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Recommended Citation

Buss DG, Sharma A, Giuliano E, Mohan RR. Gene delivery in the equine cornea: a novel therapeutic strategy. *Vet Ophthalmol.* 2010;13(5):301-306. doi:10.1111/j.1463-5224.2010.00813.x.

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Buss DG, Sharma A, Giuliano E, Mohan RR. Gene delivery in the equine cornea: a novel therapeutic strategy. *Vet Ophthalmol.* 2010;13(5):301-306. doi:10.1111/j.1463-5224.2010.00813.x.

which has been published in final form at DOI: [10.1111/j.1463-5224.2010.00813.x](https://doi.org/10.1111/j.1463-5224.2010.00813.x). This article may be used for non-commercial purposes in accordance with [Wiley Terms and Conditions for Self-Archiving](#).

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Published in final edited form as:

Vet Ophthalmol. 2010 September ; 13(5): 301–306. doi:10.1111/j.1463-5224.2010.00813.x.

Gene delivery in the equine cornea: a novel therapeutic strategy

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Abstract

Objective—To determine if hybrid adeno-associated virus serotype 2/5 (AAV5) vector can effectively deliver foreign genes into the equine cornea without causing adverse side effects. The aims of this study were to: (i) evaluate efficacy of AAV5 to deliver therapeutic genes into equine corneal fibroblasts (ECFs) using enhanced green fluorescent protein (EGFP) marker gene and (ii) establish the safety of AAV5 vector for equine corneal gene therapy.

Animal Material—Primary ECF cultures were harvested from healthy donor equine corneas. Cultures were maintained at 37⁰C in humidified atmosphere with 5% CO₂.

Procedure—AAV5 vector expressing EGFP under control of hybrid cytomegalovirus (CMV) + chicken β-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—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—Tested AAV5 vector is effective and safe for gene therapy in ECFs *in vitro*.

Clinical Relevance—Tested AAV5 vector has potential to extend a novel gene therapy approach to treat equine corneal disease *in vivo*.

Introduction

The cornea is an ideal target tissue for gene therapy because of its accessibility and immune-privileged nature.^{1–3} The success of gene therapy is contingent upon two variables: the vector and the technique employed to deliver a vector into targeted tissue. Multiple viral and non-viral vectors have been developed for somatic gene therapy. In general, viral vectors offer several fold higher transduction efficiencies and long-term gene expression compared to non-viral vectors.^{1, 2} In the last two decades, efficacy of various viral vectors such as adeno, adeno-associated (AAV), retro, lenti, and herpes simplex, have been examined for introducing DNA into corneal cells *in vitro*, *in vivo* and *ex vivo*.^{1–4} Most of these studies

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used fluorescent or non-fluorescent marker genes to track the level and duration of transgene expression in cornea.² Viral vectors have been associated with toxicity and immunogenicity; however, previous studies have demonstrated AAV to be non-pathogenic and safe for corneal gene therapy in both the rabbit and mouse.^{1, 2, 5} To our knowledge no studies have been conducted to test the efficacy of AAV for the equine cornea.

Corneal disease is a significant cause of decreased or absent vision in horses world-wide.⁶ Vision loss in a horse frequently results in ocular pain, decreased performance, an increased safety risk and in some cases, the horse's very survival. Inflammation of the cornea as the result of ocular trauma, autoimmune or neoplasia are common causes of decreased corneal transparency.⁷ However, corneal ulceration and ocular trauma are the most significant pathologic entities affecting the equine eye.⁶ Corneal ulceration, particularly deep corneal infections can rob a horse of its vision, leaving it unable to fulfill the tasks that they are asked to complete. Treatment of corneal ulceration can be costly, labor intensive, and prolonged.⁸ Conventional treatment of corneal ulceration utilizes topical and systemic therapeutic agents including antimicrobials/antifungals to eliminate and control infectious agents, anticollagenases to decrease stromal degradation, mydriatics for ciliary spasm and stabilization of the blood-aqueous barrier, and non-steroidal anti-inflammatory drugs to alleviate ocular pain.^{6, 8, 9} Presently, pharmacological agents effective in the reduction of corneal fibrosis and specifically used to reduce corneal scarring, thus improving long-term visual outcome, are not routinely used in the management of equine corneal ulcers in veterinary medicine.

