Trauma-Induced Vestibular Dysfunction: Improved Repair Under Local Treatment with α1-Antitrypsin

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One Sentence Summary: Local clinical-grade α1-antitrypsin therapy, unlike dexamethasone, restores vestibular function in trauma-induced inner ear injury in mice.

Abstract:

Background: Alpha1-antitrypsin (AAT) is a circulating tissue-protective molecule that rises during inflammatory conditions and promotes inflammatory resolution. Its local concentration in

the human perilymph inversely correlates with severity of inner ear dysfunction; concomitantly, mice that overexpress AAT and undergo inner ear trauma rapidly restore vestibular function. Locally applied AAT has yet to be examined in this context, nor has it been directly compared to anti-inflammatory corticosteroid treatment. **Aim**: To characterize vestibular recovery in a mouse model of unilateral labyrinthotomy under local AAT and dexamethasone treatments. **Methods**: Wild-type mice underwent unilateral inner ear injury. Nine microliters of either saline, clinical-grade AAT (180 μg/site), dexamethasone (4 mg/site) or both, were applied locally on days 0, 1 and 2 (n=5/group). Vestibular function was assessed for 7 days. In vitro, human epithelial gap closure assay was performed using A549 cells in the presence of AAT and dexamethasone. **Results**: Upon labyrinthotomy, all groups depicted severe vestibular dysfunction. Saline-treated mice sustained the longest impairment. That group and the dexamethasone group displayed partial to no recovery, while AAT-treated mice exhibited complete recovery within 7 days; at this time-point, dexamethasone-treated mice exhibited 50% recovery ($p<0.0001$). In vitro, co-treatment with AAT and dexamethasone resulted in a gap closure dynamic that was superior to AAT alone at 6 hours, and superior to DEX alone at 48 hours. **Conclusion**: Locally applied AAT treatment is superior to locally applied dexamethasone in promoting vestibular recovery in vivo. Ongoing studies are to explore the potential advantage of AAT combined with early, low-dose dexamethasone therapy.

INTRODUCTION

In the context of trauma–induced vestibular dysfunction, the mechanism of damage to neuronal afferent terminals in the inner ear remains poorly documented. In particular, information on the changes that take place at the primary vestibular endings during the first hours following insult, is lacking. A consistent feature, however, relates to insufficient repair of pivotal cellular structures due to excessive inflammation (1).

Steroid therapy is typically indicated in patients with Meniere's disease, idiopathic abrupt sensorineural hearing loss, vestibular neuronitis, autoimmune inner ear disease and noise-induced hearing loss. Steroids are also used during surgical procedures in the inner and middle ear, as in the case of cochlear implantation and stapes surgery. This strategy is justified by an effort to protect inner ear functionality in the face of excessive tissue-driven inflammation. In addition, applying local steroid therapy both minimizes the side effects of the systemic approach and results in higher concentrations of the drug in the inner ear (2, 3). However, corticosteroid therapy severely affects inner ear tissue repair by blocking initiatory inflammatory signals (4).

Alpha1-antitrypsin (AAT) is a circulating serine protease inhibitor that inhibits cell-injury–related proteases, such as tissue-degrading neutrophil elastase, cathepsin G and proteinase-3, as reviewed in (5). AAT reduces the production of injurious reactive oxygen species (ROS), as well as inhibits apoptosis of multiple cell types, such as neutrophils and epithelial cells. It is therefore not unexpected that AAT is associated with a beneficial reduction in bacterial burden and promotes wound healing. Its levels rise appropriately both locally and systemically, during hypoxia, inflammatory surges, acute phase responses and during the 3rd trimester of a healthy pregnancy. Aside from a sharp decline in the levels of endogenous human AAT (hAAT)

in the case of genetic AAT deficiency, AAT *insufficiency* is presently emerging as an entity relevant to several medical conditions (6).

Two recent studies depict a potentially protective role for AAT in the inner ear (9, 10). In one, endogenous AAT levels were determined in perilymphatic fluids collected from patients during cochlear implantation and were found to inversely correlate with hearing loss severity (10). In the other, using an inner ear injury animal model, augmented circulating levels of AAT were found to protect from tissue damage and had led to improved vestibular rehabilitation (9). Importantly, administration of local recombinant AAT to the middle ear was proven safe and feasible (7, 8). However, it has not yet been established whether *local* AAT therapy, with or without further steroidal treatment, addresses vestibular rehabilitation.

