Research Article

Iota-carrageenan protects the ocular surface from desiccation-induced cell death and tissue damage in vitro *and* ex vivo

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1. Abstract

Iota-carrageenan is a natural polymer with moisturizing, mucoadhesive and shear-thinning properties. In this study, we aimed to evaluate the protective effects of iota-carrageenan on ocular surface against dehydration, to demonstrate its suitability for the use in lubricant eye drops. We utilized a human epithelial corneal cell culture model to test if pre-incubation with iota-carrageenan solution could protect cells from desiccation-induced cell death, to compare its effect with other natural polymers commonly used in artificial tear products, and to determine the optimal iota-carrageenan concentration. An *ex vivo* porcine eye model was established to confirm the protective effect of iota-carrageenan against dehydration on ocular tissue. Pre-incubation with 1.2 mg/ml iota-carrageenan increased the survival half-life of human corneal epithelial cells upon dehydration by three-fold; the effect was in the same range as observed for large molecular weight hyaluronic acid, and superior to all other tested natural polymers. The highest tested concentration of iota-carrageenan, 1.6 mg/ml, extended the cellular survival half-life by eight-fold while maintaining healthy cellular morphology. Repeated *ex vivo* instillation of an iota-carrageenan-based ophthalmic formulation into porcine eyes significantly protected the ocular surface from desiccation-induced corneal damage, as shown by corneal fluorescein staining These data suggest that Iota-carrageenan effectively moisturizes and protects the ocular surface, supporting its potential as a promising novel ingredient for eye drops in the management of dry eye disease.

2. Introduction

Dry eye disease (DED), also termed dry eye syndrome (DES) or keratoconjunctivitis sicca (KCS), is a multifactorial disease of the ocular surface characterized by a loss of homeostasis of the tear film and tear film instability. Symptoms include ocular dryness, irritation, redness, discharge, and blurred vision, can range from mild and occasional to severe and continuous, and have been shown to negatively impact work productivity and daily activities (Kanellopoulos and Asimellis, 2016; Liu et al., 2022; Sheppard et al., 2023).

The tear film of the eye, consisting of the lipid layer, the aqueous layer, and the mucous layer, is responsible for the maintenance of the corneal epithelium. The aqueous layer is responsible for the osmolality of the ocular surface and changes thereto lead to a disturbed homeostasis of the tear film. The mucous layer has viscoelastic and lubricating properties and detains the moisture on the corneal epithelium and is responsible to form a barrier against drugs, toxins, virus particles or allergens (Račić and Krajišnik, 2023; Willcox et al., 2017).

Mild to moderate cases of DED are usually treated with lubricant eye drops, gels or ointments that can be easily purchased over the counter. The majority of the available OTC lubricating products are gel-like ophthalmic formulations composed of ingredients that contain multifunctional large biopolymers such as hyaluronic acid, carboxymethyl cellulose or hydroxypropylmethylcellulose in order to increase the viscosity of the tear film, to moisturize and prolong the residence time of an aqueous layer on the cornea (Cassano et al., 2021; Račić and Krajišnik, 2023; Zhang et al., 2017). An ideal compound for the treatment of DED that aims to enhance tear film stability should possess properties similar to the mucous layer and in addition should be mucoadhesive to remain on the ocular surface. Here, we propose iota-carrageenan as a promising, safe and effective new alternative for the treatment of DED. Carrageenans are natural polysaccharides built of D-galactose and 3, 6-anhydro-D-galactose units which are extracted from certain red seaweeds of the Rhodophyceae

class. In the food, cosmetic and pharmaceutical industry, carrageenans are widely utilized due to their excellent physical functional properties, such as gelling, thickening, emulsifying and stabilizing abilities (Li et al., 2014). There are three main types of carrageenan based on the number of sulfate groups attached to the galactose molecule, which differ with regard to their solubility and their gelling and thickening capacity: kappa- (one sulfate group per two galactose molecules), iota- (two sulfate groups per two galactose molecules) and lambda- (three sulfate groups per two galactose molecules) carrageenan. The toxicological profile of carrageenans has been thoroughly evaluated, and they have been established to have minimal or no adverse physiological effects (Cohen and Ito, 2002). No health hazards have been documented for iota-carrageenan so far, and it has been designated as “Generally Recognized As Safe” (GRAS status) by the FDA for food and topical applications (U.S. Food & Drug Administration, SCOGS (Select Committee on GRAS Substances), 2020).

Iota-carrageenan is certified for marketing in the EU, parts of Asia and Australia under the brand name Carragelose® as a component of nasal sprays, throat sprays and lozenges. Previous pre-clinical and clinical studies led by us and others have demonstrated the broad, non-specific antiviral and barrier-forming properties of iota-carrageenan (Bichiri et al., 2021; Eccles et al., 2010; Fazekas et al., 2012; Fröba et al., 2021; Graf et al., 2018; Grassauer et al., 2008; Koenighofer et al., 2014; Ludwig et al., 2013; Morokutti-Kurz et al., 2021; Unger-Manhart et al., 2024).

In addition, iota-carrageenan has great potential to be used in lubricating and moisturizing eye drops based on the following properties: Firstly, it forms an *in situ* gelling system upon exposure to physiological conditions (Akash Muktiram Shendge*, Vikas B Wamane, Jayshree R. Shejul, 2024; Rupenthal et al., 2011) and hence retains moisture on the ocular surface due to its excellent water-binding capacity (Thommes et al., 2009), counteracting evaporation. Secondly, it is a viscosity enhancer that exhibits viscoelastic behavior (Hilliou, 2021; HOSSAIN et al., 1997). Thirdly, its mucoadhesive properties are similar like that of the mucous layer (Račić and Krajišnik, 2023; Vigani

et al., 2019; Volod'ko et al., 2024). The second and third property additionally improves the retention time on the ocular surface and thereby may provide a prolonged stay on the ocular surface compared to aqueous solutions. Fourthly, it is a polymer with non-Newtonian properties and as such it is per definition shear-thinning, hence reducing resistance against movements of the eyelid when blinking (Ludwig, 2005). Finally, iota-carrageenan acts locally and is not absorbed by the body, therefore it may be used also during pregnancy and lactation.

