Title: ***In vitro*** **inhibition of the CFTR ion channel in the cervix thickens cervical mucus**

Running Title: CFTR inhibition thickens cervical mucus in vitro

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Abstract

Study question: Does the inhibition of Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) alter cervical mucus produced by endocervical cells *in vitro*?

Summary answer: Inhibition of CFTR dehydrates and thickens *in vitro* endocervical secretions.

What is known already: Cervical mucus changes throughout the menstrual cycle in response to hormonal fluctuations, helping to regulate fertility and access of pathogens to the reproductive tract. CFTR is an anion channel that plays a critical role in mediating epithelial mucus secretions.

Study design, size, duration: *In vitro* experiments conducted with control and experimental arms.

Participants/materials, setting, methods: Primary endocervical cells obtained from rhesus macaques *Macaca mulatta* were treated with vehicle controls or CFTR inhibitor Inh-172. Hydration and viscosity of secreted mucus was measured through air surface liquid and particle tracking microrheology.

Main results and the role of chance: Endocervical cells treated with CFTR inhibitor Inh-172 demonstrated dehydrated, thicker mucus secretions compared to control ($p<.01$).

Limitations, reasons for caution: *In vitro* models do not fully recapitulate cellular mucus secretions.

Wider implications of the findings: Cervical mucus plays an important role in fertility regulation. Our studies suggest that CFTR may be an important mediator of those changes and provide experimental evidence for the infertility phenotype seen in women with cystic fibrosis. Additionally, assays used in these studies are useful endpoints for studying cervical mucus regulation *in vitro*.

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39 **Introduction**

40 Epithelial cells, lining the endocervical canal, control sperm and pathogen entry into the female
41 reproductive tract through cervical mucus secretion. Over the course of the menstrual cycle, fluctuations
42 in ovarian sex-steroids, estradiol (E2) and progesterone (P4), regulate the secreted mucus (Moghissi *et*
43 *al.*, 1972; Najmabadi *et al.*, 2021). When E2 levels peak during the ovulation window, cervical mucus
44 increases in quantity and hydration, and decreases in viscosity, creating a permeable gel that allows
45 sperm to move through to the upper tract. Under the influence of P4, following ovulation during the
46 luteal phase, cervical mucus increases in concentration, becomes more viscous, and blocks sperm from
47 entering the reproductive tract (Moghissi, 1972).

48 Cervical mucus is a heterogenous mixture of water, mucin and non-mucin proteins, lipids,
49 carbohydrates, and ions. Outside the cervix, other epithelia, including the lung, GI tract, and eye, also
50 produce mucus that maintain organ health and functions. Mucins and proteins found in mucus are
51 retained across organ systems (Thornton *et al.*, 2008). The Cystic Fibrosis Transmembrane Conductance
52 Regulator (CFTR) ion channel has been shown to be a critical regulator of mucus characteristics in all
53 mucus secreting epithelia. Mutations in the CFTR gene lead to dysfunctional channel activity and the
54 autosomal recessive disease, cystic fibrosis (CF). CF is characterized by systemically thickened secretions
55 that can cause multi-organ dysfunction in the airway, GI tract, and reproductive tract. In the female
56 reproductive tract, CF is notable for thickened endocervical mucus as well as sub-fertile and infertile
57 phenotypes (Anthony *et al.*, 1970; Kopito *et al.*, 1973).

58 The CFTR channel moves chloride and bicarbonate (ions) into the lumen, promoting hydration
59 and the thinning of mucus through several mechanisms. In conjunction with the epithelial sodium
60 channel (ENaC) and other apical ion channels, CFTR maintains an ionic gradient for water to move
61 osmotically (Saint-Criq and Gray, 2017). CFTR has also been shown to play a critical role in hydration of
62 mucins. Intracellularly, large mucin glycoproteins are tightly packaged, with cations shielding their

63 negatively charged carbohydrate-rich ends. Upon release, luminal anions remove the cations resulting in
64 the expansion and unfolding of the mucins. In their expanded form, mucins readily bind water
65 (Muchekehu and Quinton, 2010). When CFTR is dysfunctional, mucins are unable to fully expand and
66 bind water, leading to the dehydrated, thickened mucus seen in CF (Clunes and Boucher, 2007).

67 Previous studies have demonstrated that CFTR in the cervix is both functionally and hormonally
68 regulated (Han *et al.*, 2022). While these findings may suggest that CFTR plays a role in regulating mucus
69 changes in the cervix, it remains unclear if CFTR directly alters mucus consistency and hydration. CFTR
70 inhibition of endocervical cells and its effect on mucosal thickness and hydration has yet to be
71 demonstrated in an experimental model. Elucidating the function of CFTR in cervical mucus regulation
72 would identify its role as a target for both fertility and contraceptive studies in the cervix.

73 Prior studies using 2D cultures of the endocervix lack experimental endpoints for measuring
74 mucus changes in response to perturbation experiments. To overcome this limitation, we adapted from
75 airway studies of mucus, airway surface liquid (ASL) as a method for measuring mucus hydration *in vitro*.
76 ASL measurements correlate tightly to interfacial hydration *in vivo* (Tarran and Boucher, 2002; Harvey *et*
77 *al.*, 2011; Haq *et al.*, 2016). ASL hydration in normal airway epithelia (Tarran and Boucher, 2002;
78 Myerburg *et al.*, 2006) is lost in CF cells due to mucosal dehydration (Matsui *et al.*, 1998) and can be
79 restored in mutant cell lines with novel CFTR modulator therapies (Haq *et al.*, 2016; Berg *et al.*, 2019).
80 While ASL can be directly measured using confocal microscopy in the XZ axis (Tarran and Boucher, 2002),
81 ASL can also be assessed by measuring the edge-meniscus formed under brightfield microscopy that
82 allows 96-well experimental measurement and replication (Harvey *et al.*, 2011). Brightfield meniscus
83 width is a valid measurement of ASL and can be used for high throughput studies of CFTR modulator
84 therapies (Berg *et al.*, 2019).

85 While ASL is a measure of liquid-ion transport functionality, it does not provide a direct readout
86 of the biophysical status of the mucus in the lumen. Multiple studies have shown that mucus

87 concentration is the primary driver of aberrant biophysical properties (i.e. viscoelasticity) observed in CF
88 (Button *et al.*, 2012; Hill *et al.*, 2018). This has been demonstrated with classical rheometric and
89 microscale techniques relevant to the biophysics experienced by sperm within mucus, generally termed
90 particle tracking microrheology (PTMR). PTMR exploits the thermal diffusion of small (~1 µm)
91 fluorescent probes to sense the viscoelasticity of the mucus network.