Numerous studies have demonstrated AAV to be a safe and efficient method for gene therapy in a variety of tissues.^{1, 2, 4, 10-12} Among recombinant viral vectors, AAV has shown the greatest potential for efficient gene delivery into ocular cells.^{2, 3, 13} AAV can transduce dividing and non-dividing cells as well as extend long-term transgene expression.² Although there are more than 100 serotypes of AAV, serotypes 1-9 have been the most commonly examined for gene therapy.^{14, 15} AAV serotypes 2 and 5 have demonstrated a varied degree of successful transgene delivery into photoreceptors and retinal pigment epithelial cells of the rodent, canine and primate *in vivo*.^{14, 15} Preclinical and clinical gene therapy trials have been undertaken for several retinal diseases, including ocular albinism, retinitis pigmentosa and Leber congenital amaurosis.¹⁶⁻²⁰ Compared to literature reports of retinal gene therapy, limited studies have been performed to evaluate the usefulness of AAV vectors in corneal gene therapy. The efficacy of AAV2 and AAV5 to deliver genes into the corneal stroma of rabbits and rodent *in vivo* has been established.²

Evidence suggests that the major determinant of cellular tropism and transduction efficiency of AAV is attributable to the capsid protein.²¹ Many hybrid AAV vectors have been engineered using capsid protein of AAV serotypes 1-9. AAV5 has proven superior for transgene delivery into the retina and cornea compared to AAV2.¹⁵ Our ongoing gene therapy studies in mouse and rabbit corneas have demonstrated that AAV5 and AAV8 are relatively more efficient at delivering genes into corneal stromal cells compared to AAV2, 6 and 9 serotypes (unpublished data, in press, Brain Research Bulletin). In this study we tested the hypothesis that AAV5 vector could effectively deliver a foreign gene into ECFs without causing adverse side effects. Specifically, the aims of this study were to: (i) evaluate efficacy of AAV5 expressing enhanced fluorescent green protein (EGFP) marker gene for delivering therapeutic genes into ECFs and (ii) establish the safety of AAV5 vector for equine corneal gene therapy.

Materials and Methods

Equine corneal fibroblast culture

Full-thickness 6-mm axial corneal buttons were aseptically harvested from healthy research horses undergoing humane euthanasia for reasons unrelated to this study. Slit-lamp biomicroscopy was performed by a veterinary ophthalmologist (EAG) prior to euthanasia to insure that harvested corneal tissues were collected from horses free of any anterior segment disease. Corneal samples were washed with serum-free Dulbecco's modified Eagles medium supplemented with antibiotics (penicillin-streptomycin). The epithelium and endothelium were removed by gentle scraping with a #15 scalpel blade. Using sharp dissection, the remaining corneal stroma was sub-sectioned into 2mm sections and placed on tissue culture dishes in a humidified CO₂ incubator at 37 degrees Celsius in Dulbecco's modified Eagles medium (DMEM) containing 10% fetal bovine serum for several weeks to obtain primary cultures of ECFs.

AAV vector production

AAV serotype 5 expressing EGFP under control of hybrid cytomegalovirus (CMV) + chicken β -actin (CBA) promoters (AAV5-pTRUF11-EGFP) was utilized for all experiments and obtained from Prof. Gregory Schultz and Prof. William Hauswirth, University of Florida, Gainesville, Florida. Construction of an AAV5 vector containing the enhanced green fluorescent protein (EGFP) reporter gene was performed according to standard helper-dependent techniques using adenovirus free system.¹

AAV vector and ECF transduction

Seventy-percent confluent cultures of ECFs in 12-well plates were used for all the experiments. Cultures were serum deprived for 2 hrs prior to viral transduction (viral titer of 10⁹ genomic copies/micro liter) and two microliters of AAV serotype 5 expressing EGFP marker gene under control of hybrid CMV + CBA promoters were added to ECF cultures via topical application in serum free medium at a multiplicity of infection (MOI) of 10⁵ and 2 \times 10⁵. After 6 hrs, the medium containing viral vector was replaced with fresh serum-containing medium and the cells were incubated for 2 days prior to microscopic evaluation of fluorescence.

