for 5 min with 0.25% riboflavin with 0.01% BACS, 9 mW UVA for 10 min or (vii) 12.5 min. Enzymatic resistance was assessed by daily measurement of a corneal button placed in pepsin solution and measurement of corneal button dry weight after 11 days of digestion. This study revealed that the enzymatic resistance was greater in CXL corneas than non-irradiated corneas ($p < 0.0001$). Epithelium-off CXL showed the greatest enzymatic resistance ($p < 0.0001$). The prolonged iontophoresis protocol was found to be superior to all other trans-epithelial protocols ($p < 0.0001$). A 25% increase in UVA radiance significantly increased corneal enzymatic resistance ($p < 0.0001$). In conclusion, although epithelium-on CXL appears to be inferior to epithelium-off CXL in terms of enzymatic resistance to pepsin digestion, the outcome of epithelium-on CXL may be significantly improved through the use of higher concentrations of riboflavin solution, a longer duration of iontophoresis and an increase in UVA radiance.

1. Introduction

Over the past decade, riboflavin/UVA corneal cross-linking (CXL) has become an established treatment to halt the progression of keratoconus and other corneal ectasias. Riboflavin is a hydrophilic molecule, with a molecular weight of 340 Da, while, the corneal epithelium is lipophyllic, with a decreasing permeability to molecules over 180 Da (Huang et al., 1989). Therefore in the "gold-standard" CXL protocol the epithelium is removed from the central corneal to allow adequate stromal absorption of riboflavin prior to UVA irradiation. Spoerl et al. confirmed this need for epithelial removal, reporting no changes in corneal biomechanics when CXL was performed with the epithelium intact (Spoerl et al., 1998). On this basis, the epithelium was removed in the first published clinical studies (Caporossi et al., 2006; Gkika et al., 2011; O'Brart et al., 2011; Richoz et al., 2013; Wittig-Silva et al., 2014; Wollensak et al., 2003). However, epithelial debridement is associated with a number of adverse events, including severe ocular pain in the immediate post-operative period, delayed visual rehabilitation and the risks of scarring, infectious and non-infectious keratitis (Koller et al., 2009).

As a result of such considerations, a number of trans-epithelial CXL techniques have been postulated. The first epithelium-on CXL (epi-on-CXL) protocols included the use of multiple topical applications of tetracaine 1% to disrupt epithelial tight junctions (Boxer Wachler et al., 2010; Chan et al., 2007) and partial epithelial debridement in a grid pattern (Rechichi et al., 2013). More recently, novel formulations of riboflavin have been developed to facilitate

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epi-on-CXL. Laboratory studies have shown that riboflavin preparations in which trometamol (Tris-hydroxymethyl-aminometane) and sodium ethylenediaminetetraacetic acid (EDTA) have been added facilitate stromal absorption when used in conjunction with superficial/grid pattern epithelial trauma (Alhamad et al., 2012). Similarly, preparations without dextran but with sodium chloride 0.44% and benzalkonium chloride (BAC) have been shown to facilitate trans-epithelial riboflavin stromal absorption (Kissner et al., 2010; Raiskup et al., 2012). Clinical studies with such formulations have demonstrated equivocal results with some suggesting similar efficacy to epithelium-off CXL (epi-off-CXL) (Filippello et al., 2012; Magli et al., 2013), and others showing less pronounced effects (Buzzonetti and Petrocelli, 2012; Kocak et al., 2014; Koppen et al., 2012; Leccisotti and Islam, 2010). As riboflavin is negatively charged at physiological pH and soluble in water, iontophoresis as a means of enhancing trans-epithelial absorption has also been postulated. In vitro studies of CXL using iontophoresis-assisted delivery (Ion-CXL) of riboflavin 0.1% with a current of 0.5–1 mA for 5–10 min have been encouraging, demonstrating enhanced trans-epithelial riboflavin absorption and corneal tissue biomechanics (Cassagne et al., 2016; Lombardo et al., 2014; Mastropasqua et al., 2014; Touboul et al., 2014; Vinciguerra et al., 2014a). Early clinical studies have reported cessation of progression and improvements in keratometric and visual parameters (Bikbova and Bikbov, 2014; Buzzonetti et al., 2015; Vinciguerra et al., 2014b).

In addition to the desire to keep the epithelium intact, the current "gold-standard", epi-off-CXL protocol, involves a 30 min application of riboflavin followed by a 30 min irradiation with 370 nm UVA light, with an intensity of 3 mW/cm^2, necessitating in excess of one hour treatment time. In an attempt to reduce treatment times, the use of accelerated CXL (A-CXL) procedures using the same energy dose but higher UVA intensities and shorter exposure times have been investigated. Published clinical studies of A-CXL protocols are relatively few. However, those using 7 mW for 15 min or 9 mW for 10 min have reported improvements in corrected distance acuity and a reduction in topographic keratometry at up to 46 months follow-up with no adverse events associated with the high fluences used (Cinar et al., 2014; Cummings et al., 2016; Kanellopoulos, 2012; Kymionis et al., 2014a; Shetty et al., 2015). More recently, it has also been demonstrated that the efficacy of A-CXL may be further improved by increasing the UVA exposure time and the overall cumulative dosage (Aldahlawi et al., 2016; Kymionis et al., 2014b; Sherif, 2014).