92 In this investigation, we utilized both ASL measurements of mucus hydration and PTMR
93 measurements of viscosity in response to hormonal changes and CFTR inhibition. We hypothesized that
94 selectively inhibiting CFTR with CFTR Inhibtor-172 (Inh-172) would result in decreased mucosal
95 hydration, and increased mucus viscosity in vitro.

96 **Methods**

97 *Primary cell culture*

98 Rhesus macaque cervical tissue was obtained from the Oregon National Primate Research
99 Center tissue distribution program. Endocervical epithelia were scalpel separated from the cervical
100 stroma, enzymatically digested, and cultured using conditionally reprogramming conditions as
101 previously described (CRECs) (Han *et al.*, 2020). Expanded cells were passaged at 80% and placed onto
102 HTS Transwell® 96-well and 12-well Permeable Supports (0. 4 µm)(Corning, New York) in a serum-free
103 media (ReproLife CX Lifeline Cell Technology, Frederick, Maryland), supplemented with 1M calcium
104 chloride and 10nM E2 and taken to an air-liquid interface (ALI).

105 *Airway surface Liquid*

106 After 10-14 days at an ALI (when transepithelial electrical resistance >300 Ω), we performed the
107 air-surface liquid assay on treatment naïve cells. For ASL, we adapted an assay variation used for high-
108 throughput screening in human bronchial epithelial (HBE) cultures that measures the resorption of
109 added saline to the apical (mucus) side of ALI culture (Berg *et al.*, 2019). Cultures that create more

110 dehydrated mucus resorb more saline over time. To do this, we used two different versions of the
111 resorption assay. In a version similar to the previously published HTS, phosphate-buffered saline (PBS; 8
112 μL per well) (Thermo-Fisher Scientific) was added to the apical surface and images were taken using
113 brightfield microscopy (2x PlanApoλ NA 0.10 magnification, BZ-X Series, Keyence, Itastca, Illinois).
114 Images were taken pre-treatment, immediately post-treatment (0 hours), 24, 48, 72, and 96 hours.
115 We additionally assessed these changes using an apical addition of 8 μL per well of 100 mM
116 mannitol in PBS. Mannitol is a sugar alcohol that demonstrates osmotic based expansion of ASL in HBE
117 cells and has been studied in clinical trials as potential treatment for CF lung disease (Jaques *et al.*, 2008;
118 Keyrouz *et al.*, 2008). We used this combined method of assessing ASL (mannitol+ PBS) as we found that
119 it more robustly defined changes between controls and treatment in ASL studies (Locke *et al.*, 2014).
120 Following image acquisition, we used custom FIJI (Schindelin *et al.*, 2012) scripts to automatically
121 calculate the change in fluid meniscus. The scripts use pixel thresholding to determine the size of the
122 meniscus by measuring the annulus; the annulus is the area of dark fluid meniscus that emanates from
123 the edge of the well to the lighter center region (Figure 1A,B). This area increases with greater apical
124 hydration and decreases with reduced hydration. The metrics were reported as magnitude change from
125 baseline (time=0 hours) in downstream analysis and statistics. More negative values correspond to
126 greater reduction in the meniscus from the 0Hr time point (time of apical application of PBS). Thus,
127 larger change in meniscal reduction indicates increased dehydration of the cell. As part of our custom
128 scripts, we additionally adjusted for overlying artifacts (i.e. overlaying cellular mucus or debris) in the cell
129 cultures using thresholding automatic identification of outliers based on a pre-determined 95%
130 confidence interval. Using the 96-well plate, treatment conditions were repeated using two different
131 plates to account for edge effects with a total of either 48 or 96 wells per condition. Experiments
132 represent at least three or more separate primary cell culture experiments.
133 *Air Surface Liquid Baseline Assays*

134 We began by demonstrating the ability of our assay to measure apical hydration using mannitol
135 only treatments (Myerburg *et al.*, 2010). Cells were treated with vehicle or mannitol (100mM and
136 300mM) in PBS (8 μL) applied apically on day 1. We also sought to demonstrate that ASL measurements
137 responded to hormonal regulation of mucus. Clinically, E2 peaks during ovulation and results in thinner,
138 hydrated mucus. During the luteal phase, P4 dominates, and mucus becomes thicker and more
139 dehydrated. Thus, we would expect E2 to increase ASL relative to control, and P4 to decrease ASL
140 relative to control (Moghissi, 1972). To do this, we treated differentiated cells with E2 (10 nM) or E2/P4
141 (10 – 14 days of 10 nm E2 priming followed by 1nM E2 and 100 nM P4) basolaterally and compared the
142 responses to the vehicle only controls (Nussey and Whitehead, 2001; Hombach-Klonisch *et al.*, 2005;
143 Buckner *et al.*, 2011).

144 *ASL Measurement of CFTR Inhibition*

145 CFTR was inhibited with two small molecule CFTR inhibitors: Inh-172 (5 or 10μM) or GlyH-101
146 (5μM) (MilliporeSigma, Burlington, Vermont)(Verkman *et al.*, 2013). The cells were dosed in the
147 basolateral compartment of the Transwell® every other day.

148 *Microrheology*

149 To use PTMR for measuring viscosity, 1 µm diameter carboxylated FluoSpheres™ (ThermoFisher,
150 Fremont, CA), henceforth referred to as "beads", were added to the lumen of cell cultures. The thermally
151 driven motion of the beads was tracked to determine the viscoelastic properties of the luminal mucus.
152 Bead motion was recorded for 10s at a rate of 60 frames/s using a 40x air objective on a Zeiss AxioVert
153 A1 Fluorescent microscope (Zeiss, Jena, Germany). Individual bead trajectories were measured
154 automatically using a custom Python program that uses TrackPy (Markovetz *et al.*, 2019) for bead
155 localization and tracking. Bead motion was converted into mean squared displacements (MSD) and
156 complex viscosity (η^*) values in accordance with the mathematics described previously (Hill *et al.*, 2014;
157 Markovetz *et al.*, 2019).

158 *Statistics*

159 One-way ANOVA was administered using SAS statistical software (Cary, NC). A general linear
160 model testing least squares means for effect was generated and p-values less than 0.05 were considered
161 significant. "N" was denoted as the number of primary cell cultures used for 96-well experiments.
162 Magnitude or reduction in the meniscus from baseline are expressed at means ± SEM.

163 **Results**

164 *Air-surface liquid response to hormonal manipulation*

165 When treated with mannitol (100mM and 300mM), compared to vehicle-only controls, cultures
166 demonstrated a dose-dependent increase in apical hydration at 24 and 48 hours post treatment ($P<0.05$)
167 (Figure 2). Treatment with 10 nM E2 resulted in greater ASL hydration when compared to the control
168 condition ($p<0.001$) (Figure 3A). Similarly, treatment with 100nM P4 resulted in greater reduction in ASL
169 than 10 nM E2 at every time point ($P<0.05$) (Figure 3B).