AAV safety

Light microscopy images were obtained to observe any phenotypic or morphologic changes in ECF cultures after the application of AAV5.

TUNEL assay was used to detect fibroblast apoptosis due to AAV vector transduction. ECF cells were fixed in Paraformaldehyde at room temperature for 5 minutes and then washed in phosphate buffered saline. A fluorescence-based TUNEL assay was used according to the manufacture's instructions using ApopTag apoptosis detection kit (Chemicon international, Temecula, CA, USA). Cellular viability was assessed using a trypan blue (Sigma-Aldrich, St. Louis, USA) administered 48 hrs after transduction. Trypan blue dye staining dead cells blue and viable cells staining white were counted microscopically in a hemocytometer.

AAV transduction efficiency

EGFP transduced ECF cells and DAPI-nuclei in culture were counted using direct fluorescence microscopy at 100X magnification in ten randomly selected, non-overlapping areas for each treatment. Analysis was performed on a PC computer using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at <http://rsb.info.nih.gov/nih-image>). Percent transduction efficiency was

calculated by dividing the total number EGFP positive cells by that of the total number of DAPI stained nuclei.

Statistical Analysis

Results were expressed as mean \pm standard error of the mean (SEM). Statistical analysis was performed using two-way analysis of variance (ANOVA) followed by Bonferroni test for comparing data between control and AAV vector.

Results

AAV safety

Cultured ECFs demonstrated a classic spindle shaped morphology. No observable changes in light microscopic morphology were detected at any time point after successful transduction with AAV5 (Figure 1A–D). Throughout the study period, both the control and the AAV transduced populations continued to demonstrate a healthy spindle shaped morphology and progressed normally to confluence.

Trypan blue exclusion assay demonstrated cellular viability of AAV-treated and untreated ECFs between 96–98% at all three tested time points (8hrs, 24hrs and 48hrs) after AAV transduction. Recall that transduction efficiency was established at the 48 hour time point. The results of cellular viability determined with trypan blue assay are shown in Figure 2. The safety of AAV5 vector for ECF was further established by determining its effects on cell death with TUNEL assay. No detectable cellular apoptosis was detected using this method (results not shown).

AAV transduction efficiency

We were able to demonstrate the successful transduction of a foreign gene, EGFP, into ECF cells with topically applied AAV5 via fluorescent microscopy. Figure 3A–C demonstrates green fluorescence in a spindle shaped morphology in successfully transduced ECF cells against the blue DAPI-stained nuclei of the ECF cells. Quantification of this data was completed at 100x magnification using the previously described method and is displayed in Figure 3D. This data demonstrates a significant transduction efficiency of 13.1% using AAV5 in ECF *in vitro*.

Discussion

Corneal gene therapy represents a novel approach to the potential treatment of a wide variety of keratopathies. It may provide an opportunity to target the specific underlying cause of a corneal disorder as opposed to simply treating the symptoms as with conventional pharmaceuticals. Furthermore, its therapeutic benefits are evident long-term, even after a single treatment versus the required repeated applications for long periods with conventional small molecule drug therapy.² Gene therapy for equine corneal disease is a new application of this developing technology. Common equine corneal diseases include traumatic, inflammatory, neoplastic and infectious keratidities. For many of these conditions, particularly when severe, therapeutic goals are directed at preserving the globe and relieving ocular discomfort and to a lesser extent toward re-establishing clear corneal transparency. Many of these keratidities require labor-intensive treatments which can be difficult for both horse and owner. Gene therapy could provide an alternative to traditional small molecule therapy or be used adjunctively to improve corneal transparency and long-term visual success.