The suitability of iota-carrageenan for ophthalmic applications has already been demonstrated in various contexts: Carrageenans have been tested and proposed as promising carriers for ocular drug delivery (Bonferoni et al., 2004; Omran et al., 2023; Volod'ko et al., 2024). A commercial iota-carrageenan-containing viscous gel has been proven to be safe and effective when used to protect and hydrate the corneal surface during ophthalmic surgery (Giardini et al., 2018; Mencucci et al., 2019). Hence, the potential of iota-carrageenan in multiple ophthalmic therapeutic areas may deserve further attention. But to date, no study has evaluated the suitability of iota-carrageenan as lubricant and moisturizing compound for treatment of DED.

The present research evaluated the moisturizing properties and the protective effects of iota-carrageenan against air-induced desiccation. Using both *in vitro* cell culture and *ex vivo* porcine eye model systems, we assessed the protective effect of iota-carrageenan in comparison to established substances commonly used for the treatment of DED. Additionally, we demonstrated a concentration-dependent protective effect of iota-carrageenan against dehydration and determined the concentration of iota-carrageenan for use in an ophthalmic formulation.

108 # 3. Methods

109 **3.1 Cell culture**

110 We have adapted a cell culture model that has previously been used in the development of DED
111 treatments to assess prevention of cell death from desiccation (Hill-Bator et al., 2014). Immortalized
112 Human Corneal Epithelial Cell Line (IM-HCEpiC; Innoprot, Derio (Bizkaia), Spain; # P10871-IM) were
113 cultured according to the supplier's instructions. Briefly, cells were maintained in IM-Ocular
114 Epithelial Cell Medium (Innoprot; P60189) supplemented with growth factors and antibiotics in
115 standard tissue culture flasks. Cells were incubated at 37°C in a humidified atmosphere containing
116 5% CO_2. The culture medium was exchanged with fresh medium every 2-3 days, and cells were
117 passaged upon reaching 80-90% confluency using trypsin-EDTA.

118 **3.2 *In vitro* dehydration assay**

119 Human immortalized cornea epithelial cells (IM-HCEpiC) were seeded into 96-well plates (TPP;
120 92696) at a density of $1x10^4$ cells/well on the day before the experiment. The next day, medium was
121 replaced with the sample solutions described below or with fresh cell culture medium only as
122 control, and incubation under cell culture conditions was continued for 2 h. Subsequently,
123 supernatants were removed, and culture plates were placed without lid in a dry incubator at 33°C
124 for defined timespans of 2-40 min to allow dehydration. The following controls were included in the
125 experiment: i) internal positive control (iPC) – cells were incubated with medium but not submitted
126 to dehydration; ii) sample positive control (sPC) – cells were incubated with respective sample
127 solution but not submitted to dehydration; iii) negative control (NC) – cells were incubated with cell
128 culture medium and submitted to dehydration. Cell viability following dehydration was assessed
129 fluorometrically using a resazurin based assay (Alamar Blue, Acros; 189900010). Cells were
130 incubated with a 1x Alamar blue solution for 30 min at 37°C in a humidified atmosphere containing
131 5% CO_2. After incubation, the fluorescence of reduced resazurin was measured using a fluorescence

132 plate reader (BMG Fluostar Omega) with excitation at 544 nm and emission at 590 nm. Cell viability
133 was calculated for each sample relative to the corresponding sPC viability. For each tested sample
134 solution, a dose-response analysis was performed and the respective cellular survival half-life, i.e.,
135 the time point when 50 % of the metabolic signal was still detectable, was calculated based on the
136 dose-response curves. The goodness-of-fit of the dose-response analysis is expressed as confidence
137 interval of the calculated half-life.

138 **3.3 Sample solutions used in the in vitro dehydration assay**

139 For comparison of the dehydration-protective effect of natural polymers, the following substances
140 were dissolved in distilled water (aqua destillata, AD; Aqua Braun; B.Braun; 0082479E) and then
141 mixed 1:1 with cell culture medium to achieve a final assay concentration of 1.2 mg/ml: fucoidan
142 (two types: derived from Fucus vesiculosus (Sigma; F8190) and from Undaria pinnatifida (Sigma;
143 F8315)), dextran sulfate sodium salt (Sigma; D6001), carboxymethyl cellulose (Hercules; 7MF
144 Pharm), hydroxypropyl methylcellulose (Fagron; 102517), hyaluronic acid (three different molecular
145 weight species: 300-500 kDa (Sigma; 00941), 750-1000 kDa (Sigma; 53163) and 1.2-1.5MDa (Sigma;
146 53747), kappa-carrageenan (Du PontNutrition), and iota-carrageenan (Du PontNutrition).

147 For investigation of an iota-carrageenan dose effect, iota-carrageenan was dissolved in AD at a
148 concentration of 3.2 mg/ml and this stock solution was further diluted with AD to prepare solutions
149 with concentrations down to 0.15 mg/ml. These iota-carrageenan solutions were mixed 1:1 with cell
150 culture medium to obtain the following final assay concentrations of iota-carrageenan: 1.6, 1.4, 1.2,
151 1.0, 0.8, 0.6, 0.3, 0.15, and 0.075 mg/ml.

152 **3.4 Cytoskeletal and nuclear staining after treatment and dehydration**

153 Cell treatment and dehydration: IM-HCEpiC were seeded at a density of $8x10^4$ cells/well on
154 coverslips (VWR; ECN631-1578) in a 24-well plate (TPP; 92024) the day before the experiment. Cells
155 were incubated for 2 h with 3.2 mg/ml iota-carrageenan in an isotonic buffer, mixed 1:1 with cell

156 culture medium to give a final assay concentration of 1.6 mg/ml. After the 2 h incubation, specific
157 wells were designated for dehydration condition. Cell culture medium and treatment solution was
158 aspired from designated wells. Subsequently, the plate was placed without lid in a dry incubator at
159 33°C for 10 or 30 min. Following dehydration, cells were washed with PBS (Sigma; D8537), fixed with
160 Roti Histofix (Roth; P087.6) for 10 min, washed with PBS (3x), permeabilized with 0.1% Triton X-100
161 (Serva Feinbiochemica; 37240) for 5 min, and washed with PBS (3x). Coverslips were then stained for
162 1 h with 1x Phalloidin-iFluor 488 (Abcam; AB176753) plus 1 µg/ml of DAPI (Sigma; D9542) in PBS plus
163 1% BSA (Roth; 8076.4) on parafilm in a humidified petri dish. Coverslips were washed with PBS (3x)
164 and mounted on glass slides (Henry Schein; 900-2573) with mounting medium (Roti-Mount Aqua;
165 Roth; 2848.1) or epifluorescence microscopy (Axio ExaminerD1 with axiocam 202 camera; Carl
166 Zeiss).

