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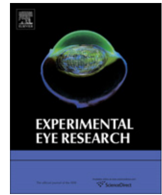

Corneal wound healing is modulated by topical application of amniotic fluid in an ex vivo organ culture model

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ABSTRACT

The purpose of this study was to evaluate the effects of topical human amniotic fluid (HAF) and equine amniotic fluid (EAF) on corneal reepithelialization and stromal wound healing.

New Zealand white rabbit corneas ($n = 52$) were placed in an ex vivo air-interface organ culture. An 8.5 mm-diameter mark in the center of the cornea was produced with a hand trephine to select the area for epithelial scraping. A number 15 surgical blade was used to remove the epithelial layer within the demarcated area in a standardized fashion. The corneas were assigned to one of four treatment groups ($n = 8$): fetal bovine serum (FBS), HAF, EAF, and a control group that was exposed to phosphate buffer solution (PBS). Corneal epithelial defects were imaged every 8 h for 72 h after the application of a 30 μl drop of 0.015% fluorescein. Five corneas of each treatment group were used for histology, proliferation, and apoptosis assay at 72 h after the epithelial defect was created.

There was no significant difference in the mean rate of closure of the corneal epithelial defect between FBS treated corneas and controls ($P > 0.06$). The mean epithelial defect area (MEDA) was significantly smaller in the EAF group as compared to control corneas at 24 h ($P = 0.016$), 40 h ($P = 0.032$), 64 h ($P = 0.008$) and 72 h ($P = 0.007$) following epithelial scrape. The MEDA in the HAF group was significantly smaller at 16 h ($P = 0.008$), 64 h ($P = 0.0072$), and 72 h ($P = 0.016$) compared to the control group. The MEDA in the HAF and EAF groups was smaller at all time points as compared to the FBS group, but the difference was not significant. At histology, the mean keratocyte density was significantly higher in the anterior stroma in the HAF ($P < 0.001$) and EAF groups ($P = 0.001$) as compared to control group. The number of BrdU positive keratocytes was significantly higher in the superficial and deep stromal sub-areas in the HAF group as compared to control ($P < 0.001$ and $P = 0.002$, respectively). EAF and FBS treated corneas also showed a higher number of BrdU positive cells compared to control, but this difference was not significant. Finally, we did not observe any difference in the amount of TUNEL positive keratocytes among the different groups.

Our data indicates that the topical application of HAF and EAF is associated with accelerated reepithelialization in this cornea organ culture model. Similarly, corneal keratocyte density appears to be less affected after epithelial injury using this treatment.

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1. Introduction

Human amniotic membrane (HAM) was first used in ophthalmology by de Rotth (1940) to repair conjunctival defects. Since then, HAM has been used to treat a wide variety of corneal disorders such as persistent epithelial defects (Shukla, 1962; Batmanov et al., 1990), neurotrophic corneas (Kruse et al., 1999; Lee and Tseng, 1997), chemical injuries (Sorsby and Symons, 1946; Sorsby et al., 1947), recurrent erosion syndrome and persistent epithelial defects associated with cicatricial conditions (Lee and Tseng, 1997). Positive effects on corneal reepithelialization, inflammatory response, and scar formation have been reported with the use of HAM (Kim and Tseng, 1995; Meller and Tseng, 1998). In a randomized prospective study of rabbit corneas undergoing photorefractive keratectomy excimer laser Choi et al. (1998) demonstrated a reduction of haze in the postoperative period after amniotic membrane was applied to the wound area; they hypothesized that this may be due to a reduction in inflammatory cell infiltration in the early postoperative period. Moreover, Woo et al. (2001) created large

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epithelial defects on rabbit corneas using excimer laser, reporting faster rate of reepithelialization when the corneas were treated with amniotic membrane.

Most of the proteins present in HAM have also been found in HAF (Zhang et al., 2001). It has been reported that HAF is effective in the reduction of corneal opacity and scar formation in mouse corneas after alkali corneal burn induction (Herretes et al., 2006). In addition, reports indicate that amniotic fluid accelerates recovery of corneal sensitivity through nerve regeneration and decreases corneal scar formation after photorefractive keratectomy (Lee and Kim, 1996). Potential therapeutic effects on corneal wound healing for HAM and HAF are therefore expected to be similar.

Based on these observations, our objective with this study was to evaluate the effects of topical application of HAF and EAF to modulate corneal healing. To test this hypothesis, we utilized an established air-interface organ culture technique with New Zealand white rabbit corneas (Chuck et al., 2001).

2. Methods

2.1. Rabbit globe harvest

The eyes of 26 adult New Zealand white rabbits were enucleated immediately following euthanization and were placed in 50 ml tubes (Becton Dickinson and Company, Franklin Lake, NJ) containing Dulbecco Modified Eagle Medium (Gibco BRL, Life Technologies Inc., Rockville, MD). The tubes were placed in a container with ice for preservation (4 °C). A circular central epithelial defect was created on each cornea 2 h after enucleation. The sclero-corneal ring was excised from each globe with conjunctiva attached using curved scissors and leaving 3–5 mm of sclera.