This is the first study to evaluate the transfection effectively AAV into ECFs. AAV5 was selected in this study because of its availability, enhanced transgene delivery, and low cellular toxicity. As this was the initial testing of AAV on ECFs, an *in vitro* model was used. *In vitro* models are economical, practical, easily maintained, and allow the investigator precision in control of experimental conditions. The transduction efficiency of AAV5 serotype determined *in vitro* for various cell types such as lung epithelium, skeletal muscle, gastrointestinal cells, and corneal fibroblasts was similar to that studied *in vivo*.^{22, 23, 24} Thus, it is reasonable to conclude that an *in vitro* model can provide highly relevant and beneficial information regarding the efficacy of the AAV vector for gene therapy *in vivo*.²⁵ Based on our earlier *in vivo* studies performed in mouse and rabbit corneas with selected AAV5 vector, we speculate that tested AAV5 vector will demonstrate similar gene transfer efficacy into equine stroma *in vivo* without causing significant side effects.^{1, 2} Further studies examining the potential of selected AAV5 vector to deliver therapeutic genes into the equine cornea *in vivo* are warranted.

Many laboratories, including ours, have demonstrated that recombinant AAV2 vectors can efficiently transport foreign genes in the cornea, retina, and various other ocular tissues.^{2, 14, 15} However, enhanced gene transfer has been noted with the hybrid AAV vectors developed subsequently by combining AAV2 genome with capsid proteins of other AAV serotypes (1–9). The hybrid AAV vectors were found superior to the conventional recombinant AAV vectors in regards to transduction efficiency and safety.^{2, 11, 23, 26} Also, hybrid vectors demonstrated unique tropism and variable transduction efficiencies for differing target tissues. For example, AAV 2/9 hybrid vector was more efficient at gene delivery in cardiac muscle cells compared to AAV2/1, AAV2/6, AAV2/7 and AAV2/8.¹⁸ Gao *et al*²⁷ found that AAV 2/8 had a 200 fold higher transgene delivery into liver cells compared to AAV2 vector. Interestingly, AAV2/6 hybrid vector was more efficient at gene delivery into muscle cells than the AAV2/5 or AAV2 vector.²² This pattern of cellular tropism and subsequent differences in gene efficiency has been documented with AAV in ocular tissues. The corneal stroma, photoreceptors, and retinal pigmented epithelium showed higher levels of delivered-gene expression with AAV2/5 compared to the AAV2 vector.¹⁵ The current literature suggests that further evaluation using different hybrid AAV vectors developed by combining AAV2 genome with various AAV serotypes capsids (AAV1-9) for the ECFs is also warranted.

A transduction efficiency of 13.1% was noted for the ECFs with the tested hybrid AAV2/5 vector in this study. Due to lack of experimental evidences at this point it is unknown whether this rate of transduction is of therapeutic value for the equine cornea. We hypothesize that moderate transduction efficiency ranging from 12–20% will be sufficient to treat corneal diseases in horse. Future investigations will test this hypothesis. Based on the results of our gene transfer investigations performed in mice and rabbit corneas *in vivo*, and human corneas *ex vivo* we speculate that for achieving maximum gene transduction in the equine cornea *in vivo* direct contact of AAV to the stroma for a short duration (2–10 minutes) may be required. AAV applied topically on the mouse or rabbit cornea with intact epithelium showed considerably low AAV penetration in the stroma compared to the corneas that received AAV after removing the epithelium¹. Also, corneas receiving AAV after epithelial removal showed transduction predominantly in anterior stroma and minimal in the posterior stroma. Gene therapy to treat corneal ulceration and scarring is clinically relevant as most myofibroblast formation is observed in the anterior stroma²⁸ except in the cases of very severe, deep ulceration. The immunogenicity of tested AAV5 vector *in vivo* also needs to be established. Of note, tested AAV5 vector did not cause clinically significant immune reactions in mouse or rabbit eyes *in vivo*, suggesting that similar results could be observed *in vivo* in horses.² In this *in vitro* study, testing of AAV5 resulted in no detectable adverse effects (i.e. loss of cellular viability, cell death, and/or phenotype change). Future

studies may examine the effects of different viral concentrations, viral incubation times, and comparison of the transduction efficiencies of several serotypes.