167 **3.5 *Ex vivo* dehydration assay**

168 The induction of ocular surface damage by desiccation is based on a published model (Choy et al.,
169 2004). Porcine eyes including the surrounding tissue of the eye socket were extracted from skulls of
170 pigs (German-Large-White) slaughtered at a local slaughterhouse. Eyes were examined for corneal
171 damage by distributing one drop (25 µL) of sodium fluorescein (Fluka, 46960; 0.1% w/v in PBS) on
172 the ocular surface and scoring the surface damage under a black light torch l λ_{Em}: 395 nm according
173 to the Oxford grading scale. Only undamaged eyes (Oxford scale grading 0 or 1) were used for the
174 experiment. Eyes judged as undamaged were pinned open and incubated in a laminar flow hood for
175 6 h at room temperature and approx. 50% humidity. Every 15 min during this 6 h period, a single
176 drop of sample solution (described below) or 0.9% NaCl (B.Braun, 3570130) (negative control), was
177 dropped onto the cornea and spread across the surface by blinking with the nictitating membrane.
178 At the end of the 6 h dehydration period, eyes were again submitted to Oxford grading after
179 instillation of 1 drop of sodium fluorescein (0.1% w/v in PBS). Next, eyeballs were excised from the
180 surrounding tissue and immersed into sodium fluorescein (0.1% w/v in PBS) for 3 min, followed by

181 ten rinsing steps in PBS. Corneas were dissected, and 0.8 cm diameter punches were taken from the
182 center. Fluorescein was extracted from cornea punches in Triton X-100 (Sigma, T8787-50mL) (1 %
183 v/v in PBS) at 4°C for 48 h. Relative fluorescence units of extracts were measured at Ex/Em 485/520
184 nm using a BMG Fluostar Omega plate reader, and fluorescein concentration [µg/g] of each sample
185 was calculated based on a 20-point standard curve with two-fold serial dilutions. P values were
186 derived from one-way ANOVA with Tukey's post-hoc test.

187 **3.6 Sample solutions used in the *ex vivo* dehydration assay**

188 Iota-carrageenan dissolved in isotonic buffer composed of disodium-EDTA (Titriplex III) 1.00 mg/ml,
189 citric acid monohydrate 0.46 mg/ml, disodium hydrogen phosphate dihydrate 3.67 mg/ml, sorbitol
190 40.00 mg/ml, at concentrations 0.6 mg/ml, 2.0 mg/ml, and 3.2 mg/ml.

191 # 4. Results

192 Based on the inherent properties of iota-carrageenan and its demonstrated suitability for
193 ophthalmic applications [Bonferoni et al., 2004; Giardini et al., 2018; Mencucci et al., 2019; Omran et
194 al., 2023; Volod'ko et al., 2024], we set out to examine the potential of iota-carrageenan for use in
195 the treatment of DED.

196 To quantify and contextualize the moisturizing capacity of iota-carrageenan, we employed a cell
197 culture dehydration assay using human immortalized cornea epithelial cells (IM-HCEpiC). Cells were-
198 preincubated for 2 h with iota-carrageenan or other polymers that are currently used in products for
199 the treatment of DED, all at a final assay concentration of 1.2 mg/ml. Subsequently, cells were
200 submitted to dehydration for various time periods, and viability after dehydration was determined
201 fluorometrically using a resazurin based assay. Cellular survival half-lives upon dehydration, i.e. the
202 duration of dehydration when cell viability reached 50% of the respective non-dehydrated control,
203 were calculated for each sample. As shown in **figure 1**, pre-incubation of IM-HCEpiC with iota-
204 carrageenan protected cells from desiccation-induced cell death and led to a threefold increase of
205 the cellular survival half-life upon dehydration. This effect was specific to iota-carrageenan and was
206 not observed with kappa-carrageenan. The magnitude of effect was comparable with that of large
207 molecular weight hyaluronic acid (1.2-1.5 MDa) and was superior to the effect of all other tested
208 polymers, including fucoidan, dextran sulfate, carboxymethyl cellulose, hydroxypropyl
209 methylcellulose, as well as low and medium molecular weight hyaluronic acid (0.3-1 MDa).

210 Next, we aimed at determining if there is a concentration dependence of iota-carrageenan for
211 protection against dehydration, again using the IM-HCEpiC *in vitro* dehydration model. In this case,
212 cells were-preincubated for 2 h with various concentrations of iota-carrageenan spanning final assay
213 concentrations from 1.6 mg /ml down to 0.075 mg/ml, followed by dehydration for various
214 durations, and viability assessment based on resazurin (Alamar Blue). Our results demonstrate that
215 pre-incubation with iota-carrageenan enhanced cell viability after dehydration in a dose-dependent

216 manner. As shown for selected concentrations in **Figure 2a**, cells pre-incubated with medium alone
217 could compensate for only a 2 min dehydration period without loss of viability and had already lost
218 50% viability within less than 5 min of dehydration. In contrast, cells pre-incubated with the highest
219 tested concentration of 1.6 mg /ml iota-carrageenan were able to withstand dehydration and
220 maintain high viability levels of > 90% for at least 20 min and showed an increase in half-life survival
221 by eight-fold in comparison to the negative control. Lower iota-carrageenan concentrations had
222 similar, though proportionally weaker effects. Iota-carrageenan concentrations below 1 mg/ml led to
223 markedly reduced viability already after 5-10 min of dehydration but still extended the dehydration
224 survival half-life, albeit to a lower extent. Concentrations below 0.15 mg/ml yielded similar results as
225 the negative control (data not shown). In sum, the protective effect of iota-carrageenan against
226 dehydration shows a clear dose-dependence.

227 As shown in **figure 3,** dehydration caused cells to shrink and subsequently die, leading to reduced
228 cell density, when they had been pre-treated with medium only, while pre-incubation with 1.6
229 mg/ml iota-carrageenan prevents these changes and maintains cell density and healthy cell
230 morphology.