170 *ASL response to CFTR Inhibition*

171 10μM Inh-172 resulted in statistically significant ASL dehydration by 48, 72, and 96 hours
172 ($p<0.01$), while 5μM GlyH-101 had a smaller but still significant dehydration effect ($p<0.05$), compared to
173 the control condition (Figure 4A,B). Using mannitol in conjunction with PBS or the inhibitors amplified
174 the dehydration response (Figure 4B). The reduction of ASL volume by Inh-172 was visually notable
175 (Figure 5), and the resultant increase in mucus viscoelasticity was quantified by PTMR in n=6 cultures
176 and found to be significant ($p<0.01$)(Figure 6).

177 **Discussion**

178 Using two different phenotypic assays measuring mucus properties, we demonstrate that
179 inhibition of CFTR *in vitro* changes secreted mucus by endocervical epithelial cells. Specifically, our
180 results are consistent with the *clinical* findings that mucus is more hydrated in response to E2,
181 dehydrated when exposed to P4, and that CFTR inhibition results in abnormal, dehydrated, thickened

182 mucus secretions similar to that seen in cystic fibrosis (Kopito *et al.*, 1973). Combined with prior studies
183 that demonstrate the hormonal regulation of CFTR during the menstrual cycle, these findings further
184 support the integral role of CFTR in endocervical mucus changes.

185 Additionally, this study translates two assays, ASL and PTMR, into endocervical mucus studies
186 (Harvey *et al.*, 2011; Markovetz *et al.*, 2019). New to endocervical mucus studies, these assays provide
187 experimental endpoints that directly reflect mucus changes secreted by cultures, correlated to
188 biophysical properties of mucus itself. ASL, in particular, has potentially high experimental utility as it has
189 been both well validated in mechanistic studies of mucus regulation and used in a high-throughput
190 fashion for drug screening (Berg *et al.*, 2019). PTMR is a useful technique for this study for two reasons:
191 it operates and measures forces at the same scale as sperm in the cervical mucus environment, and it
192 has been shown that there is a strong correlation between *in vitro* mucus models and *in vivo* mucus in
193 the airway (Markovetz *et al.*, 2019). Uniquely, the reduced ciliary activity/presence in the CREC cultures
194 allows for PTMR to be performed *in situ*, further increasing its utility here.

195 Both assays provide potential endpoints for future studies of mucus *in vitro*. One challenging
196 characteristic of studying endocervical mucus is the lack of valid small animal models. Classic
197 mammalian models, such as rodents, are poor options for studying cervical mucus changes or lower
198 tract fertility as they lack a mucus secreting endocervix and sperm is ejaculated directly into the uterus
199 (Cunha *et al.*, 2019). In larger mammalian models, experimental rigor is challenging due to biological
200 variability, semi-quantitative nature of mucus appraisal, and invasiveness for mucus collection. An *in*
201 *vitro* model provides a tool for mechanistic studies to determine the key regulators of mucus changes
202 such as CFTR, but also a platform for drug discovery. With recent advances in primary cell culture such
203 as conditional reprogramming, there is further potential for study of genetic differences and
204 personalization of drugs response (Liu *et al.*, 2017).

205 Our previous studies demonstrate that CFTR is a hormonally regulated ion channel with E2 (mid
206 cycle) conditions increasing CFTR expression and, conversely, P4 (luteal) conditions decreasing CFTR
207 expression (Han *et al.*, 2022). In CF, the CFTR gene mutation produces a defective channel, resulting in
208 thick, viscous cervical mucus. Prior to the drug discovery and development of small molecules that
209 modulate mutant CFTR function, women with CF had known infertility/subfertility phenotypes that were
210 largely attributed to these mucus changes, preventing sperm penetration (Hodges *et al.*, 2008). In our
211 study, we used inhibitors to mimic CFTR mutation, recapitulating this phenotype *in vitro*. Thus, our study
212 is one of the first to experimentally confirm the relationship of CFTR inhibition and mucus changes using
213 loss of function experiments *in vitro*. Additionally, it provides evidence that CFTR is likely a critical
214 mediator of mucus changes in normal menstrual cycles.

215 Our *in vitro* study does not fully recapitulate either tissue level characteristics of epithelial
216 biology nor the full content of mucus secretions produced *in vivo*. However, the conditionally
217 reprogrammed platform used in this investigation provides a robust model where we can investigate
218 epithelial changes under multiple treatment conditions, while evaluating mucus production and quality.
219 We have previously demonstrated in proteomic studies, high similarity between the mucus produced in
220 vitro using this cellular system to *in vivo* mucus (Han *et al.*, 2020, 2022; Park *et al.*, 2022), and epithelial
221 only cell models have been used to high success in disease investigations and drug discovery in other
222 mucosal systems (Gentzsch *et al.*, 2017; Liu *et al.*, 2017).

223 The extent to which CFTR is responsible for mucus changes in the cervix is not fully quantified by
224 our studies. CFTR, while a critical channel with a well-known mutated human phenotype, is part of a
225 large ecosystem of ion channels. There are other ion channels, such as the epithelial sodium channel
226 (ENaC) that are hormonally active and may be involved in mucus hydration (Roberts *et al.*, 2023).
227 Investigating the relative contributions of different ion channels in mucosal hydration could provide a
228 more comprehensive understanding of female fertility and the development of nonhormonal drug

therapies. Additionally, future studies might develop our findings further by examining the effect of CFTR inhibition on fertility and mucus consistency and hydration in *vivo* using a whole animal model.

Conclusion

Cervical mucus produced by the epithelia in the endocervix is a hormonally induced regulator of female fertility. In this investigation we validate and employ two different techniques, ASL and PTMR, to measure the viscosity and hydration of endocervical cell mucus. We determined that the inhibition of CFTR using small molecule inhibitors results in significant dehydration and increased viscosity of cervical mucus *in vitro*. These findings are consistent with the physiology seen clinically and predicted by molecular studies of CFTR gene expression.

Author contribution statement

Rachel Looney: performed experiments, analyzed data, wrote paper

Mackenzie Roberts: performed experiments, analyzed data, wrote paper

Matthew Markovetz: performed experiments, analyzed data, wrote paper

Rachelle Godiah: performed experiments, analyzed data, wrote paper

Shan Yao performed experiments, analyzed data

Kirsti Golgotiu: performed experiments, analyzed data, wrote paper

Shuhao Wei: performed experiments, analyzed data

Chris Cellucci: performed experiments, analyzed data

Leo Han: conceived the study, performed experiments, analyzed data and wrote the paper

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Declaration of interest

There is no conflict of interest to report.