Gene therapy provides many possible therapeutic options depending on the gene being transduced. Potential areas of therapy include inserting the decorin gene into ECF.²⁹ The decorin gene is known to silence the effects of TGF β , which causes transformation of ECF to myofibroblasts and haze in the cornea.²⁸ The reduction in myofibroblast formation could then reduce the development of a corneal scar, a very common sequela to equine corneal ulceration. Another approach would be to deliver fibroblast growth factor (FGF2) gene with AAV in the face of a developed corneal scar or chronic corneal ulcer to reduce the established corneal scar. FGF2 has been shown to revert myofibroblasts to corneal fibroblast using an *in vitro* model.³⁰ Additional possibilities include modification of cytokine production in the cornea. For example Morrison *et al.*, used an AAV vector to increase IL-10 in the lung of pigs with *Actinobacillus pleuropneumoniae* and found a reduction in mortality and morbidity due to the decrease in macrophage activation which typically leads to significant lung pathology.³¹ In cases of severe equine infectious keratitis, specifically where keratomalacia is present, the immune response is fulminant and possibly over stimulated. Delivering a therapeutic gene to increase expression of IL-10 could reduce the immune response and if so, have clinical relevance. Greenberger *et al.*, used an AAV vector to increase IL-12 gene expression in tracheal epithelial cells of mice infected with *Klebsiella*. IL-12, a pro-inflammatory cytokine stimulates an immune response. In certain cases of equine corneal disease there appears to be a lack of an immune response. As a result these cases can take months to heal and may require surgical intervention to aid in the process. An AAV vector which could introduce an IL-12 gene to increase an immune response may be of benefit. The advantages of gene therapy by delivering a gene or gene products such as Flt23K, endostatin, kringle-5 domain of plasminogen, angiostatin, IL12, IL10 etc. to reduce corneal neovascularization has been demonstrated using experimental animal models.³² Additionally, gene therapy has huge potential for developing vaccines by delivering DNA encoding for HSV-1 glycoproteins, such as glycoprotein (g) D, gB1, or a cocktail of glycoproteins in an effort to increase immunity against HSV-induced ocular keratitis.³² This same biotechnology has the potential to be applied to equine patients inflicted with equine herpes keratitis.

To our knowledge, this is the first study to demonstrate that AAV5 can effectively transduce ECFs, making AAV-mediated gene therapy a plausible method for treatment of equine corneal disease.

Acknowledgments

Funding for this project was provided through a Clinician Scientist Grant (EAG, DGB, RRM) at the University of Missouri, College of Veterinary Medicine; RO1-EY-017294 (RRM) grant from the National Eye Institute, National Institutes of Health, Bethesda, MD; and an Unrestricted grant from the Research to Prevent Blindness (RRM), New York, NY. Authors thank Drs. Cecil Moore and Jacqueline Pearce for their useful suggestions.