231 Next, we applied a porcine *ex vivo* model to validate the results obtained *in vitro*. **Figure 4a-c**
232 illustrates the experimental set-up. Eyes were extracted from the skulls of freshly slaughtered swine.
233 After transport to the laboratory, eyes were visually inspected for ocular surface damage, and only
234 undamaged eyes were used. Eye lids were pinned open and eyes were dehydrated by exposure to
235 laminar air flow for 6 h. During this period, eyes were treated in 15 min intervals with 1 drop of
236 either 0.9% NaCl or of 0.6, 2.0 or 3.2 mg/ml iota-carrageenan dissolved in isotonic buffer. In contrast
237 to the *in vitro* cell culture experiment where iota-carrageenan was dissolved in AD and 1:1 mixed
238 with cell culture medium to maintain cell viability, for *ex vivo* application iota-carrageenan was
239 dissolved in an isotonic buffered solution compatible for ophthalmic application, i.e., with neutral pH
240 containing sorbitol, di-sodium hydrogen phosphate dihydrate, citric acid monohydrate, and sodium-

241 ethylenediaminetetraacetic acid (EDTA) in AD. After 6 hours of dehydration, eyes were stained with
242 fluorescein to assess and quantify corneal damage caused by the desiccation. Fluorescein penetrates
243 poorly into the lipid layer of the corneal epithelium, and therefore, does not stain intact cornea, but
244 can only penetrate and stain damaged cornea where cell-to-cell junctions are disrupted (Srinivas and
245 Rao, 2023). As shown in **figure 5a**, no obvious cornea damage was seen before dehydration. After 6
246 h dehydration, a very small extent of damage was observed in eyes repeatedly treated with iota-
247 carrageenan eye drops during dehydration. In contrast, the control eyes treated with 0.9% NaCl had
248 acquired widespread corneal damage, as indicated by white arrows. The extent of damage can be
249 quantified by dye extraction from cornea punches after fluorescein staining. **Figure 5b** shows the
250 amount of fluorescein extracted from cornea punches, expressed as percentage of NaCl treated
251 control eyes. The amount of dye that penetrates the corneal epithelium and hence can be extracted
252 from cornea samples is proportional to the corneal damage acquired during dehydration. Repeated
253 treatment with iota-carrageenan protected porcine eyes from dehydration-induced cornea damage.
254 With all three tested concentrations of iota-carrageenan, the amount of extracted dye, i.e., of
255 corneal damage, was significantly reduced compared to control eyes treated with physiologic saline
256 solution. As shown before *in vitro*, there was a clear trend towards dose dependence of the effect,
257 even though the difference in effect size between concentrations was just short of being statistically
258 significant (p value for comparison between 0.6 and 3.2 mg/ml = 0.059) based on the available
259 number of biologic replicates. The baseline value has been derived from eyes not subjected to
260 dehydration and demonstrates the effectiveness of the dehydration treatment for inflicting corneal
261 damage.

262 In sum, our data demonstrate the capacity of iota-carrageenan to protect against dehydration in an
263 *in vitro* cell culture system of human cornea cells as well as in an *ex vivo* animal model. Based on
264 these *in vitro* and *ex vivo* results, 3.2 mg/ml was determined to be the most effective concentration
265 of iota-carrageenan to protect ocular cells and tissue from dehydration-induced damage.

266 ## 5. Discussion

267 To restore and maintain a healthy ocular surface, it is essential that both the cornea and conjunctiva
268 remain continuously hydrated - a function normally provided by the tear film. Recent consensus
269 identifies tear film instability as the core mechanism underlying DED. This understanding has led to
270 the development of tear film-oriented diagnosis, which seeks to clarify the specific causes of DED in
271 individual patients by focusing on the distinct layers of the tear film. Consequently, tear-film-
272 oriented DED therapy focuses on restoring tear film homeostasis and ensuring adequate lubrication
273 of the ocular surface (Dash and Choudhury, 2024; Safir et al., 2024; Shimazaki, 2018).

274 The research presented in this article investigated the protective effect of iota-carrageenan against
275 air-induced desiccation and evaluates its potential as an ocular lubricant for the relief of mild to
276 moderate DED.

277 Our data show that in a cell culture model of corneal epithelial cells, pre-treatment of cultured cells
278 with an iota-carrageenan solution dramatically enhanced cell survival after dehydration. The
279 magnitude of the protective effect was comparable to high molecular weight hyaluronic acid, and
280 was superior to all other tested natural polymers, including other widely used ingredients of eye
281 drops used for DED treatments. Moreover, our results highlighted significant differences in the
282 effectiveness of hyaluronic acid based on its molecular weight. The protective effect of iota-
283 carrageenan was dose-dependent, as higher concentrations correlated with increased survival rates.
284 Moreover, iota-carrageenan significantly inhibited the desiccation-stress induced accumulation of
285 corneal damage in an *ex vivo* porcine eye model also in a dose dependent manner. In sum, the data
286 presented here suggest that iota-carrageenan has a moisturizing and protective effect that prevents
287 cornea cells from dehydration and, in consequence, from dehydration-induced cell death and tissue
288 damage. Of note, the highest concentration used in the *ex vivo* experiment (3.2 mg/ml; maximum
289 possible concentration for technical reasons) exceeds the highest concentration in the cell culture
290 experiment described above (1.6 mg/ml). This was intended to compensate for the dilution with the

291 lacrimal fluid and the rapid restoration of the tear film that washes away a considerable proportion
292 of the administered solution (Gaudana et al., 2010).

293 The texture properties of carrageenans have long been known and exploited in the food and the
294 cosmetics industry. Carrageenans are known to retain moisture, enhance viscosity combined with
295 shear thinning behavior, and have mucoadhesive properties that are also exploited in various forms
296 of drug delivery (Bonferoni et al., 2004; Volod'ko et al., 2024). We hypothesize that these properties
297 together contribute to the effect observed in our experiments: Carrageenan forms a mucoadhesive
298 in situ film on the cellular monolayer and on the *ex vivo* porcine eye, it enhances viscosity of the
299 solution, improves surface retention, and counteracts dehydration due to water-retention. We claim
300 that these mechanisms should work equally well in the human eye, however this shall be subject to
301 further studies.