Data Availability Statement

The data underlying this article will be shared on reasonable request to the corresponding author.

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351 **Figure Legends**

352 **Figure 1:** (A) This figure illustrates the XZ- plane of a Transwell® during an experiment. The apical
353 meniscus view shows the change in meniscus corresponding to CFTR function. (B) This figure
354 demonstrates the image obtained from brightfield microscopy used in ASL studies. The cells can be seen
355 in the center of the field forming a monolayer. The darker outer ring is the meniscus or annulus formed
356 by mucus and PBS on the apical surface of the Transwell®.

357 **Alt text:** (A) A cross-sectional diagram showing an experimental setup of endocervical epithelial cells)
358 within a liquid-filled container. To the right of the container, there are two circular illustrations labeled
359 "Apical Meniscus View." One circle represents a hydrated, "unimpaired" meniscus, while the other
360 shows a dehydrated, "impaired" meniscus. (B) A grayscale circular image resembling a microscope view,
361 featuring concentric rings with distinct boundaries outlined in white. The distance between edge of the
362 well and the lighter region is the annulus.

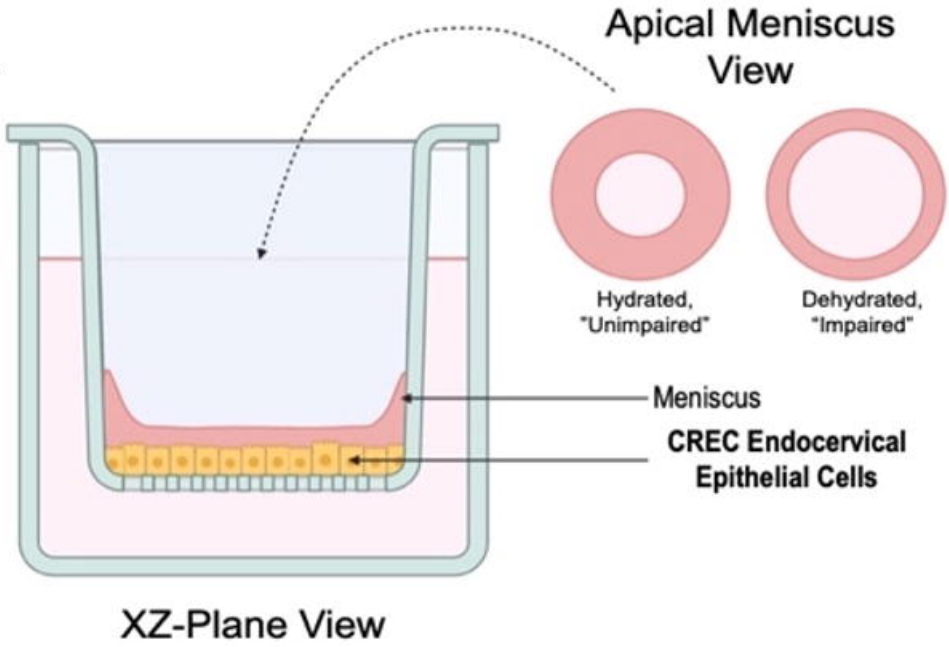
363 **Figure 2**: Effect of mannitol on ASL hydration. Across all time points (24, 48 hours from administration)
364 mannitol (100 mM and 300 mM) reduced the measured change in meniscus from baseline compared to
365 the control ($P<0.05$).

366 **Alt text**: Bar chart showing reduction in meniscus from baseline for various treatment groups over time.

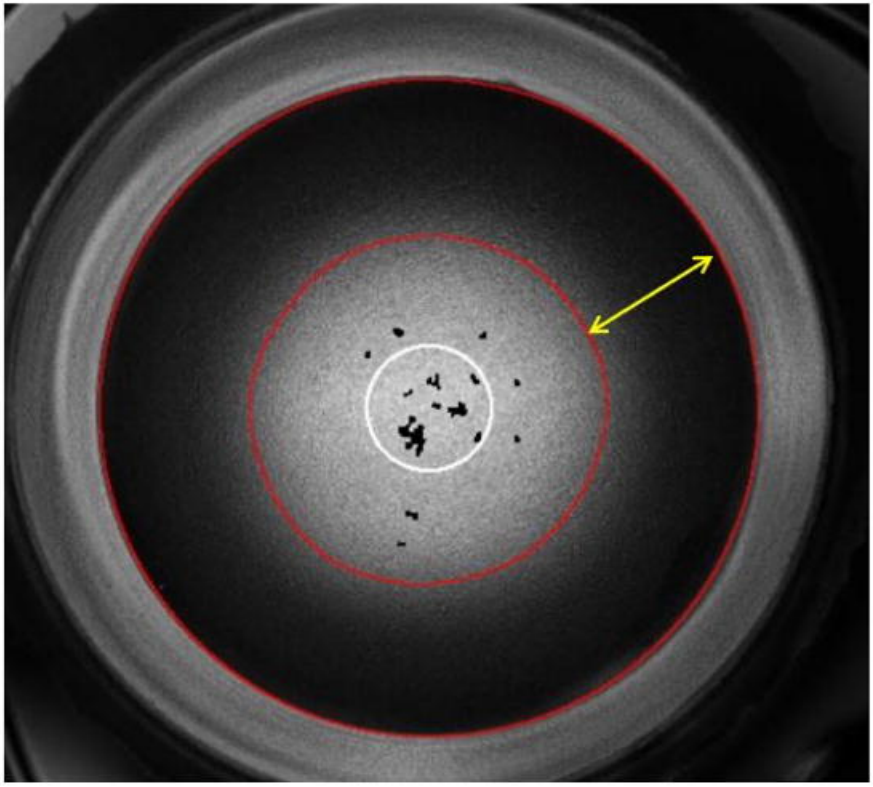
367 **Figure 3**: (A). Effect of E2 on ASL hydration. Across all time points (24, 48, 72, 96 hours from
368 administration) E2 (10 nM) reduced the measured change in meniscus from baseline compared to the
369 control ($P<0.001$). (B). Effect of E2 and P4/E2 on ASL hydration. Across all time points (24, 48, 72, 96

