Topical Quercetin And Resveratrol Protect the Ocular Surface In Experimental Dry Eye Disease

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Running head: Topical polyphenols for dry eye disease

Abstract

Purpose: To determine the anti-inflammatory effect of quercetin (QCT), resveratrol (RES), and their combination in a dry eye disease (DED) model.

Methods: DED was induced by exposing mice to a desiccating stress (DS); 0.01%QCT, 0.1%RES, 0.01%QCT+0.1%RES (QCT+RES) or vehicle were topically applied. Additionally, $CD4^+$ T cells isolated from DS-exposed mice were transferred to athymic recipient mice. Corneal fluorescein staining, tear production and tear cytokine levels were evaluated in DS-exposed mice, and conjunctival $CD4^+$ T cell infiltration was evaluated in recipient mice.

Results: Corneal staining increased in DS-exposed mice, being reduced by QCT ($p<0.001$) and QCT+RES ($p<0.05$). DS induced an increase of IL-1α concentration that was reduced by QCT, RES and QCT+RES ($p<0.05$, 0.01 and 0.01, respectively). $CD4^+$ T cells increased in recipients of DS-exposed mice ($p<0.05$), and was lower in recipients of QCT- and RES-treated mice ($p<0.05$).

Conclusion: The anti-inflammatory effect of polyphenols suggests their topical application for DED.

INTRODUCTION

Dry eye disease (DED) is a prevalent ocular surface disorder affecting from 8 to 14 % of the worldwide population.[1,2] Although the complete cellular and molecular mechanisms underlying DED are not totally understood,[3] it has been shown that immune-mediated inflammation plays a key role in this disease.[4,5] It has been found an inflammatory cell infiltration (e.g. $CD4^+$ T cells) within the conjunctiva and lacrimal gland tissues[6,7] correlating with an elevated tear pro-inflammatory cytokine/chemokine levels,[8] increased cell apoptosis in conjunctiva,[9] and a decrease of tear production coupled with decreased conjunctival goblet cell density in DED patients.[10]

Anti-inflammatory drugs are routinely used in the treatment of DED. Topical corticosteroids improve both the signs and symptoms of DED patients,[11,12] however their use is restricted by their long-term side-effects.[13]

To date, very few drugs, such as cyclosporine A and lifitegrast have been approved for the treatment of DED. Cyclosporine A is a fungal antimetabolite that inhibits IL-2 activation which is necessary for T cells replication by binding to cyclophilin for inhibiting calcineurin.[14,15] In DED, cyclosporine A decreases some inflammatory markers and immune cells,[10] however it is no available in some countries yet. Lifitegrast is a novel small molecule integrin antagonist optimized for ocular use that mimics intercellular adhesion molecule (ICAM)-1 which is expressed on T cells and in inflamed epithelium in DED.[16]

Nowadays there is a growing interest in the use of naturally-occurring polyphenols (e.g. flavonoids or stilbenes) in immune-based diseases due to their several biological properties, such as anti-inflammatory and antioxidant effects, among others.[17]

26 Quercetin (QCT) is a flavonoid-based polyphenolic compound found in a
27 variety of foods including apples, berries, onions and tea, as well as many
28 seeds. QCT has several biological properties, such as anticancer and
29 neuroprotective effects.[18] Among these properties, several studies have
30 demonstrated its potential as an anti-inflammatory compound, inhibiting many
31 kinases[19] as well as interfering with cytokine/chemokine production.[20]

32 Resveratrol (RES) is a non-flavonoid-based polyphenolic compound
33 highly enriched in grapes, peanuts and a wide variety of food sources. Like
34 QCT, RES has attracted considerable interest because of its cardioprotective,
35 neuroprotective and chemopreventive effects.[21] But RES is also well-known by
36 its antioxidant and anti-inflammatory effects with numerous mechanisms of
37 action, interacting with inflammatory cells, such as T cells.[22]

38 A great number of scientific publications have described the properties
39 and biomedically relevant activities of QCT and RES on several diseases (see
40 reviews[23] and[24] for more details). Our group has demonstrated in a previous
41 study that QCT, RES and a combination of both compounds decrease the
42 inflammatory response of ocular surface epithelial cells in-vitro.[25] Thus, the aim
43 of this work was to corroborate in-vivo the anti-inflammatory effect of QCT and
44 RES separately and in combination (QCT+RES) for the treatment of DED using
45 a well-characterized murine model of DED[26] followed by an adoptive transfer
46 model.[27]

47

48 **MATERIALS AND METHODS**

49

50 **Animals**

51 Female C57BL/6 and female T cell deficient nude C57BL/6 mice, both 8-10
52 weeks old, were purchased from Taconic Farms (Oxnard, CA, USA). Animal
53 studies approval was obtained from the Institutional Animal Care and Use
54 Committee at Allergan. All studies adhered to the Association for Research in
55 Vision and Ophthalmology (ARVO) statement for the use of animals in
56 ophthalmic and vision research.