302 Hyaluronic acid containing eye drops have become the first choice for tear substitution in many
303 countries. According to literature, the average molecular weight of hyaluronic acid determines the
304 therapeutic efficacy of such eye drops (Salzillo et al., 2016), and high molecular weight hyaluronic
305 acid has enhanced mucoadhesion and moisturizing capacity compared to the low molecular weight
306 form (Guarise et al., 2023). Hyaluronic acid is a viscosity enhancer used to form gel-like ophthalmic
307 solutions which may cause, especially with high molecular weight, blurred vision and a foreign body
308 sensation, especially immediately after instillation (Müller-Lierheim, 2020). In contrast, in situ gelling
309 systems are generally considered more comfortable because they are installed as low-viscosity
310 liquids causing minimized immediate vision disruption (Cassano et al., 2021). In our experiments, the
311 viscosity of the high molecular weight hyaluronic acid solution was 9 mPas and that of the iota-
312 carrageenan solution 2 mPas, measured at 33°C and a shear rate of 10 1/s with an Rotavisc lo-vi
313 (IKA) with ELVAS-1 extension (data not shown). We have shown *in vitro* that iota-carrageenan is
314 equal to high molecular weight (1.2-1.5 MDa) hyaluronic acid in terms of cytoprotection under
315 dehydration at a concentration of 1.2 mg/ml and therefore expect similar therapeutic efficacy of

316 iota-carrageenan-based eye drops as reported for hyaluronic acid-based eye drops. It is important to
317 note that our *ex vivo* model represents only a simplified approximation of the eye *in vivo*, where
318 continuous secretion from lacrimal and Meibomian glands as well as regular blinking occur. To
319 partially compensate for the lack of natural blinking, our experimental set-up included mechanic
320 blinking with the lid to facilitate the distribution of test solutions. No tears replenishment was
321 performed in the *ex vivo* eyes which on one hand prevented wash out of the treatment but also led
322 to rapid cornea dehydration. We tried to compensate for this with repeated installations of the
323 respective test solutions. Nevertheless, this set up does not sufficiently consider the natural tear
324 clearance and the washout processes that occur *in vivo* .

325 Further studies are required to shed light on the safety, tolerability and therapeutic effectiveness of
326 iota-carrageenan eye drops in the treatment of DED in humans.

327 6. Conclusions

328 Iota-carrageenan exhibits significant dose-dependent protection against desiccation-induced cell
329 death and damage in both in vitro cultured human corneal cells and an ex vivo porcine eye model.
330 Iota-carrageenan closely mimics natural tear fluid due to its mucoadhesive properties, enabling it to
331 substitute lost tear fluid. By lubricating and hydrating the cornea and conjunctiva, it supports a
332 healthy ocular surface and additionally protects the eye surface from dehydration-induced damage.

333 Our findings position iota-carrageenan as a promising novel ingredient for DED-targeted eye drops.
334 Its dual capacity to replace tear fluid and shield the ocular surface from dehydration underscores its
335 potential as an advanced therapeutic agent.

7. Declarations

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7.2 Statement of Ethics

Regulations on the protection of animals used for scientific purposes do not apply to the present study, since tissues of animal origin used in this study were derived from the local slaughterhouse as waste product from food production under adherence to national laws on the protection of animals in connection with slaughtering.

7.3 Funding Sources

This research was sponsored by Marinomed Biotech AG.

7.4 Data Availability Statement

Data related to this manuscript can be made available from the corresponding author upon reasonable request.

7.5 Author Contributions

SRN: conceptualization, project administration, supervision, data analysis, writing – review and editing; AR: methodology, supervision, data analysis, writing – review and editing; CK: methodology, data analysis, writing – review and editing; AD: methodology, data analysis, writing – review and editing; MKS: methodology, data analysis, writing – review and editing, HD: data analysis, visualization, writing – original draft, writing – review and editing; EPG: conceptualization, supervision, data analysis, writing – review and editing.

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Iota-carrageenan protects the ocular surface from desiccation-induced cell death and tissue damage in vitro *and* ex vivo

Figure Captions

Figure 1: Iota-carrageenan (white column) protect corneal cells from dehydration. IM-HCEpiC were pre-incubated with natural polymers in cell culture medium at a concentration of 1.2 mg/ml for 2h and then dehydrated for 2-40 min by removing the medium and placing the culture plates without lid in a dry incubator. Viability was assessed after dehydration photometrically using a resazurin based assay. Survival half-life was defined as time after which 50% of cells were still metabolic active. Error bars represent 95% CI (n= min. 3). NC = negative control, cells pre-treated with cell culture medium w/o polymers, followed by dehydration.

Figure 2: Iota-carrageenan protects corneal cells from dehydration in a concentration-dependant manner. IM-HCEpiC were pre-incubated with indicated iota-carrageenan concentrations in cell culture medium for 2h and then dehydrated for 2-40 min by removing the medium and placing the culture plates without lid in a dry incubator.

a: Viability was assessed photometrically using a resazurin based assay after dehydrating cells for indicated time periods. Viability is given relative to the viability of the respective sample positive control (i.e., cells pre-treated with same iota-carrageenan concentration as respective sample but did not undergo dehydration). Error bars represent the standard deviation across all replicates (n = 6-12). The horizontal red line represent 50% cell viability.

b: Survival half-life defined as time after which 50% of cells were still metabolic active after pretreatment with the tested concentrations of iota-carrageenan. Error bars represent 95% CI.

NC = negative control, cells pre-treated with cell culture medium w/o iota-carrageenan, followed by dehydration.

Figure 3: Pre-treatment with iota-carrageenan eye drops protects corneal cells from dehydration-induced morphological changes. IM-HCEpiC were pre-incubated with 3.2 mg/ml iota-carrageenan mixed 1:1 with cell culture medium (giving a final iota-carrageenan concentration of 1.6 mg/ml) for 2h and then dehydrated for 10 or 30 min. Actin fibers were stained with phalloidin (green) and nuclei with DAPI (blue) to visualize morphology. Scale bare is 20 µm.

Figure 4: Experimental set-up of induction and detection of ocular surface damage in a porcine *ex vivo* model. Eyes extracted from freshly slaughtered swine were dehydrated by exposure to laminar air flow for 6 h under repeated instillation of 0.9% NaCl or iota-carrageenan in isotonic buffer. Eyes were visually inspected for ocular surface damage before and after dehydration. Corneal damage was quantified by dye extraction from cornea punches after fluorescein staining of eyeballs.

a-c Handling of porcine eyes. **a:** Porcine eye were pinned open and exposed to air for 6 h. Every 15 min, 1 drop of 0.9% NaCl or iota-carrageenan solution was instilled into the eye and evenly spread by closing and opening the nictitating membrane. **b:** Immersion of eyeballs in 0.1% fluorescein solution. **c**: Washing of fluorescein-stained eyeballs. After fluorescein staining, corneas were dissected, circular punches were taken, and fluorescein was extracted from punches and quantified.