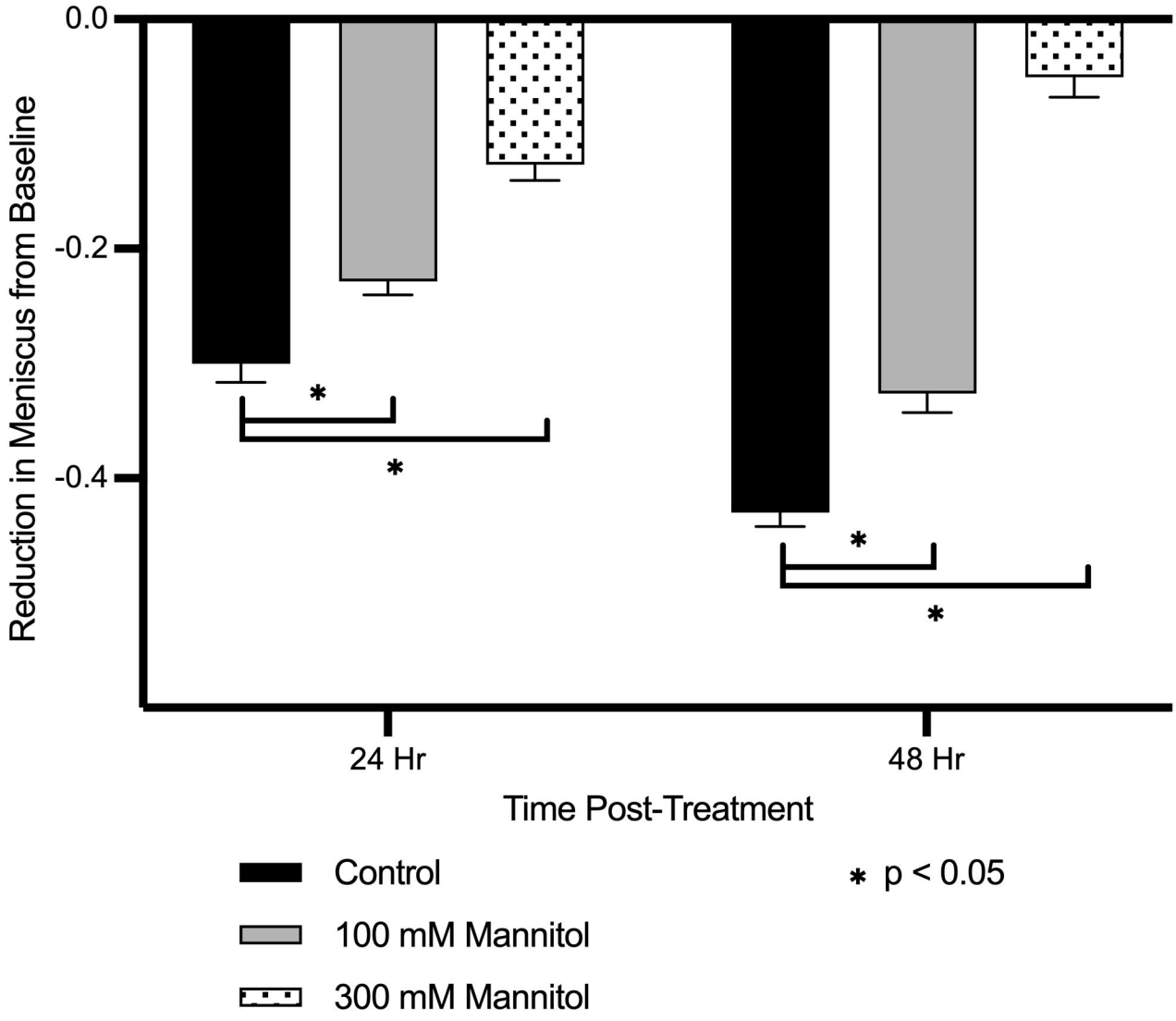
370 hours from administration) 100 nM P4 (after E2 priming) increased the measured change in meniscus
371 from baseline as compared E2 only ($P<0.05$).
372 **Alt text**: Bar charts showing reduction in meniscus from baseline for various treatment groups over time.
373 **Figure 4**: (A) Effect of InH-172 and GlyH-101 on ASL Hydration. Across time points 48, 72, and 96 hours
374 from administration, 10 μM Inh-172 and 5 μM GlyH-101 increased the measured change in meniscus
375 from baseline compared to the control ($P<0.01$, $P<0.05$ respectively). (B) Effect of Inh-172 and Mannitol
376 on ASL Hydration. Across time points 48, 72 and 96 hours from administration, 5 μM Inh-172 with 100
377 mM mannitol increased the measured change in meniscus from baseline compared to the control
378 ($P<0.05$, $P<0.01$, $P<0.01$ respectively).
379 **Alt text:** Bar charts showing reduction in meniscus from baseline for various treatment groups over time.
380 Chart A compares three groups, and chart B compares two groups.
381 **Figure 5**: Representative images obtained at 0 hr and 96 hr with control and Inh-172. Visually greater
382 reduction in meniscus in Inh-172 condition is seen.
383 **Alt text:** A collection of four images depicting a single culture well. The dark region represents the
384 meniscus which decreases over time in both conditions.
385 **Figure 6:** Effect of 10 μM Inh-172 on mucus complex viscosity (η^*). Treatment with Inh-172 for 72 hours
386 increased mucus secretion η^* ($p<0.01$).
387 **Alt text:** a graph demonstrating the difference between DMSO and Inh-172 on complex viscosity.