57

58 **Desiccating stress-induced DED and topical treatments**

59 Experimental DED was induced in mice as previously described.[26,27] Up to five
60 mice were placed in a cage with a perforated plastic screens on two sides of the
61 cage. Mice were exposed to desiccating stress (DS) during 10 days by placing
62 them in a controlled-environment chamber with airflow from fans for 10 h a day,
63 room relative humidity at 20 % and temperature maintained at 23°C. A
64 continuous dose of 0.1 mg/day scopolamine hydrobromide (Sigma-Aldrich, St.
65 Louis, MO, USA) was administered by subcutaneously implanted osmotic pump
66 (Alzet®, Cupertino, CA, USA). Control mice were kept in a non-stressed
67 environment between 50 and 80 % relative humidity and temperature of 21 ºC
68 to 23 ºC, without exposure to forced airflow.

69 QCT and RES were purchased from Sigma-Aldrich and were dissolved in
70 a vehicle (β-cyclodextrin). Doses for topical application of QCT and RES (0.1
71 and 0.01 %, respectively) were chosen based on our in-vitro experiments,[25] a
72 preliminary in-vivo study (unpublished data), and on previous studies using
73 other polyphenols.[28,29]

74 Mice were randomly divided into 6 groups (n=9 in each group), according
75 to DS exposure and topical treatments: Control group (no DS and no topical

76 treatment), DS (no topical treatment), DS + vehicle, DS + 0.01 % QCT, DS +
77 0.1 % RES, DS + 0.01 % QCT + 0.1 % RES. The treatments started one day
78 before DS was set up and were administered topically in both eyes (5 µL/eye)
79 three times a day (9 AM, 1 PM and 5 PM).
80
81 **Corneal fluorescein staining**
82 Corneal staining was used to evaluate corneal epithelial damage caused by DS
83 after 9 days. A dose of 5 µL of 0.125 % fluorescein sodium salt (Sigma-Aldrich)
84 was applied into the lateral conjunctival sac of the mouse and both corneas
85 were examined 1 min later, using a stereo microscope under cobalt blue light
86 with a yellow filter to enhance observation of the fluorescence. Superficial
87 punctate staining was recorded in a masked fashion using the National Eye
88 Institute grading system, scoring 0 to 3 for each of 5 areas of the cornea:
89 central, superior, inferior, nasal and temporal.[30] Total corneal fluorescein
90 staining score was calculated as the sum of all areas of each eye, and medians
91 were obtained from both eyes of each mouse.
92
93 **Aqueous tear production**
94 Tear production was performed the day before DS induction and after 9 days of
95 DS using phenol red-impregnated cotton threads (Zone-Quick; Lacrimedics,
96 Eastsound, WA, USA). The thread was held with sterilized jeweller forceps and
97 placed in the lateral cantus of the right eye for 30 s. Threads turn from yellow to
98 red on absorption of tears. Wetting of the thread was measured using the
99 millimeter scale on the cotton thread package.
100

101 **Tear collection and measurement of cytokine and chemokine levels**

102 Tear samples were collected on day 6 in mice exposed to DS. A volume of 1.5
103 μL of cytokine assay buffer (Beadlyte; Millipore, Billerica, MA, USA) was instilled
104 into each eye, and immediately 1 μL/eye of tear fluid and buffer were collected
105 from the tear meniscus in the lateral cantus with a glass capillary tube and a
106 microcap (Drummond Scientific, Broomall, PA, USA). Then, the tear sample
107 from both eyes was pooled and diluted in 8 μL of ice-cold Beadlyte assay buffer,
108 and stored at -80 ºC until the time of assay. The level of selected cytokines and
109 chemokines in the tears was evaluated using a multiplex bead analysis with x-
110 MAP® technology.