Increased resistance of the corneal stroma to enzymatic digestion following standard (Hayes et al., 2013; Spoerl et al., 2004) and accelerated (Aldahlawi et al., 2015) epi-off-CXL has been demonstrated by a number of investigators. It is likely that the improved enzymatic resistance is an important factor in protection against disease progression, since an increase in proteinase activity and a reduction in proteinase inhibitor activity has been identified in keratoconic corneas (Zhou et al., 1998). In order to investigate the efficacy of a number of different epi-on-CXL protocols, we compared the enzymatic resistance of corneal tissue cross-linked using epi-off-CXL with that of corneas treated with partially disrupted-epithelium CXL (dis-CXL), existing epi-on-CXL protocols (involving different riboflavin formulations and modes of delivery) and a prolonged iontophoresis CXL protocol with 0.25% riboflavin (TC-ion-CXL) that we have recently developed. In addition to this we examined the effect of increasing the cumulative UVA dosage on the enzymatic resistance of corneas treated with iontophoresis-assisted epi-on-CXL. Pepsin was selected as the enzyme of choice for this study in favour of collagenase, as it is a non-specific endopeptidase that can break down both collagen and proteoglycan core proteins, both of which are believed to be sites of riboflavin/UVA induced cross-links (Hayes et al., 2013). Porcine eyes were used as, unlike human cadaver eyes and rabbit eyes which are of limited availability in the UK, they were readily available in the fresh state and in the large numbers required for this study. However, due to the porcine cornea having a thicker epithelium than the human cornea, the results presented herein should be regarded as a conservative assessment of the effectiveness of trans-epithelial CXL.

2. Materials and methods

2.1. Specimen preparation

A total of one hundred and fourteen fresh porcine cadaver eyes with clear corneas and intact epithelium were retrieved from a local European Community licensed abattoir within 6–8 h of death. Due to the large number of eyes and treatment groups involved, it was necessary to split the study into two runs. Run 1 examined the effectiveness of dis-CXL and epi-on-CXL protocols at increasing corneal enzymatic resistance to digestion with pepsin, whilst run 2 examined the effect on enzymatic resistance of increasing the UVA dosage by 25% from 5.4 J/cm^2 to 6.75 J/cm^2 during iontophoretic epi-on-CXL. In both runs, the effectiveness of the standard epi-off CXL protocol at increasing enzymatic resistance was also examined for comparative purposes. The 11 treatment groups are described below and summarised in Table 1.

Run 1:

Each treatment group consisted of six eyes.

i) Epi-off standard protocol (Group 1: Epi-off-ribo; Group 2: Epi-off-CXL)

Complete corneal epithelial debridement was performed in groups 1 and 2 using a single edged razor blade. These eyes then received 0.1% riboflavin eye drops containing 20% dextran T-500 solution (Mediocross D®, Peschke Meditrade, Huenenberg, Switzerland) every 5 min for 30 min. The central 9 mm region of corneas in group 2 was then exposed to 365 nm UVA light with a fluence of CXL 9 mW/cm^2 for 10 min using a CCL-365 Vario™ cross-linking system (Peschkmed, Huenenberg, Switzerland). During irradiation, riboflavin was re-applied at 5 min intervals. Group 1 served as a non-irradiated control.

ii) Epi-disrupted protocol (Group 3: Dis-ribo; Group 4: Dis-CXL)

Partial epithelial disruption was performed in groups 3 and 4, by making 64 full-thickness epithelial punctures with a 25 gauge needle in an 8 × 8 grid pattern. The corneas were then soaked in riboflavin 0.1% dextran-free solution (Vitamin B2 Streuli, Uznach, Switzerland) for 30 min and rinsed with phosphate-buffered saline (PBS) for 5 min. Group 4 was then irradiated with 9 mW UVA for 10 min with PBS applied at 5 min intervals to keep the corneal surface moist. Group 3 served as a non-irradiated control.

iii) Epi-on and high riboflavin concentration protocol (Group 5: Medio-ribo; Group 6: Medio-CXL)

Groups 5 and 6 received 0.25% riboflavin with 1.2% hydroxypropylmethyl cellulose (HPMC) and 0.01% BAC (Mediocross TE®, Peschketrade, Huenenberg, Switzerland) every 5 min for 30 min. This was followed by a 5 min rinse with PBS. Group 6 was then irradiated with 9 mW UVA for 10 min with PBS applied at 5 min intervals. Group 5 served as a non-irradiated control.

iv) Epi-on, high riboflavin concentration and prolonged iontophoresis (St. Thomas-Cardiff) protocol (Group 7: TC-ion-ribo; Group 8: TC-ion-CXL)

Groups 7 and 8 received iontophoresis assisted delivery of 0.25% riboflavin with 1.2% HPMC and 0.01% BAC (Mediocross TE®, Peschketrade, Huenenberg, Switzerland) using a current of 1 mA for 5 min. The corneas were then soaked with this riboflavin