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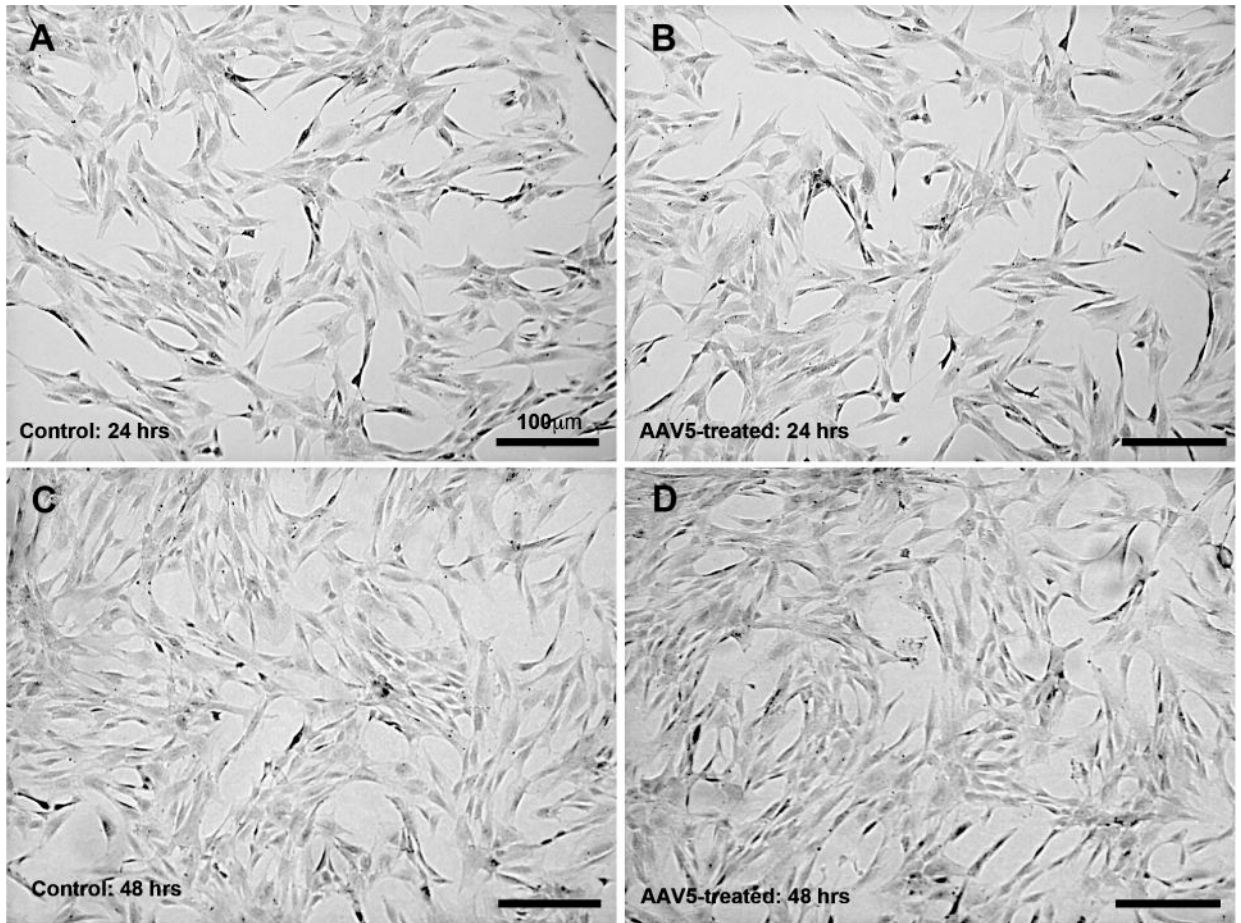


Figure 1.

Light microscopy images of both ECFs alone and ECFs after application of AAV5 at two time points (24 hrs and 48 hrs post application). All images demonstrate the classic normal, spindle shape morphology of ECFs. ECFs alone at 24 hrs (A), ECFs + AAV5, 24 hrs after application of AAV5 (B), ECFs alone at 48 hrs (C), and ECFs + AAV5, 48 hrs after application of AAV5 (D). Calibration bar is 100 microns.

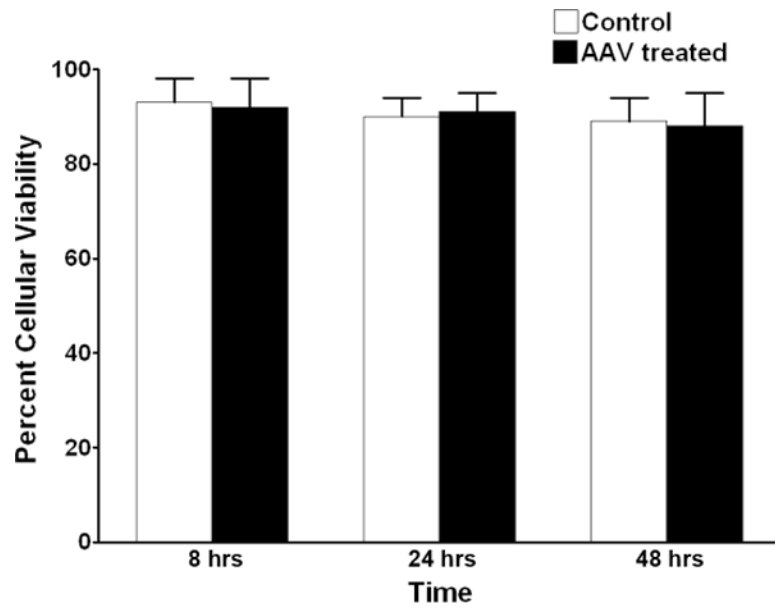


Figure 2. Percent viability of control ECFs and ECFs transduced with AAV5 over three time points: 8 hrs, 24 hrs and 48 hrs after AAV5 application.