111 Cytokine/chemokine tear levels were assessed using a Milliplex 15-plex
112 (IFN-γ, IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12(p70), MMP-9, IL-13, IL-17, IP-
113 10/CXCL10, MCP-1/CCL2, RANTES/CCL5 and TNF-α) mouse
114 cytokine/chemokine immunobead-based assay (Millipore) and analyzed on a
115 Luminex 200 instrument (Luminex Corporation, Austin, TX). The minimum
116 detectable level for each cytokine/chemokine, based on the manufacturer
117 specifications, was: 1.1 pg/mL for IFN-γ, 10.3 pg/mL for IL-1α, 5.4 pg/mL for IL-
118 1β, 1.0 pg/mL for IL-2, 0.4 pg/mL for IL-4, 1.1 pg/mL for IL-6, 2.0 pg/mL for IL-
119 10, 4.8 pg/mL for IL-12(p70), 0.38 pg/mL for MMP-9, 8.3 pg/mL for IL-13, 0.5
120 pg/mL for IL-17, 0.8 pg/mL for IP-10/CXCL10, 6.7 pg/mL for MCP-1/CCL2, 2.7
121 pg/mL for RANTES/CCL5 and 2.3 pg/mL for TNF-α. When a cytokine level was
122 not detectable in a sample, the minimum detectable level was used in the
123 analysis. Statistical analysis was restricted to molecules with percentage of
124 detection value higher than 50 % (i.e., with < 50 % of samples falling below the

LOD) in at least one of the study groups. Data were analyzed using Milliplex analyst software V5 (Millipore).

Adoptive transfer model

The adoptive transfer model was performed as previously described.[27,31] CD4$^+$ T cells from each group of DS-exposed mice were isolated from spleens and cervical lymph nodes, and enriched using a CD4$^+$ T cell isolation kit II (MACS System, Miltenyi Biotec, Auburn, CA, USA), according to the manufacturer's instructions. Then, one donor-equivalent [defined as the number CD4$^+$ T cells remaining after the respective in-vitro manipulation of a single set of lymph nodes or spleen (approximately $5x10^6$ CD4$^+$ T cells)] of cell suspension in PBS was transferred intraperitoneally into each T-cell-deficient athymic nude C57BL/6 mouse (recipient mouse). Eight recipient mice per donor group were used. Recipient mice were sacrificed 72 h after receiving adoptively transferred CD4$^+$ T cells. Immediately, left eyeballs with attached lids were embedded in optimal cutting temperature (OCT) compound and flash frozen until use.

CD4$^+$ T cell immunohistochemistry

CD4$^+$ T cell immunostaining and counting were performed in recipient mouse samples as previously described.[27,31,32] OCT-embedded left eyeballs with attached lids from recipient mice were sectioned at 7 µm thickness with a cryostat microtome (CM3050 S; Leica Microsystems, Buffalo Grove, IL, USA), mounted on glass slides and stored at -80 ºC. On the day of use, the mounted sections were dried at 37 ºC overnight and fixed in -20 ºC acetone for 10 min at room temperature. Endogenous peroxidases were then quenched with H_2O_2 for

150 10 min, and slides were rinsed with PBS and air dried for 1 h. After that, non-
151 specific sites were blocked with 20 % normal rabbit serum (Dako, Carpinteria,
152 CA), and rat anti-mouse CD4 primary antibody (BD Biosciences, Mountain
153 View, CA, USA) was added to each slide and incubated for 1 h at room
154 temperature. After slides were extensively washed, sections were incubated
155 with biotinylated polyclonal anti-rat IgG secondary antibody (BD Biosciences)
156 for 30 min. Then, Vectastain Elite ABC reagent (Vector laboratories,
157 Burlingame, CA, USA) was added for antigen localization, following
158 manufacturer's instructions. Finally, samples were incubated with NovaRED
159 (Vector Laboratories) peroxidase substrate to give a red stain, and
160 subsequently counterstained with haematoxylin (Invitrogen, Carlsbad, CA,
161 USA). In addition, rat anti-mouse IgG isotype (BD Biosciences) was used as
162 negative control. For quantification of $CD4^{+}$ T cells, a midline section of each
163 eye was counted following the morphometric guideline of the entire superior and
164 inferior conjunctiva, starting at the limbus and spanning the entire length to the
165 tarsal conjunctiva, including the conjunctival epithelium and stroma to a depth of
166 75 μm below the basement membrane. $CD4^{+}$ T cells in the conjunctiva were
167 countered in a masked fashion with light microscopy, using an Eclipse E400
168 microscope (Nikon, Melville, NY, USA).