Figure 5: Iota-carrageenan provides dose-dependent protection of the cornea from dehydration-induced ocular surface damage in a porcine ex vivo ocular dehydration model.

a: Freshly extracted porcine eyes were examined for cornea damage under a black light torch after instilling 25 µl fluorescein solution (left column). Only undamaged eyes (Oxford scale grading 0 or 1) were used in the experiment. After 6 hours of dehydration under repeated instillation of 0.9% NaCl or 3.2 mg/ml iota-carrageenan eye drops, eyes were again visually inspected under black light after instilling of 25 µl fluorescein solution (right column). Widespread corneal damage is seen after dehydration in NaCl-treated eyes, but not in eyes treated with 3.2 mg/ml iota-carrageenan eye drops (white arrows indicate corneal damage).

b: The amount of fluorescein extracted from cornea punch samples after dehydration and immersion into a 0.1% fluorescein solution, expressed as percentage of NaCl treated controls, is proportional to the corneal damage acquired during dehydration. Error bars represent the SEM. N=number of eyes used per treatment group. * $p \leq 0.05$; ** $p \leq 0.01$;*** $p \leq 0.001$. Baseline value (=before dehydration) is significantly different from all other samples.

Fig. 1

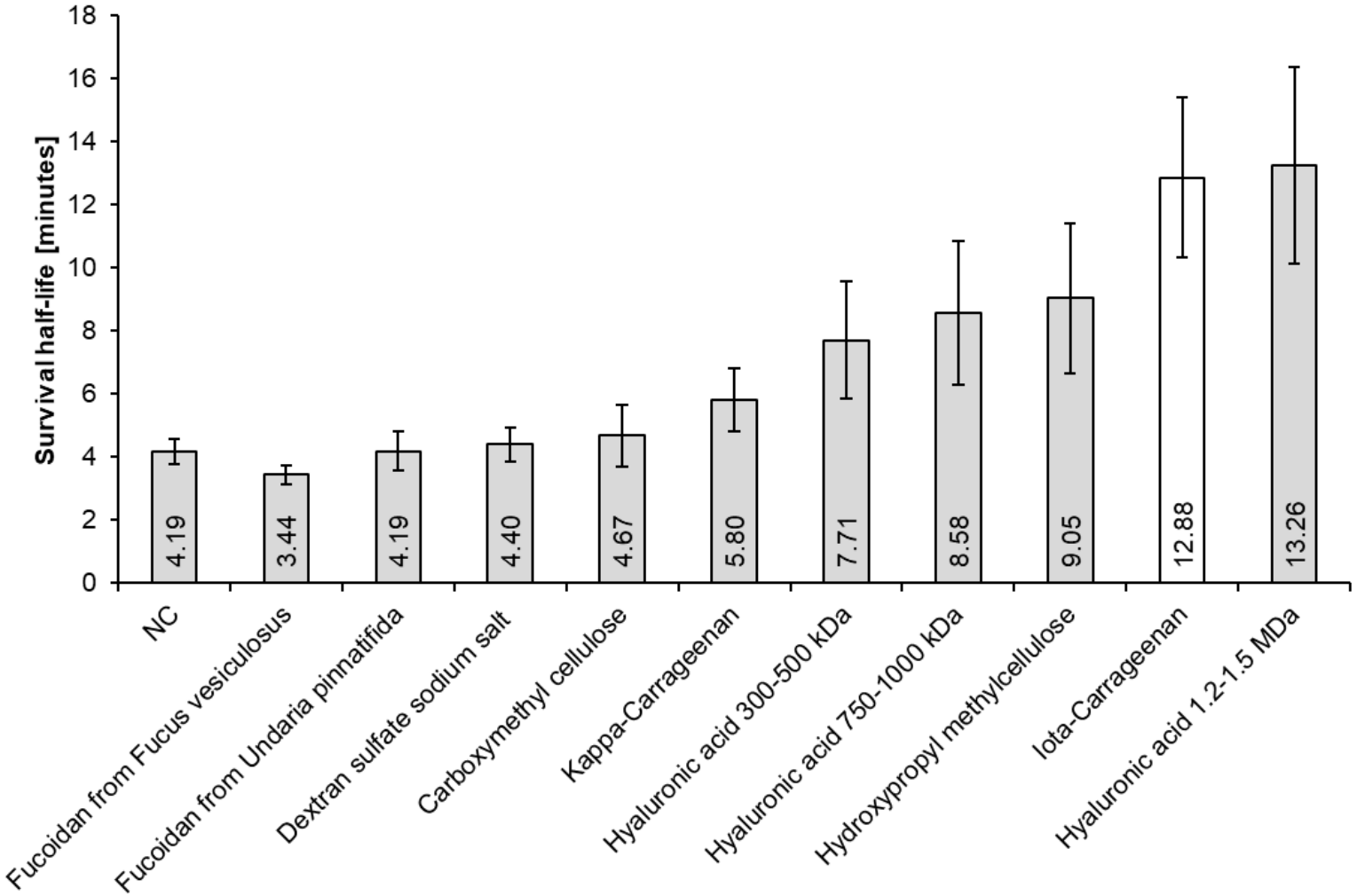

Survival half-life [minutes]
0
2
4
6
8
10
12
14
16
18
4.19
3.44
4.19
4.40
4.67
5.80
7.71
8.58
9.05
12.88
13.26
NC
Fucoidan from Fucus vesiculosus
Fucoidan from Undaria pinnatifida
Dextran sulfate sodium salt
Carboxymethyl cellulose
Kappa-Carrageenan
Hyaluronic acid 300-500 kDa
Hyaluronic acid 750-1000 kDa
Hydroxypropyl methylcellulose
Iota-Carrageenan
Hyaluronic acid 1.2-1.5 MDa