To demonstrate the proof-of-concept for the impact of locally applied clinical-grade AAT on inner ear tissue recovery and vestibular rehabilitation, a unilateral inner ear injury was induced through surgical labyrinthotomy in mice. These mice were then locally treated with either saline, dexamethasone (DEX), or clinical-grade AAT. In an epithelial gap repair model, the ability of AAT and DEX to induce a tissue repair profile was investigated, giving insight into the actions of supporting non-neuronal cells.

RESULTS

Effect of local AAT or DEX application on recovery from labyrinthotomy: General behavioral signs

Surgical labyrinthotomy was performed, in which local treatments were introduced (n = 5 per group): saline, AAT or DEX. Behavioral evaluation was performed serially for 1 week (**Fig. 1**

left). As shown, all mice experienced peak dysfunction scores by 6 hours from injury. At 24 hours, mice that received local AAT and mice that received local DEX treatments both had a significant improvement in behavioral scores compared to saline-treated mice. Subsequently, mice that received local DEX remained roughly unchanged throughout the remainder of the week, at which point their mean behavioral score was 5 out of 9, whereas mice that received local AAT treatment exhibited a steady improvement until near complete recovery by 1 week from insult (168 hours; mean score 0.5 out of 9). A statistically significant divergence between the two treatment groups was first observed 72 hrs from injury ($p = 0.0002$).

Effect of local AAT or DEX application on recovery from labyrinthotomy: Vestibular signs

In the vestibular scoring follow-up, DEX treatment afforded no advantage over saline treatment throughout the entire follow-up period (**Fig. 1** *right*). However, mice that received local AAT treatment during the surgical procedure exhibited rapid improvement as soon as 8 hours from insult, in the form of a sharp decline from a 6-hr peak score (from a score of 13 ± 2 out of 19 to 6.5 ± 2.5 out of 19, mean ± SEM). At the 8-hr time point, the difference between DEX and AAT treatments was significant (16.5 ± 0.3 out of 19 versus 6.5 ± 2.5 out of 19, mean ± SEM, $p = 0.0156$). Altogether, within 72 hours from insult, mice that received local AAT treatment during the surgical procedure had reached fully functional baseline vestibular scores, whereas saline and DEX treated mice were, at large, still lacking physiological vestibular functions.

Timeline aspects of AAT versus DEX treatment on recovery from labyrinthotomy

In noticing the differences between response to local AAT and to local DEX, the difference of means from severity of outcomes under DEX treatment and severity of outcomes under AAT treatment was calculated and plotted, whereby the status of the AAT group is set at 0 change (**Fig. 2**). As shown, general behavior within the first 24 hours from insult did not differ between the two approaches; however, days 1 through 7 from insult were typically less responsive to local DEX treatment. Regarding vestibular scores, the lack of advantage of local DEX treatment over local AAT treatment is clearly visible as early as 8 hours from insult, after which there is little to no benefit to DEX treatment over AAT treatment.

Combination of AAT and DEX treatments in an epithelial gap repair assay

In order to compare between the outcomes of treatment with DEX, AAT or both on epithelial gap repair, confluent A549 cells were disrupted uniformly and treated with 2.5% FCS in the presence of DEX (1 μg/ml) and/or AAT (10 μg/ml). Two snapshots were obtained in order to compute the extent of spontaneous closure of the gap: 6 hours and 48 hours from scratch. As shown (**Fig. 3**), between 6 hours and 48 hours from scratch, wells exhibited a reduction in gap area that reflected media conditions; medium enriched with 10% FCS allowed for a 2.0-fold reduction in gap size, medium enriched with 5% FCS allowed for a 1.6-fold reduction in gap area and the under-nourishing medium of 2.5% FCS resulted in a 1.5-fold closure. The intermediate 5% FCS profile was used to explore the impact of DEX and AAT on gap closure. As shown, the DEX treatment group exhibited a shift in gap area between 0 and 6 hours with an overall trend towards accelerated gap closure, albeit without reaching statistical significance; 48 hours after wounding, gap closure stopped at 66.7% mean area from initial wound area, a value inferior to the 2.5% FCS group at that time point. The overall advance from 6 hours to 48 hours

was 1.2-fold and did not reach statistical significance. In contrast, AAT-treated wells exhibited a similar spread of gap sizes at 6 hours to that of DEX, yet achieved a significant 2.0-fold closure at 48 hours from wounding.