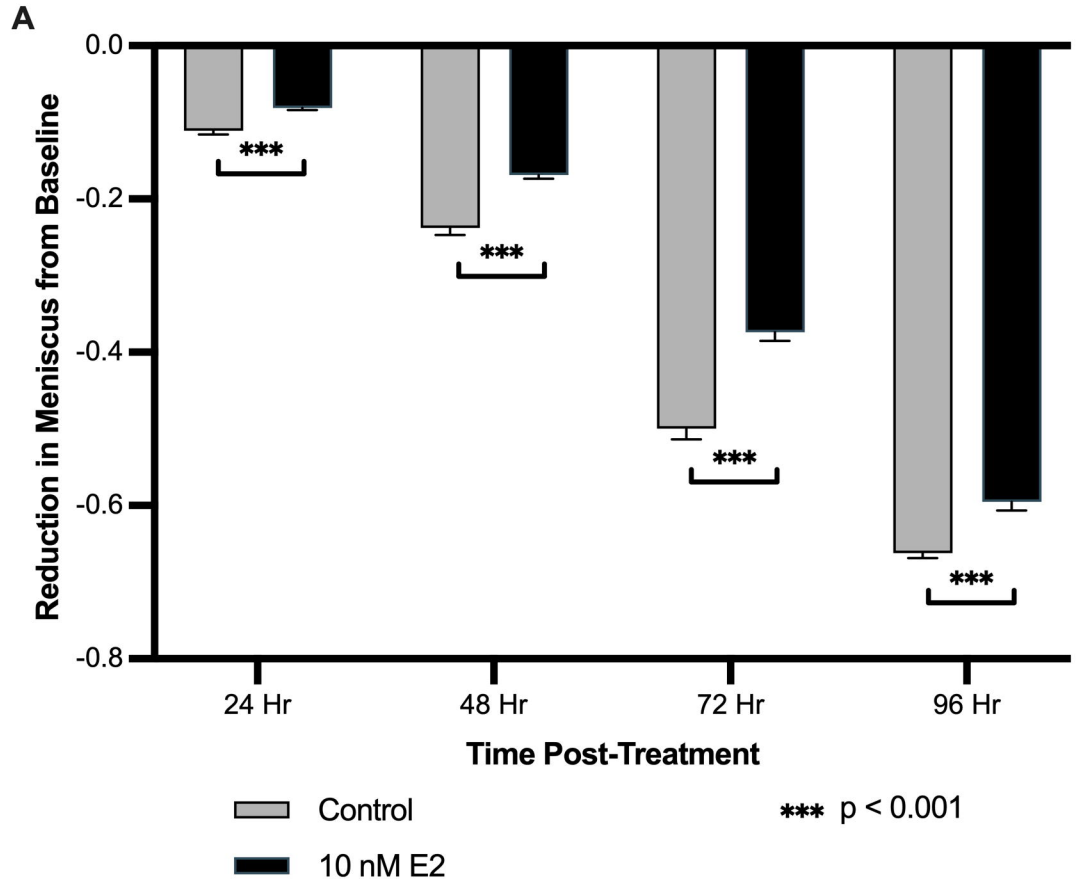
A

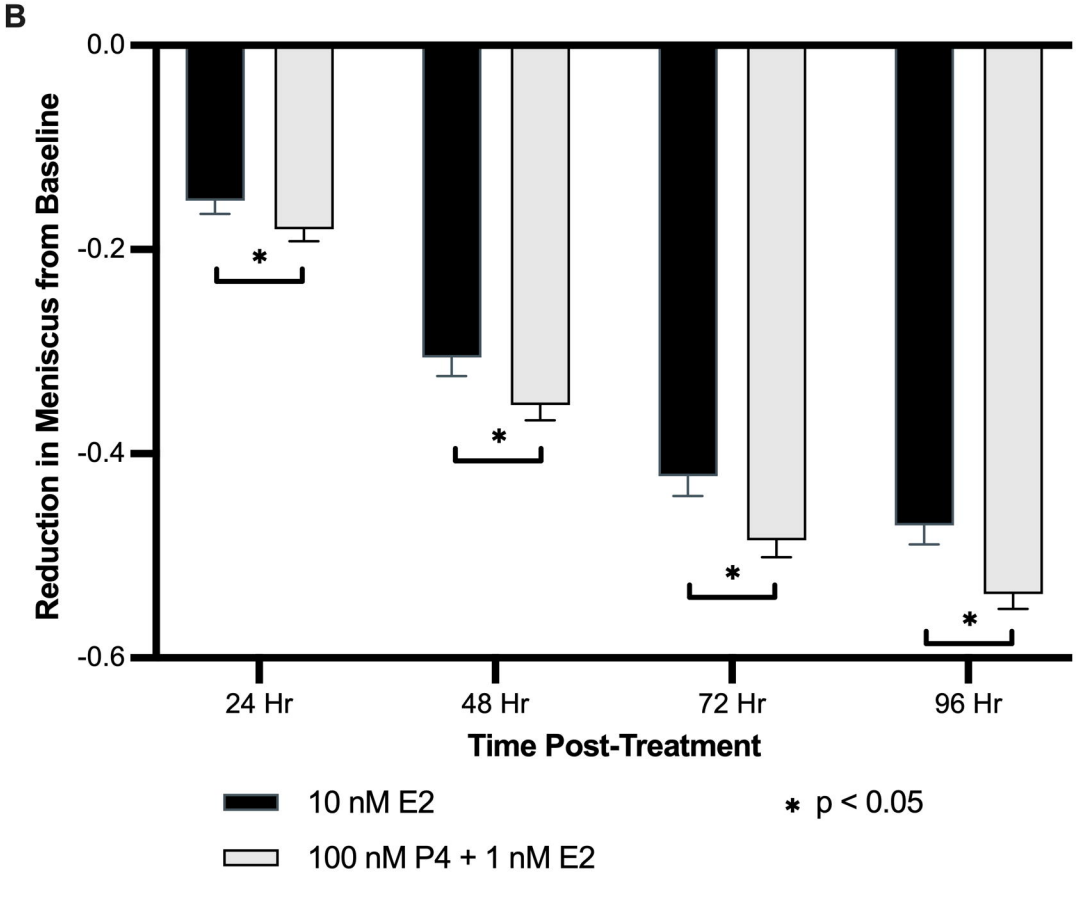

Apical Meniscus View
Hydrated, "Unimpaired"
Dehydrated, "Impaired"
Meniscus
CREC Endocervical Epithelial Cells
XZ-Plane View