Fig. 2

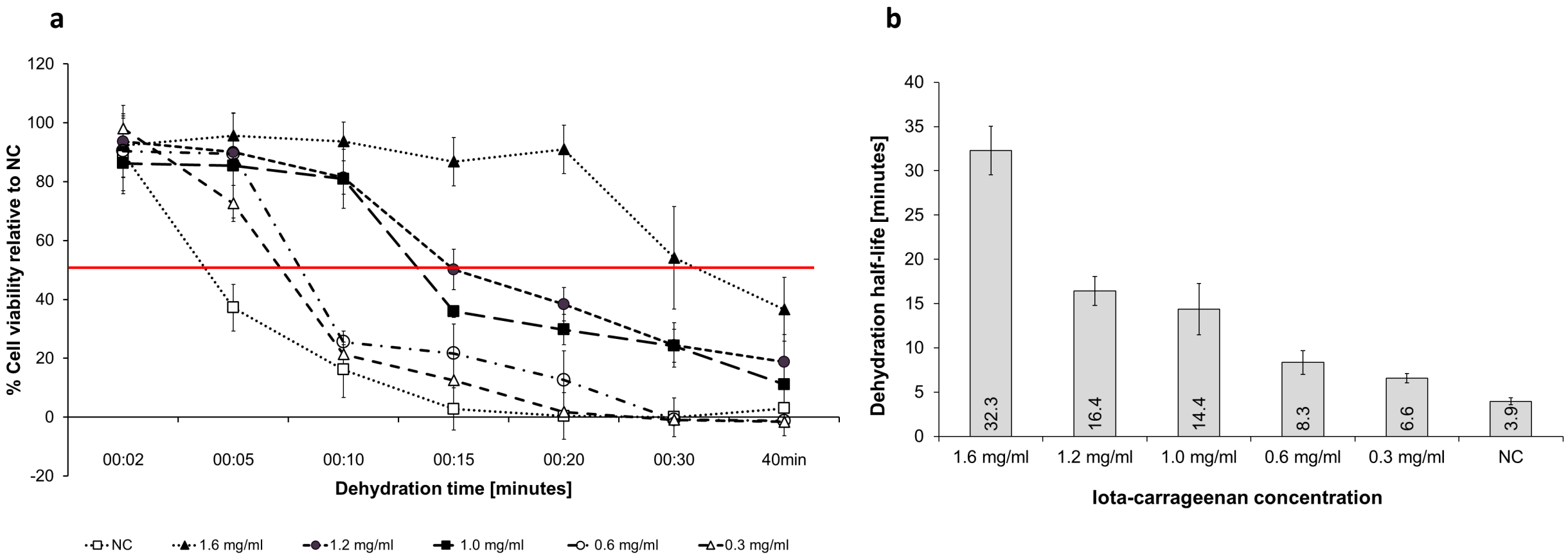

a
120
100
80
60
40
20
0
-20
% Cell viability relative to NC
00:02
00:05
00:10
00:15
00:20
00:30
40min
Dehydration time [minutes]
NC
1.6 mg/ml
1.2 mg/ml
1.0 mg/ml
0.6 mg/ml
0.3 mg/ml
b
40
35
30
25
20
15
10
5
0
Dehydration half-life [minutes]
32.3
16.4
14.4
8.3
6.6
3.9
1.6 mg/ml
1.2 mg/ml
1.0 mg/ml
0.6 mg/ml
0.3 mg/ml
NC
Iota-carrageenan concentration

Fig. 3

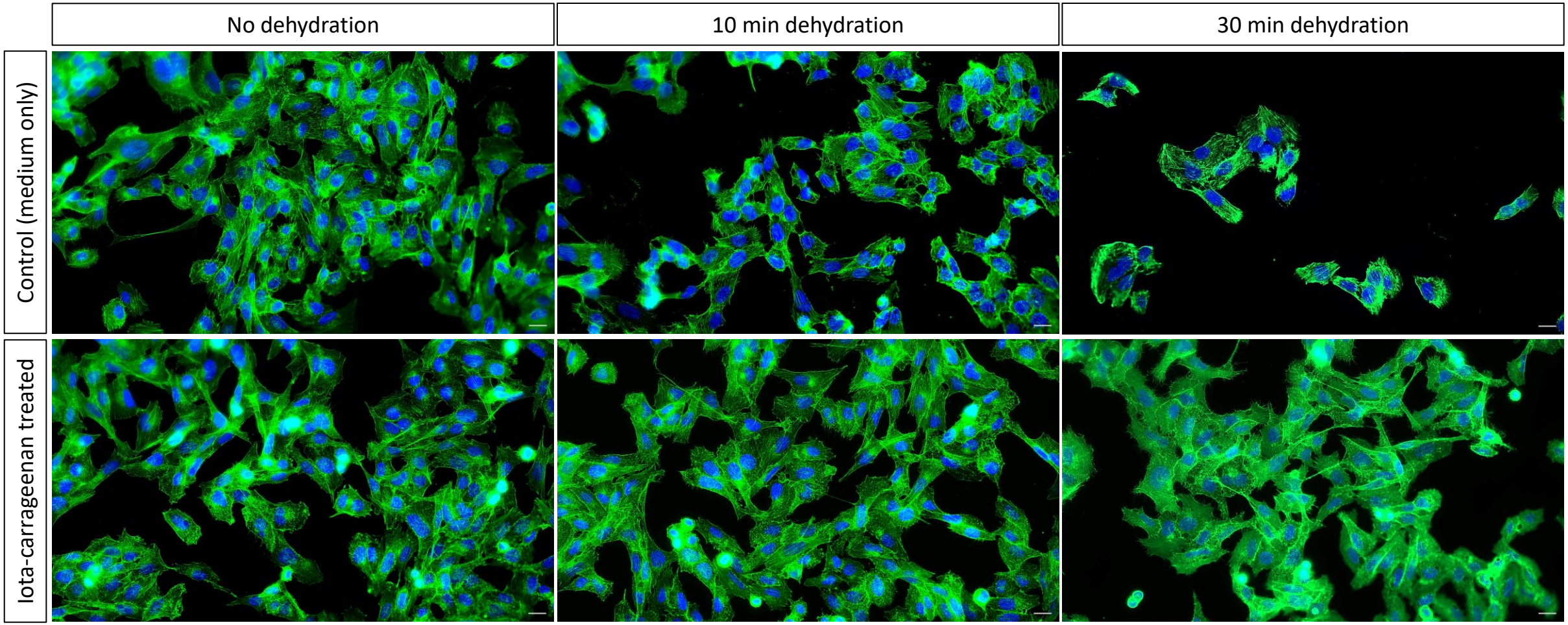

No dehydration
10 min dehydration
30 min dehydration
Control (medium only)
Iota-carrageenan treated