Upon combining both AAT and DEX treatments in the same wells, wound closure was enhanced both at 6 hours and at 48 hours from wounding, resulting in a mean gap area of 48.1%, not unsimilar to that observed in the conditions of medium enriched with 10% FCS. Of note, the combination of treatments provided a more rapid closure when compared to each single treatment, but at distinct time points; DEX plus AAT resulted in a more rapid closure at 6 hours compared to AAT alone, and a more rapid closure at 48 hours compared to DEX alone. Altogether, both AAT alone and the combination of AAT and DEX were superior to DEX alone at 48 hours.

DISCUSSION

As there are studies addressing benefits of systemic augmentation with AAT, the current study investigates the effect of locally applied AAT on functional recovery from inner ear injury in mice. The study also compares differences between the impact of local AAT and standard steroidal therapy, and challenges the possibility that, combined with local AAT therapy, standard of care local DEX treatment may display improved outcomes.

Protecting tissues from excessive inflammation using glucocorticoids results in effective blockade of inflammation, primarily via inhibiting the NF-kB pathway; yet it does not instigate processes of inflammatory resolution or tissue repair. Due to molecular inflammatory blockade at the expense of proactive tissue repair, affected tissues are at risk of functional loss. Patients with

genetic AAT deficiency exhibit signs of poor tissue repair, most typically in the form of lung alveolar wall degradation (non-smoking emphysema); for these individuals, human plasma-derived affinity-purified AAT is given in the form of weekly intravenous slow-drip infusion sessions. In recent years, interest in some potential benefits of clinical-grade AAT therapy for enhancing tissue repair outside genetic AAT deficiency, has gained attention, leaning on the assumption that AAT might be *insufficient* in several particular setups.

In the present study, mice treated with local AAT responded with accelerated vestibular function recovery times, consistent with a previous study that examined inner ear injury in transgenic mice that express elevated circulating human AAT (9). In that report, transgenic mice depicted a 50% recovery of vestibular scores within 24 hours of injury, compared to wild-type mice that scored poorly at that time point. This may indicate that, as hypothesized, AAT diverts excessive inflammation towards active tissue repair, leading to improved functional tissue reinstatement. With minimal tissue injury-related molecular signals in the presence of elevated AAT, it is presumed that the vicious cycle of cell injury and inflammation is minimized.

In exploring the effect of AAT on recovery from a unilateral inner ear injury, we observed two main levels of impact: kinetics, and amplitude. In the acute phase of tissue injury, which peaks rapidly within the first hours of vestibular impairment, mice displayed the various symptoms of vestibular dysfunction regardless of treatment grouping, and their respective intensities reached maximal scores. Subsequently, the symptoms gradually regressed, each with its own unique kinetics. Observations similar to ours have been obtained using the TTK rat model (17). It is interesting to note that post oculomotor alterations also affect various activities of general behavior. Thus, during the early acute inflammatory phase, there are significant changes in walking, vertical and horizontal exploration behaviors, as well as positioning of the

body. Most of these behavioral changes regressed in AAT-treated mice within days from injury, compared to 1-2 weeks in the control group. Indeed, vestibular compensation occurs as a process of behavioral recovery in unilateral removal of the vestibular receptors (11). However, electrophysiologic and histologic evidence shows that vestibular compensation is not due to regeneration of the vestibular receptors nor of a recovery of the 8th nerve (12, 13, 14). It is therefore generally accepted that vestibular compensation is due to some form of CNS plasticity, both at the contralateral (15) and ipsilateral (12, 16) nuclei. The local ipsilateral compensatory mechanism is most likely what we are engaging with in the present model.