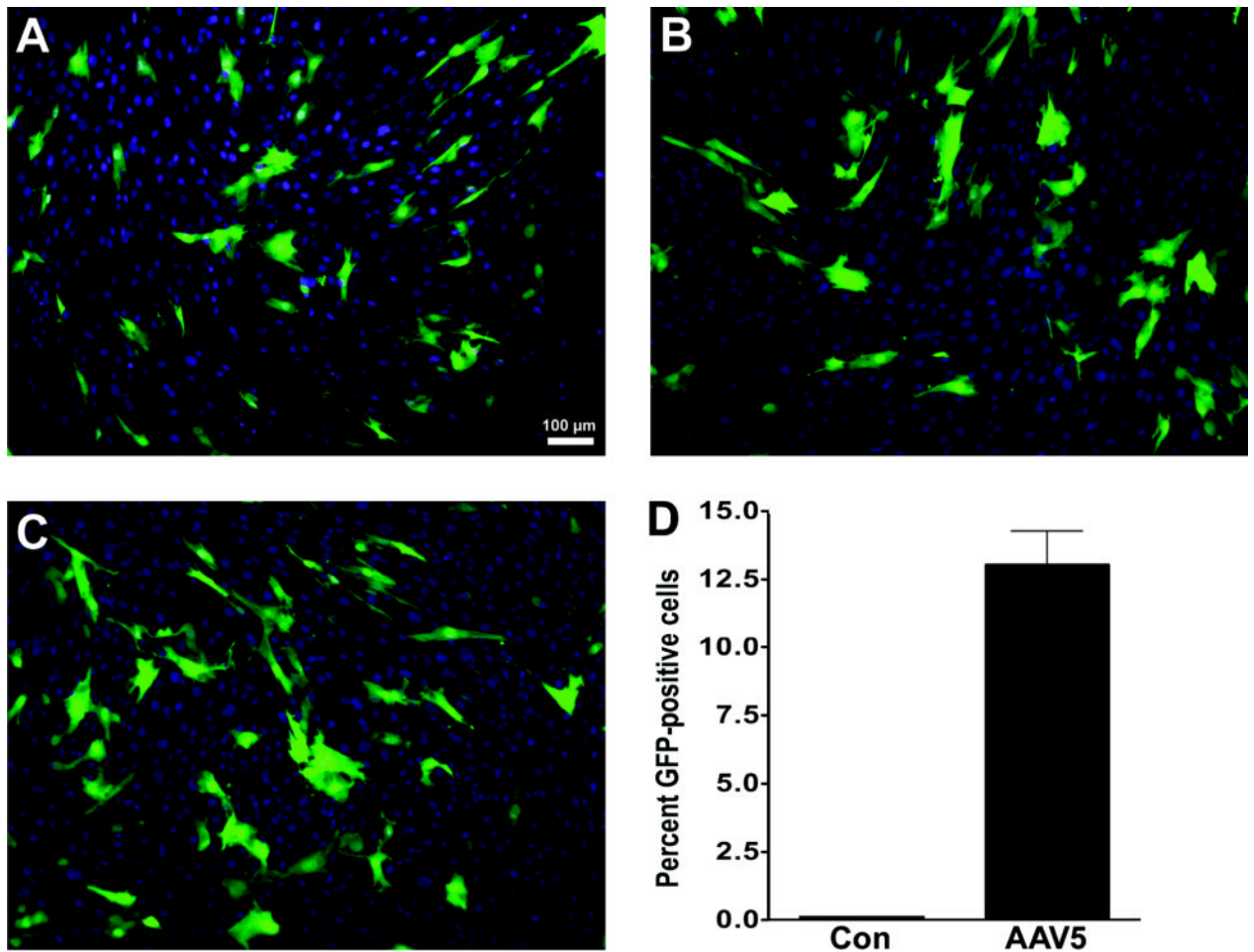


Figure 3.

Panels A–C demonstrate the successful transduction of ECF by AAV5 as evidenced by the expression of EGFP demonstrating the classic spindle shape cellular morphology. The ECF nuclei are stained by blue by DAPI. Panel D shows the quantification of the data using Image J software and expressed as percent transduction efficiency.