Fig. 4

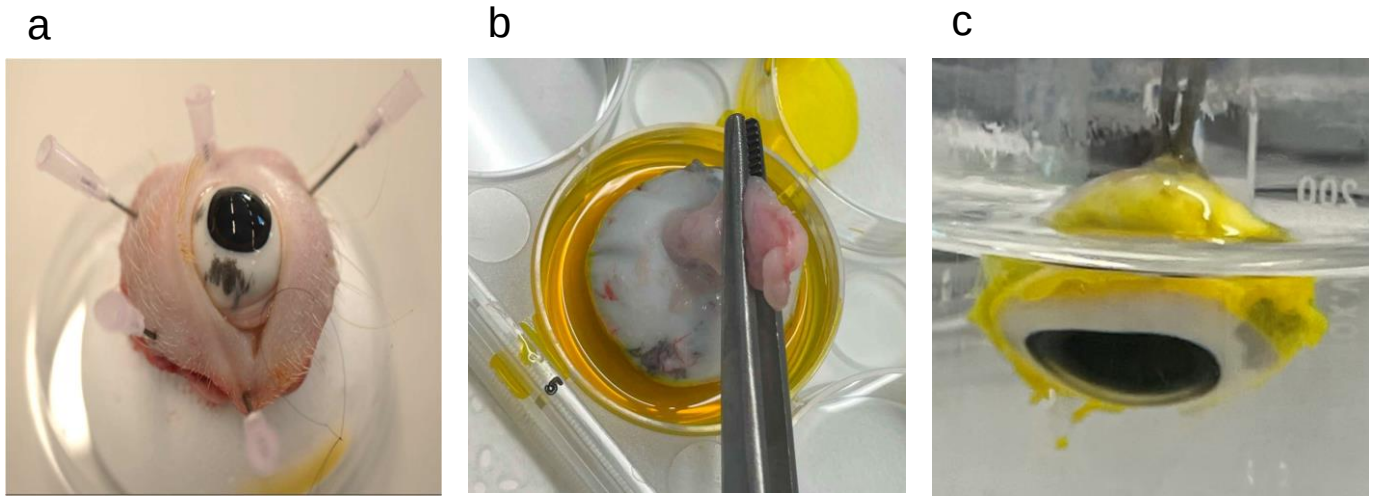
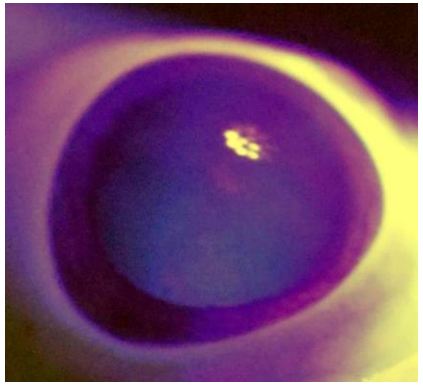
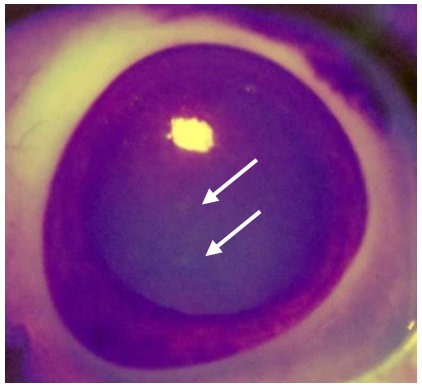
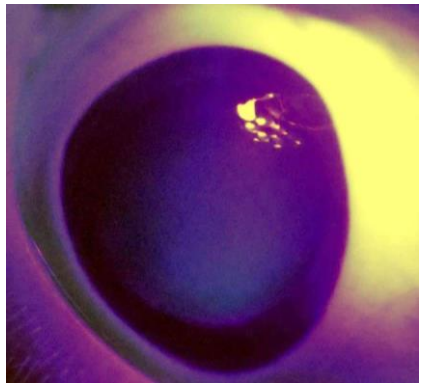
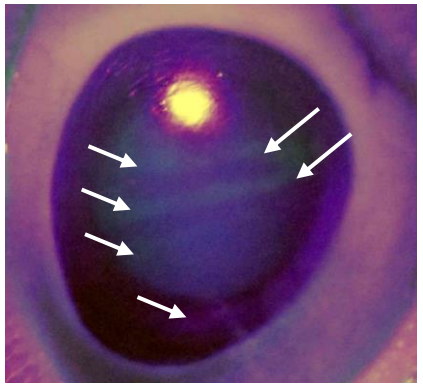
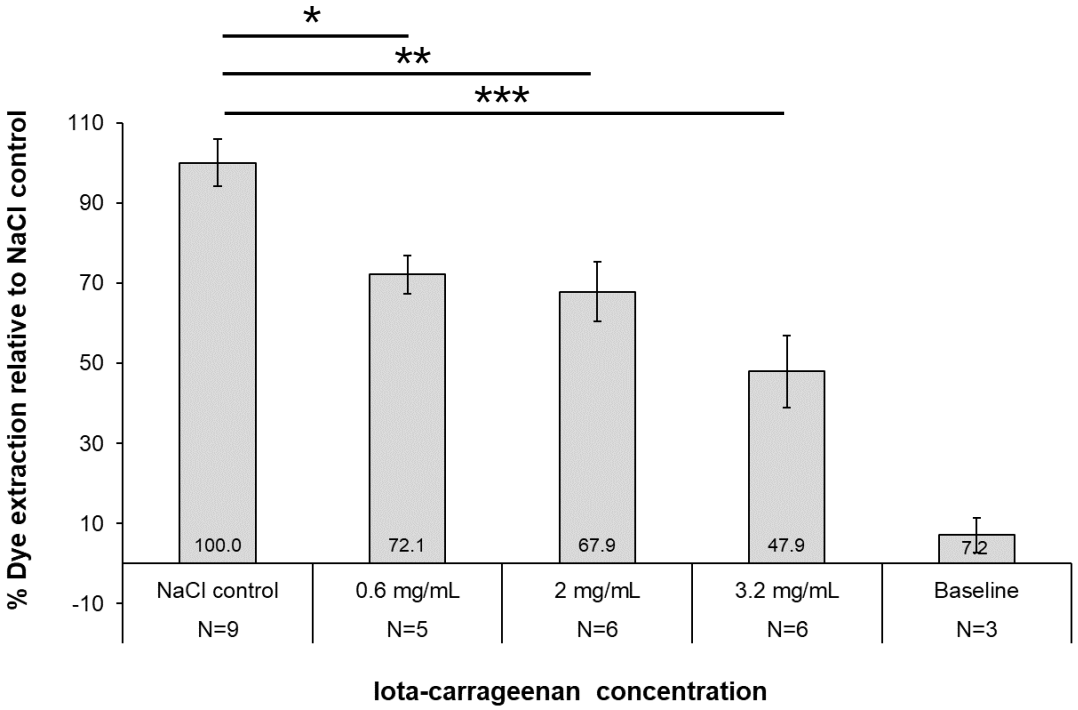


Fig. 5

a

	Before dehydration	After 6 hours of dehydration and repeated treatment
Iota-carrageenan eye drops		
0.9% NaCl		

b