According to the epithelial gap closure assay, changes in the capacity of cells to restore confluence displayed differences between AAT and DEX treatments. In the earlier hours, where inflammation is assumed to be required for instigating desired processes but also disrupts complex cell functions, DEX appeared to promote gap closure. At that time point, cell migration in wells treated with AAT resembled control conditions. However, at 48 hours from injury, the difference between DEX and AAT-treated wells was profound: in the presence of DEX, gap closure was stunted and had barely advanced from the 6-hr point. In contrast, cell migration in AAT-treated wells was as far advanced as the plentiful conditions of 10% FCS, agreeing with previously reported data using other cells types (e.g., Caco2 colon epithelial cells), drug concentrations (e.g., AAT at 0.1-0.5 mg/ml) and experimental protocols (e.g., 96-well plates) (18, 19, 20). Perhaps more intriguing is the combination of the two approaches; when cell were co-treated with AAT and DEX, the early point of 6 hours depicted a profile of gap closure that was superior to AAT alone, and the point of gap closure at 48 hours was markedly superior to that of DEX alone. Based on direct comparison between the impact of DEX and of AAT in the same system, it appears that early intervention with DEX, possibly at a low dose, may be

followed by AAT; in this manner, DEX should not interfere with the requirement of AAT to send NF-kB family member, p65, to the nucleus for advancement of the pro-resolution gene expression profile. Indeed, in the context of physiological acute phase responses, AAT does not spike but rather rise within 1-2 days, allowing for initial inflammatory pathways to instigate important biological processes.

The present study provides valuable insights that invite further exploration of tissue recovery in the context of local compensation mechanisms. The fact that delayed or insufficient tissue recovery processes, independent of direct neuronal injury, have a significant negative impact on patients' quality of life, should serve as an incentive to extend the study of complex neuronal function loss to the local supporting tissues, ideally realizing their full potential as key to functional tissue restoration. Thus, future research should examine cellular and molecular alterations in the inner ear compartment under conditions of AAT-driven enhanced tissue repair.

MATERIALS AND METHODS

Animals

Experimental protocols and animal care were in compliance with institutional guidelines and approved by Institutional Animal Care and Use committee. Animal experiments were performed on wild-type C57BL/6 mice (6-8–week old, Harlan, Israel). All animals were kept in standard animal cages under conventional laboratory conditions, and all behavior experiments were conducted during the light phase.

Surgical labyrinthotomy

All surgical procedures were performed under anesthesia induced by ketamine and xylazine. Unilateral microsurgical labyrinthotomy was performed, as described elsewhere (21). Briefly, the surgical area was shaved, disinfected and treated with topical lidocaine. A retroauricular incision was performed to expose the external ear canal, which was then opened immediately anterior to the exit point of the facial nerve. The tympanic membrane was partially removed and stapes dislocated to expose the round window. Guided by a microsurgical binocular (Zeiss), a 1-mm opening was created around the oval window, creating a fistula between the inner ear and middle ear compartments, with visible leakage of inner ear fluid. A volume of 10 µl of either saline, clinical grade AAT (Glassia®, Kamada LTD, Israel; 180 µg/site) or DEX (West-Ward Pharmaceuticals, NJ, USA; 4 mg/site) was injected through the perforation at close proximity to the site of injury while the surgeon was blinded to experimental subgrouping. The perforated tympanic membrane was then sealed by absorbable gelatin powder (Gelfoam®) and the retroauricular incision was closed using absorbable sutures. Mice were placed on a heating pad on their side with the injected ear facing upwards, and allowed to recover for at least 30 minutes. Treatments were introduced again on days 1 and 2 from surgery under mild anesthesia.

Follow-up: Behavioral parameters and vestibular signs

Spontaneous behavior was evaluated in the evenings at indicated time points from injury, during a 2-min time period for each mouse, as described elsewhere (22). The evaluator was blinded to experimental subgrouping. A general behavior score was compiled by the summation of 3 parameters, each determined on a severity scale of 0 *(assured)* to 3 *(null)*: Horizontal exploration, vertical exploration and walking. Baseline indexes were obtained as reference and graded 0.

A general vestibular score was compiled by the summation of severity scores of 6 vestibular signs: *Stereotyped rotatory movement* (circling around the hips), *head inclination* (head tilt), *muscle dystonia* (hypertonia on the side of the lesion), *tumbling* (turning around own central axis, scored from none to uninterrupted spins), *head bobbing* (abnormal intermittent backward extension of the neck, scored from none to compulsive movement) and a *tail spin test*. The first 5 parameters were scored 0 to 3 (0, no visible sign; 1, faint presence of sign; 2, clear evidence of sign and 3, maximal expression of sign). In the *tail spin test*, also known as tail hanging-landing test (22), mice were lifted above the tabletop to a height of 5 cm by the distal end of their tail, for 5 seconds. A normal response consisted of forelimbs *stretching towards the floor*, while 'vestibular' mice display repeated *upward trunk curling*. Responses were scored on a scale of 0 to 4 (0, stretching towards the floor with a maximum of a single curling act; 1, stretching toward the floor and curling up twice; 2, stretching toward the floor and curling up three times; 3, stretching toward the floor and curling up four times; 4, no stretching towards the floor with repeated curling up).