169

170 **Statistical analysis**

171 Data were expressed as median ± interquartile range using Tukey's method for
172 plotting the whiskers. The Mann-Whitney test was used for statistical
173 comparisons using the SPSS software package (SPSS version 15.0 for

174 Windows, SPSS Inc., Chicago, IL, USA). One-sided p-values less than 0.05
175 were considered statistically significant.
176
177 **RESULTS**
178
179 **Effect of topical QCT and RES on corneal fluorescein staining in DS**
180 **exposed mice**
181 After 9 days, both DS mice and vehicle-treated DS mice showed a significant
182 increase in corneal fluorescein staining ($p<0.001$), compared to control group
183 (Fig 1A). There were no differences in staining between DS mice and vehicle-
184 treated DS mice. Corneal fluorescein staining was significantly decreased in DS
185 mice groups that were treated with QCT and QCT+RES ($p<0.001$ and $p<0.05$,
186 respectively) compared to vehicle-treated DS mice (Fig 1A). Minimal scattered
187 punctate staining, or no staining, was observed on the corneas of control mice
188 after instillation of fluorescein dye (Fig 1B), whereas intense punctate
189 fluorescein staining was observed in both DS mice (Fig 1C) and vehicle-treated
190 DS mice (Fig 1D). This intense punctate staining decreased when mice were
191 topically treated three times a day with QCT (Fig 1E), RES (Fig 1F) and
192 QCT+RES (Fig 1G).
193
194 **Effect of QCT and RES on tear production**
195 Tear production was measured in DS-exposed mice, 1 day before DS challenge
196 (baseline) and after 10 days of DS (post-DS) (Fig 2). DS caused a significant
197 decrease in tear production ($p<0.001$) compared to baseline, however tear
198 production was not affected by QCT, RES or QCT+RES treatments.

199

200 **Effect of topical QCT and RES on cytokine production**

201 Six (IL-1α, IL-2, IL-4, IP-10/CXCL10, RANTES/CCL5 and TNF-α) out of the 15
202 cytokines and chemokines measured were detected in tears of DS exposed
203 mice at day 6 (Fig 3 A-F). DS significantly increased IL-1α in vehicle-treated DS
204 mice and RANTES/CCL5 in DS mice (both $p<0.05$), compared to control mice.
205 Tear levels of IL-1α significantly decreased when DS mice were treated with
206 QCT ($p<0.05$), RES ($p<0.01$), and QCT+RES ($p<0.01$), compared to vehicle-
207 treated DS mice. No significant differences were found between DS group and
208 vehicle-treated DS group, indicating that vehicle had no effect on IL-1α levels in
209 tear. Additionally, there was a trend for decrease IL-4 levels in QCT-treated DS
210 mice ($p=0.0625$), compared to vehicle-treated DS mice. Neither DS nor topical
211 polyphenols had any effect on IL-2, IP-10/CXCL10, and TNF-α tear levels.
212 Polyphenols did not decrease RANTES tear levels on DS-exposed mice.

213

214 **Effect of QCT and RES on conjunctival CD4$^+$ T cell infiltration in recipient**
215 **mice**

216 The CD4$^+$ T cell infiltration in conjunctiva of recipient of DS-exposed mice was
217 significantly increased ($p<0.05$), compared to recipients of control mice (Fig 4A).
218 No significant differences in CD4$^+$ T cell infiltration were found between
219 recipients of vehicle-treated DS mice and recipients of DS mice (vehicle had no
220 effect on CD4$^+$ T cell infiltration). Regarding topical treatments with polyphenols,
221 there was a significant decrease in CD4$^+$ T cell infiltration in conjunctiva of
222 recipients of RES-treated and QCT-treated DS mice (both $p<0.05$), compared to
223 recipients of vehicle-treated DS mice. The combination of both polyphenols was
224 not significant. Figure 4 B-H shows representative immunostaining images of

225 conjunctival $CD4^+$ T cells from each recipient mice group (including isotype
226 control).
227