2.2. Preparation of whole-organ rabbit cornea culture

With the use of a sterile blade, the ends of laboratory test tubes (2059; Falcon, Oxnard, CA) were cut at the indicator line nearest to the bottom. The cut edges of the domes were smoothed, and the domes were aseptically placed into six-well tissue culture plates (Becton and Dickinson, Franklin Lake, NJ). Each dome was glued tightly concave-side down into each well using acrylic glue (GE White All Purpose Adhesive Caulk). Dulbecco Modified Eagle Medium with antibiotic/antimycotic solution (1/200) (Mediatech, Inc., Herndon, VA) was then added to each well to just cover each dome (12 ml per well). The sclero-corneal rings were placed in the culture plates to be incubated at 37 °C with 5% CO_2. Culture medium was changed once during the experiment (36 h).

2.3. Corneal epithelial defect

Using a dissecting microscope (Stemi 200-C, Carl Zeiss Inc., Thornwood, NY) and a cold light source (Schott North America, Auburn, NY), an 8.5 mm-diameter circle located in the center of the cornea was produced with a hand trephine blade. A number 15 surgical blade (Bard–Parker, Franklin Lakes, NJ) was used to scrape and remove the epithelial layer including the basement membrane within the demarcated area in a standardized fashion. The reepithelialization process was followed by imaging every 8 h using a digital camera (Nikon Coolpix 990, Nikon Inc., Melville, NY) with a 17× macro lens attached for 72 h. A 30 μl drop of 0.015% fluorescein (Pharmaceuticals, Inc., Aquebogue, NY) was applied to stain the epithelial defect just before every imaging time point. The excess of fluorescein was rinsed away with 500 μl of phosphate-saline solution (PBS) (Invitrogen Corporation, Grand Island, NY). A separate six-well tissue culture plate was made using black tubes. The sclero-corneal rings were placed in this special designed plate during picture time to enhance the contrast during photography and to avoid mixing of fluorescein and treatment drops with culture medium. The areas of corneal epithelial defect were outlined and measured using a digital imaging software (AxioVision, Carl Zeiss Inc., Thornwood, NY). The wounded corneal surface area was calculated with a method described previously by Crosson et al. (1986).

2.4. Treatment and experimental design

After the epithelial defect was created, the sclero-corneal rings were assigned to four different groups according to the treatment administered: human amniotic fluid (HAF) ($n = 8$), equine amniotic fluid (EAF) ($n = 8$), fetal bovine serum (FBS) ($n = 8$) (Benchmark triple filtered, Gemini Bio-products, West Sacramento, CA), and control group that was exposed to PBS ($n = 8$). A 20 μl drop of the respective treatment was applied every 8 h.

2.5. Amniotic fluid

After approval of the Institutional Review Board of the Johns Hopkins University, HAF was obtained from patients with low risk pregnancies at the Department of Gynecology and Obstetrics at the Johns Hopkins Hospital. A 16–21 week amniotic fluid pool was made using 12 samples to be discarded after routine amniocentesis for karyotyping. EAF was obtained from the School of Veterinary Medicine at the University of Pennsylvania. The samples were aseptically recollected by direct amniocentesis at the moment of delivery. Both HAF and EAF samples were centrifuged at 1800 rpm for 10 min and the supernatant separated and preserved at −70 °C. At the moment of utilization, aliquots of fluid were thawed and then stored at 4 °C during treatment to minimize bacterial proliferation (Herretes et al., 2006).

2.6. Corneal histology

Twenty corneas ($n = 5$ from each group) were fixed in 10% buffered formaldehyde at 72 h after epithelial defect creation. The corneas were then immersed and oriented in paraffin. The samples were cut, mounted and stained with hematoxylin and eosin according to standard methods. Epithelial and stromal characteristics were analyzed using an inverted microscope with a 40× objective (Axiovert 200M, Carl Zeiss Inc., Thornwood, NY). Digital images of the central cornea were captured using a 10× objective. A 300 μm wide cross-section of the corneas was created on the digital images using AxioVision software. This cross-area was divided into three horizontal sub-areas (superficial, medial and deep stroma) with the same dimensions each (Fig. 1). Cell density in these areas was calculated by counting the number of cells in each section.

2.7. Cell proliferation assay

5-Bromo-2-deoxyuridine (100 μg/ml) (BrdU, Sigma–Aldrich) was added to culture wells of 20 corneas ($n = 5$ each group) 2 h before fixation. Tissue sections were processed as described by Levin and Verkman (2006). The slides were incubated with a sheep polyclonal antibody to BrdU (50 μg/ml; Abcam, Cambridge, MA). Bound antibody was detected using the Vectastain Elite ABC Kit (Vectastain; Vector Laboratories, Burlingame, CA). Digital images of the central cornea were captured using a 10× objective. BrdU positive keratocytes were counted in the superficial, medial and deep stromal sub-areas of a central corneal section divided as previously explained.

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