In vitro human epithelial gap repair assay

In-vitro scratch assay was performed using A549 cells (ATCC). Cells were grown to confluence in 24-well plates and uniform wounds were inflicted using a sterile 200-µl pipette tip, thus creating a cell-free area, as described elsewhere (23). Cultures were washed twice with complete RPMI 1640 supplemented with 2% FCS (both from Biological Industries Inc., Beit Haemek, Israel). Treatments were introduced directly onto cells in 5% FCS; control conditions included media supplemented with 2.5% FCS or enriched with 10% FCS to represent inept and plentiful repair conditions, respectively. Images were acquired immediately after wounding and

then again 6 and 48 hours later using a photomicroscope (Zeiss). Images were analyzed by ImageJ and cell-free areas were marked; outcomes are represented as percent from initial wound area per well.

Statistical analysis

Nonparametric unpaired t-tests with Welch's correction were used to assess statistical significance with 95% confidence interval. Results are shown as mean ± SEM, $p < 0.05$ was considered significant. Statistical processing was performed using GraphPad Prism software (GraphPad Software, La Jolla, CA).

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Competing interests: Authors declare that they have no competing interests.

Data and materials availability: All data are available in the main text or the supplementary materials.

Figures

Fig. 1.

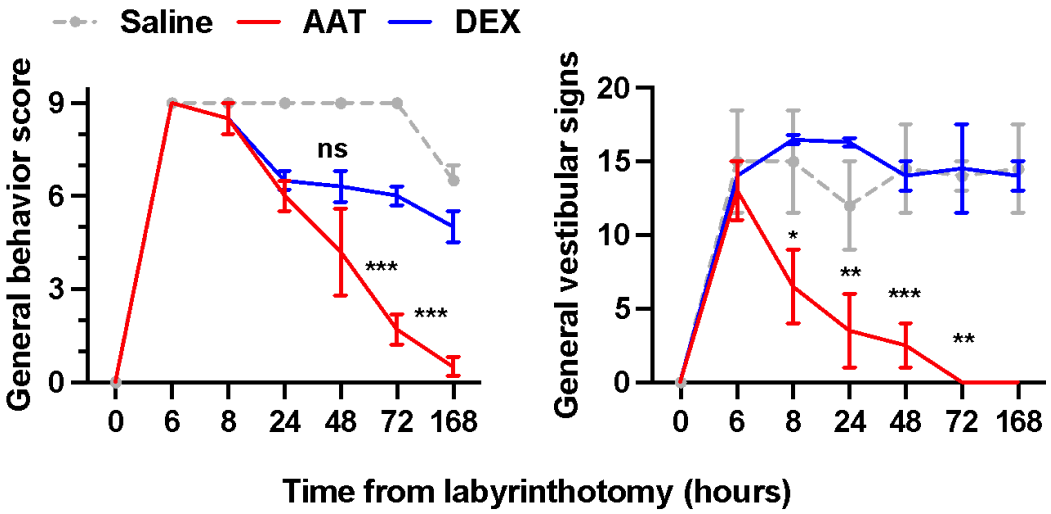

Figure 1. ***Effect of local application of AAT or DEX on recovery from surgical labyrinthotomy***. Unilateral surgical labyrinthotomy (n = 5 per group) under local treatment with saline, AAT or DEX, at time of surgery and on days 1 and 2 from surgery. Serial behavioral and vestibular scores. Mean ± SEM. Comparison between AAT and DEX groups per time point: ns, non-significant. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Fig. 2.