228 DISCUSSION

229 We present herein data demonstrating that topical treatment with QCT, RES
230 and their combination, QCT+RES, decrease the clinical signs in an
231 experimental murine model of DED. Furthermore, we have also shown that
232 QCT and RES treatments in DS-exposed mice do significantly decrease $CD4^+$ T
233 cell infiltration in athymic recipient mice. Results derived from this work also
234 corroborate those previously described by our group, in which we demonstrated
235 in-vitro the effect of QCT and RES on conjunctival and corneal epithelial cells.[25]
236 Our study has shown that fluorescein staining improves dramatically in
237 mice exposed to DS when QCT and QCT+RES are applied topically. This result
238 suggests that QCT and its combination with RES protect the ocular surface by
239 enhancing DS-exposed mouse corneal integrity. This protective effect of studied
240 polyphenols on corneal integrity agrees with a previous study by Lee et al. that
241 tested the effect of the green tea polyphenol epigallocatechin gallate in a similar
242 in-vivo DED model.[29] They applied 0.01 and 0.1 % epigallocatechin gallate
243 solutions topically to mice exposed to DS and found that topical treatments at
244 day 4, including vehicle (1 % dimethyl sulfoxide in phosphate-buffered saline),
245 have a significant decrease in corneal staining compared to the untreated
246 group. However, after 9 days of DS, only 0.1 % epigallocatechin gallate-treated
247 eyes showed a significant decrease of corneal staining. According to Chen et
248 al.,[33] Th17 pathway is involved in regulating corneal integrity in mice exposed to
249 DS, and it may be hypothesized that the protective effect of QCT and

250 QCT+RES on corneal integrity may be due at least in part to an inhibitory effect
251 upon Th17 cells.

252 We also addressed the effect of QCT and RES on inflammatory cytokine
253 levels in tears. Out of 15 cytokine/chemokine measured, six (IL-1α, IL-2, IL-4,
254 IP-10/CXCL10, RANTES/CCL5 and TNF-α) were detected and analyzed in
255 tears of DS-exposed mice at day 6. The pro-inflammatory cytokine IL-1α and
256 the chemokine RANTES/CCL5 were increased in tears of DS-exposed mice. IL-
257 1α tear levels significantly decreased in DS mice treated either with QCT, RES
258 or with both compounds combined. IL-1α is detected to be increased in tear
259 fluid of DED patients and correlates with clinical parameters of DED,[34,35]
260 particularly with fluorescein staining.[36,37] Our study agrees with this result as a
261 decreased concentration of IL-1α in tears of polyphenols-treated mice is in
262 correlation with corneal fluorescein staining results.

263 The anti-inflammatory effect of polyphenols on cytokine/chemokine
264 secretion has also been described by Lee et al.[29] They quantified the transcripts
265 encoding IL-1β, TNF-α and MCP-1 in corneas of DS-exposed mice and found
266 that treatment with topical 0.01 and 0.1 % epigallocatechin gallate decreases
267 the relative expression of IL-1β and MCP-1 transcripts, but not the TNF-α one.
268 These results suggest that the anti-inflammatory effect of topical polyphenols on
269 cytokine secretion may occur, in part, at early stages of the inflammatory
270 response.

271 Our results have also shown that QCT and RES had no effect on tear
272 production, suggesting that topical administration of polyphenols does not affect
273 lacrimal gland function. Nevertheless, an underlying finding relies on our results
274 as topical polyphenols were capable of maintaining ocular surface integrity (as

275 corneal staining results showed) even when tear film secretion is compromised.
276 This is an important finding because these compounds may help to patients
277 suffering aqueous tear-deficient dry eye, in which the lacrimal glands fail to
278 produce enough of the watery component of tears to maintain a healthy eye.
279 This lack of polyphenol effect found on tear secretion is in contrast to those data
280 found by Oh et al. who tested 0.5 % QCT eye drops in NOD.B10.H2b mice,[38]
281 which develop spontaneous Sjögren's syndrome-like autoimmune inflammation
282 exacerbated by DS.[39] In this model, tear secretion increases by topical QCT
283 when applied in mice exposed previously to DS. This difference in results may
284 be due to differences in QCT concentration used, the time when the drug is
285 administered and the DED model used in each study; Oh et al. tested a QCT
286 concentration higher than that used in this study (0.5 % vs 0.01 %). Also, it have
287 been shown that in the desiccating DED model that we have used, after the
288 removal of DS, tear volume and ocular surface parameters recover within 2
289 weeks in C57BL/6 mice (when scopolamine and air flow are removed), whilst
290 tear volume remains unchanged in NOD mice.[40] As we used C57BL/6 mice in
291 this study, this fact leaded us to apply polyphenols when mice were exposure to
292 DS in our model, whereas polyphenols can be applied after DS exposure in
293 NOD mice, as in the study by Oh. In addition, scopolamine is a tropane alkaloid
294 that inhibits acetylcholine-mediated stimulation of the lacrimal gland by blocking
295 muscarinic acetylcholine receptors, promoting pathogenic aqueous tear
296 deficiency and inflammation of the lacrimal gland.[41] Thus, these differences in
297 tear volumes may be also explained in part by the lack of muscarinic activity by
298 polyphenols when acetylcholine receptors are blocked by scopolamine (these
299 receptors were not blocked in NOD mice when QCT was applied). Although it

