

**DEVELOPMENT OF AN IN-VITRO MODEL OF EQUINE CORNEAL WOUND HEALING;  
PHARMACOLOGIC AND GENE THERAPY MODALITIES IN THE REDUCTION OF CORNEAL FIBROSIS**

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**ABSTRACT**

**Objective:** To establish an *in vitro* model for the investigation of equine corneal wound healing. To accomplish this goal, a protocol to isolate and culture equine corneal keratocytes, fibroblasts and myofibroblasts was developed. Using this model we investigated the safety and efficacy of MMC to treat corneal scarring in horses. Additionally, we determined if hybrid adeno-associated virus serotype 2/5 (AAV5) vector could effectively deliver foreign genes into the equine cornea without causing adverse side effects.

**Procedure:** Using cell culture techniques, equine corneal keratocyte, fibroblast and myofibroblast cultures were produced. The MMC dose for the equine cornea was defined with dose-dependent trypan blue exclusion and MTT assays after applying MMC to the cultures once for 2 minutes. The efficacy of MMC to control corneal scarring in horses was determined by measuring mRNA and protein expression of corneal scarring markers ( $\alpha$ -smooth muscle actin and F-actin) with western blotting, immunocytochemistry and/or quantitative real-time polymerase chain reactions. AAV5 vector expressing EGFP under control of hybrid cytomegalovirus (CMV) + chicken  $\beta$ -actin (CBA) promoter was applied topically to ECF. Expression of delivered EGFP gene in ECF was quantified using fluorescent microscopy. Using DAPI staining, the total number of cells and transduction efficiency of tested AAV vector was determined. Phase contrast microscopy, trypan blue and TUNEL assays were used to determine toxicity and safety of AAV5 for ECFs.

**Results:** Cell culture techniques were successfully used to establish a method for the isolation and culture of equine corneal keratocytes, fibroblasts and myofibroblasts. A single 2 minute treatment of 0.02% or less MMC did not alter ECF phenotype, viability, or cellular proliferation whereas 0.05% or higher MMC doses showed mild-to-moderate cellular toxicity. The TGF $\beta$ 1 at 1ng/ml showed significant myofibroblast formation in ECF under serum-free conditions. A single 2 minute, 0.02% MMC treatment 24 hours (early) after TGF $\beta$ 1 stimulation significantly reduced conversion of ECF to myofibroblasts, however, a single 0.02% MMC treatment 11 days after TGF $\beta$ 1 stimulation showed moderate myofibroblast inhibition. Topical AAV5 application successfully transduced significant numbers of ECFs. Transduction efficiency was 13.1%. Tested AAV5 vector did not cause phenotype change or significant cell death and cell viability was maintained.

**Conclusions:** Equine corneal stromal keratocytes, fibroblasts and myofibroblasts can be predictably isolated and cultured *in vitro* using this protocol. That MMC safely and effectively reduced scarring in ECF by reducing the degree of transdifferentiation of corneal fibroblasts to myofibroblasts *in vitro*. Tested AAV5 vector is effective and safe for gene therapy in ECFs *in vitro*.