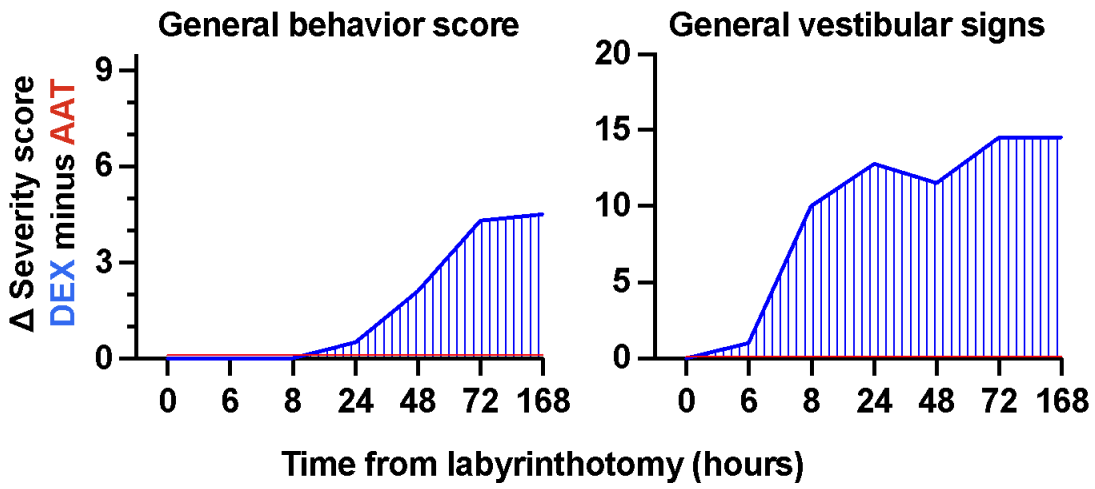

Figure 2. ***Chronicity aspects of AAT versus DEX on recovery from surgical labyrinthotomy***. Unilateral surgical labyrinthotomy (n = 5 per group) under local treatment with saline, AAT or DEX, at time of surgery and on days 1 and 2 from surgery. Calculated difference in severity of serial behavioral and vestibular scores between DEX and AAT group means.

Fig. 3.

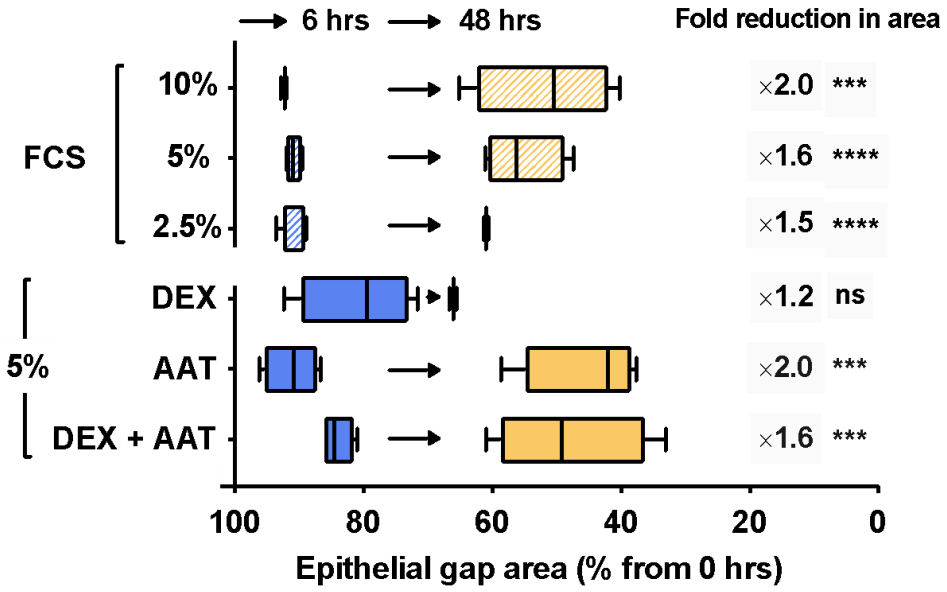

Figure 3. ***Epithelial cell gap closure assay***. Monolayers of human epithelial cell line (A549) were disrupted by direct linear scratch, set as time 0. Culture conditions of 2.5% and 10% FCS signify nutrient-poor or -rich conditions, respectively. Treatments (DEX 1 μg/ml; AAT 10 μg/ml) were applied to wells containing 5% FCS. Gap area was quantified from culture images acquired at indicated time points, represented as % from initial wound area. *Blue*, 6-hr time point; *yellow*, 48-hr time point. Box-and-Whiskers, min to max, quartiles. ns, non-significance between time points; *ns*, non-significance from control conditions. ***p<0.001, ****p<0.0001. Representative results out of three independent experiments.