300 has been shown that polyphenols are muscarinic receptor agonists,[42,43] our
301 results indicate that both polyphenols (topically and at tested doses) do not
302 have any effect on muscarinic activity when scopolamine is present, as it has
303 been shown in other in-vivo models.[44,45] Further studies are necessary to
304 warrant polyphenol effect on tear secretion on DED animal models.

305 The controlled-environment and scopolamine is used to lower aqueous
306 tear production, which contributes to upregulate pro-inflammatory cytokines
307 leading to $CD4^+$ T cells homing the ocular surface. However, the immune
308 system of this mouse can modulate its own response by generating regulatory T
309 cells, such as $CD4^+$ $CD25^+$ $Foxp3^+$ T cells,[31] maintaining a homeostatic
310 environment at the ocular surface.[27] This fact can mask the anti-inflammatory
311 effect of tested drugs for treating DED. In order to avoid this drawback, and for
312 testing whether the protective effects of polyphenols on the ocular surface of
313 DS-exposed mice were mediated by the immune system, an adoptive transfer
314 model was used.

315 Athymic nude mouse lacks a functional thymus and does not generate
316 mature T cells and normal regulatory T cells (no T cells are found in these
317 mice). When $CD4^+$ T cells isolated from mice exposed to DS are transferred to
318 athymic recipient mice, a severe DED is produced in these recipient mice ($CD4^+$
319 T cells infiltrate only at the ocular surface and lacrimal gland) although athymic
320 mice are never exposed to DS.[27] Thus, this model is a powerful tool to address
321 the anti-inflammatory effect of new treatments for DED on T-cell-specific aspect
322 (effector and regulatory). Our results have shown that recipient mice of DS-
323 exposed mice had a significant increase of $CD4^+$ T cell infiltration in the
324 conjunctiva ($p<0.05$). The $CD4^+$ T cell infiltration in the conjunctiva of recipients

325 of QCT- and RES-treated DS mice was significantly decreased, showing the
326 modulatory effect of polyphenols on immune cells. In addition, although $CD4^+$ T
327 cell infiltration in conjunctiva of recipient mice of QCT+RES-treated DS mice did
328 not significantly decrease (p=0.06), its value was clinically relevant because it
329 was similar to that found in recipients of control mice. These results are an
330 important finding because it means that QCT and RES have an anti-
331 inflammatory effect on DED; and this immunomodularory property is, in part,
332 due to the inhibition of $CD4^+$ T cells, which play an important role in the
333 development of DED.[5]

334 Nevertheless, not only $CD4^+$ T cells play a key role in DED but also other
335 immune system cells, such as professional antigen presenting cells (e.g. $CD11^+$
336 dendritic cells) which trigger T cells activation and differentiation, are involved in
337 the pathogenesis of DED.[32,46] Thus, the anti-inflammatory effect of polyphenols
338 may occur at early stages of the dendritic cell-mediated pathway. Further
339 studies are needed to elucidate the mechanism underlying corneal integrity
340 improvement by QCT and RES, and their effect on immune cells playing a
341 critical role in the in-vivo model of DED, such as Th17, $CD4^+$ $CD25^+$ $Foxp3^+$
342 regulatory T cells and antigen presenting cells.[31,32]

343 This study has some limitations. Firstly, only one concentration of QCT
344 and RES, and their combination have been tested. Our results showed the anti-
345 inflammatory and protective effects of those compounds on the ocular surface
346 in DED, however more studies are warranted to know whether other
347 concentrations of QCT, RES and their combinations could be more effective
348 than concentrations used in this study. Secondly, polyphenols were topically
349 administered in mice exposed to DS and their anti-inflammatory effects were

350 only tested in dry eyes. Although QCT and RES did not show any effect on
351 unstimulated ocular surface epithelial cells in-vitro,[25] further experiments
352 including control mice (not exposed to DS) treated with polyphenols could show
353 their effect in normal healthy eyes at the treatment doses.