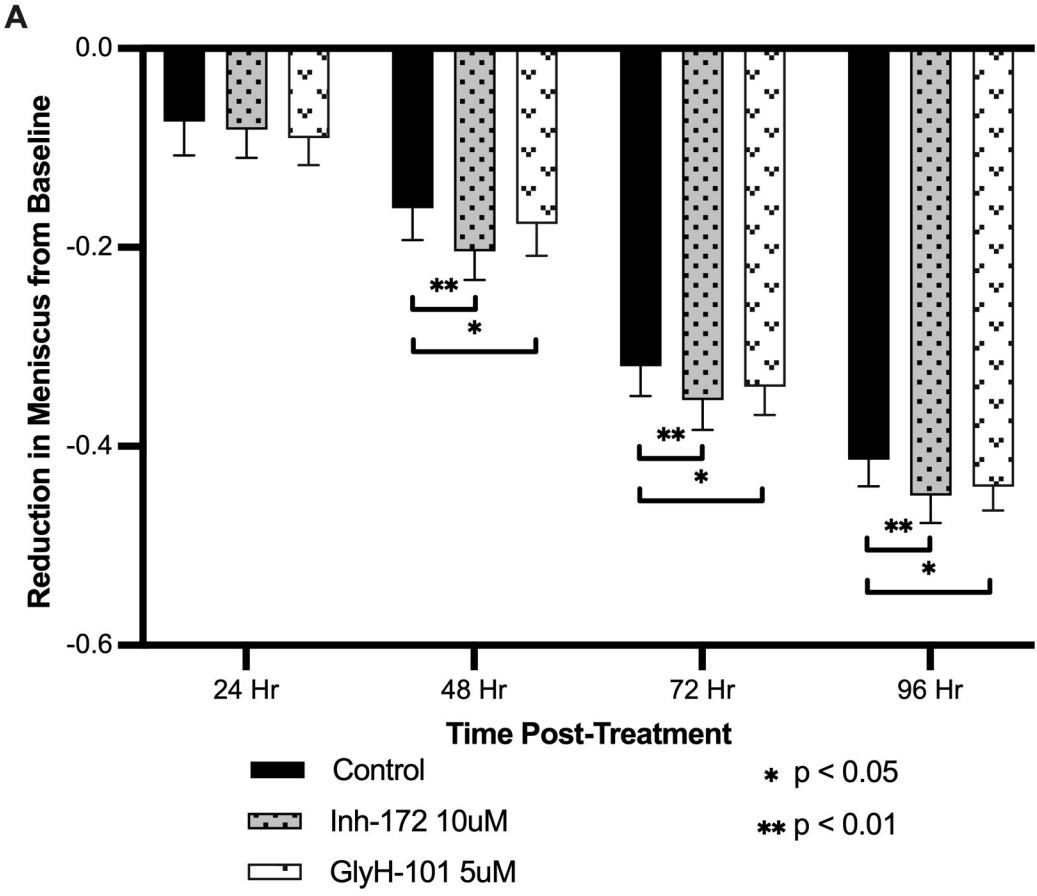
B

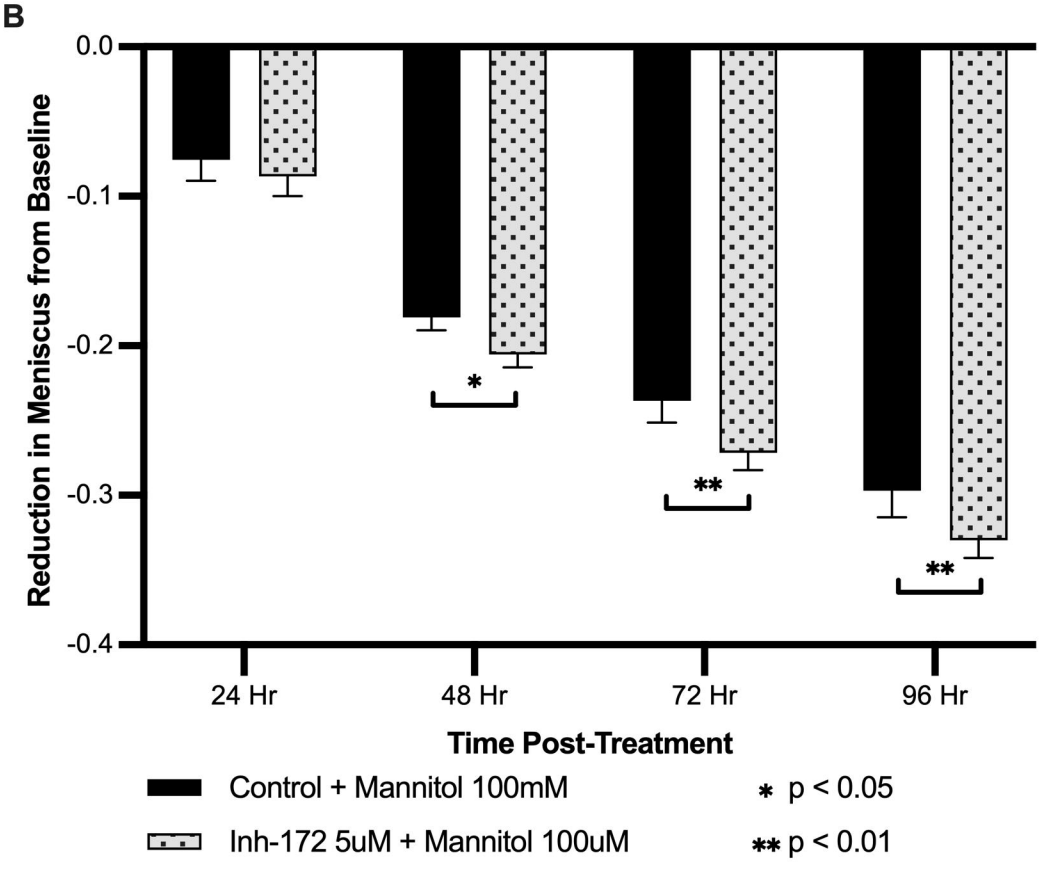


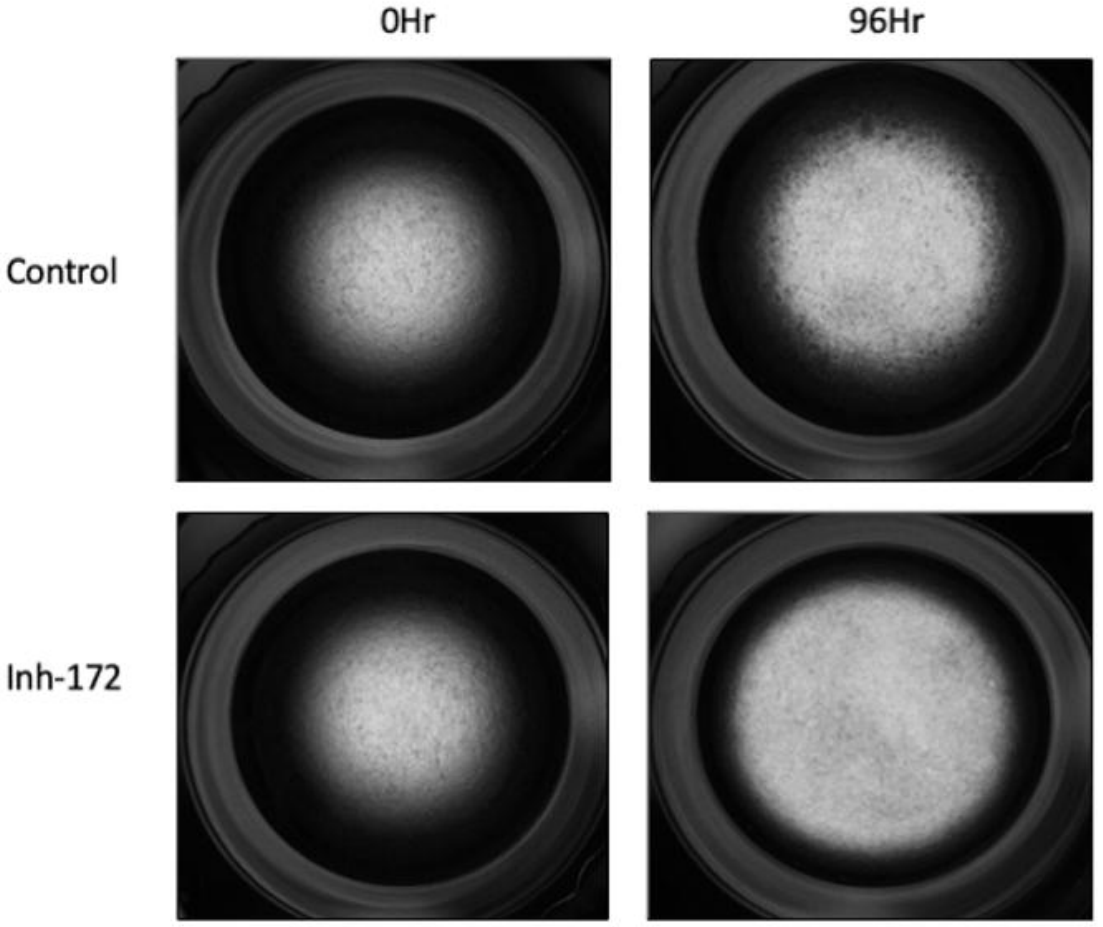

0.0
-0.2
-0.4
Reduction in Meniscus from Baseline
*
*
*
*
24 Hr
48 Hr
Time Post-Treatment
Control
100 mM Mannitol
300 mM Mannitol
* p < 0.05

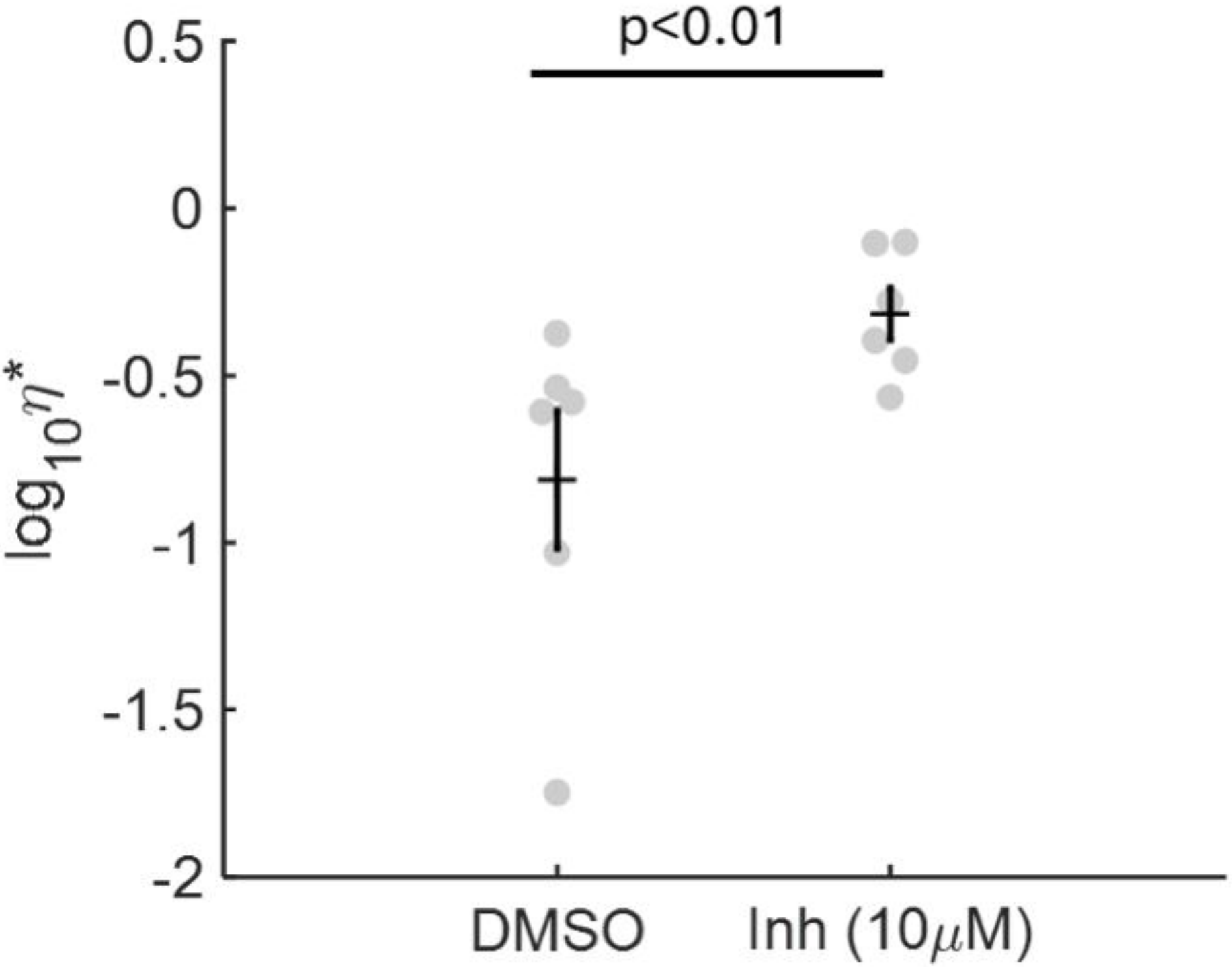

A
0.0
-0.2
-0.4
-0.6
-0.8
Reduction in Meniscus from Baseline
***
***
***
***
24 Hr
48 Hr
72 Hr
96 Hr
Time Post-Treatment
Control
10 nM E2
*** p < 0.001


B
0.0
-0.2
-0.4
-0.6
Reduction in Meniscus from Baseline
*
*
*
*
24 Hr
48 Hr
72 Hr
96 Hr
Time Post-Treatment
10 nM E2
100 nM P4 + 1 nM E2
* p < 0.05


A
0.0
-0.2
-0.4
-0.6
Reduction in Meniscus from Baseline
**
*
**
*
**
*
24 Hr
48 Hr
72 Hr
96 Hr
Time Post-Treatment
Control
Inh-172 10uM
GlyH-101 5uM
* p < 0.05
** p < 0.01


B
0.0
-0.1
-0.2
-0.3
-0.4
Reduction in Meniscus from Baseline
*
**
**
24 Hr
48 Hr
72 Hr
96 Hr
Time Post-Treatment
Control + Mannitol 100mM
Inh-172 5uM + Mannitol 100uM
* p < 0.05
** p < 0.01


0Hr
96Hr
Control
Inh-172


p<0.01
0.5
0
-0.5
-1
-1.5
-2
$\log_{10}\eta^*$
DMSO
Inh (10μM)