354 In conclusion, this work has demonstrated that topical application of 0.01
355 % QCT, 0.1 % RES or 0.01 % QCT + 0.1 % RES not only decreases the clinical
356 signs (superficial keratitis) and inflammatory response (cytokine production; e.g.
357 IL-1α) of the ocular surface in a murine model of DED, but also that this effect is
358 in part mediated by a regulatory effect on $CD4^+$ T cells, as shown the adoptive
359 transfer model results. Thus, we suggest a potential use of QCT, RES, and their
360 combination as anti-inflammatory treatments for DED and possibly of other
361 immune-based ocular surface diseases.

362

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364

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367

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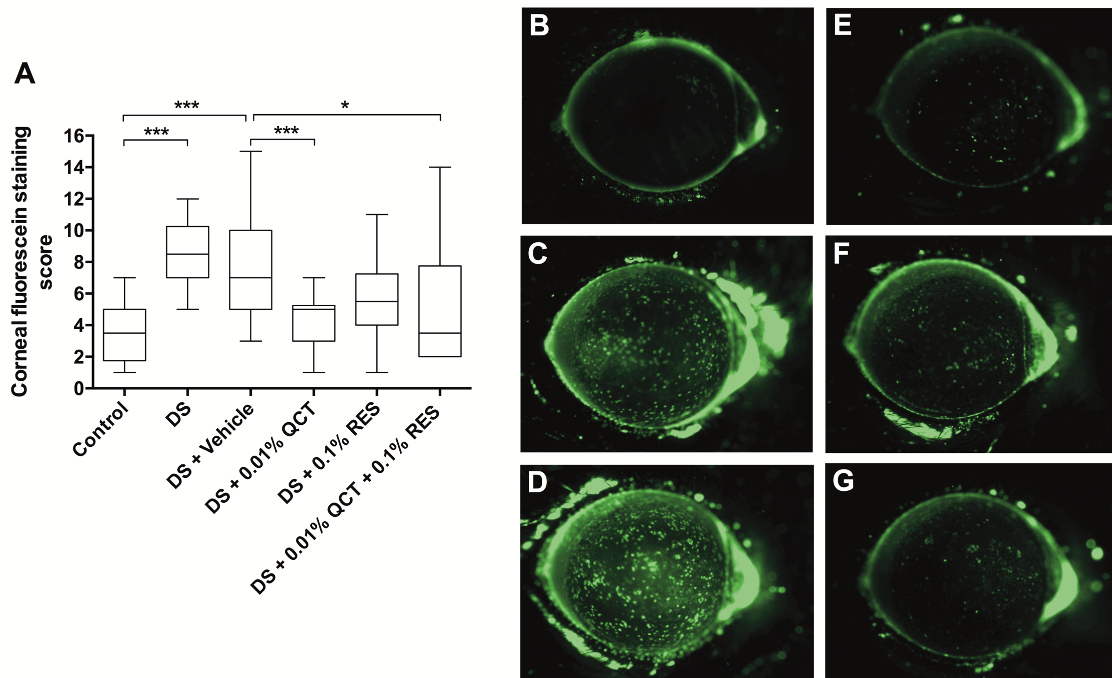

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532 **Figure 1. Effect of topical quercetin (QCT), resveratrol (RES) and**
533 **QCT+RES on corneal fluorescein staining.** Corneal epithelial damage
534 assessment by corneal fluorescein staining at day 9 in mice exposed to
535 desiccating stress (DS) (A). Representative images of corneas from each group
536 are shown on the right: control (B), DS (C), DS + vehicle (D), DS + 0.01 % QCT
537 (E), DS + 0.1 % RES (F) and DS + 0.01 % QCT + 0.1 % RES (G). Data are
538 expressed as median ± interquartile range using Tukey's method for plotting the
539 whiskers. *P<0.05, ***P<0.001.

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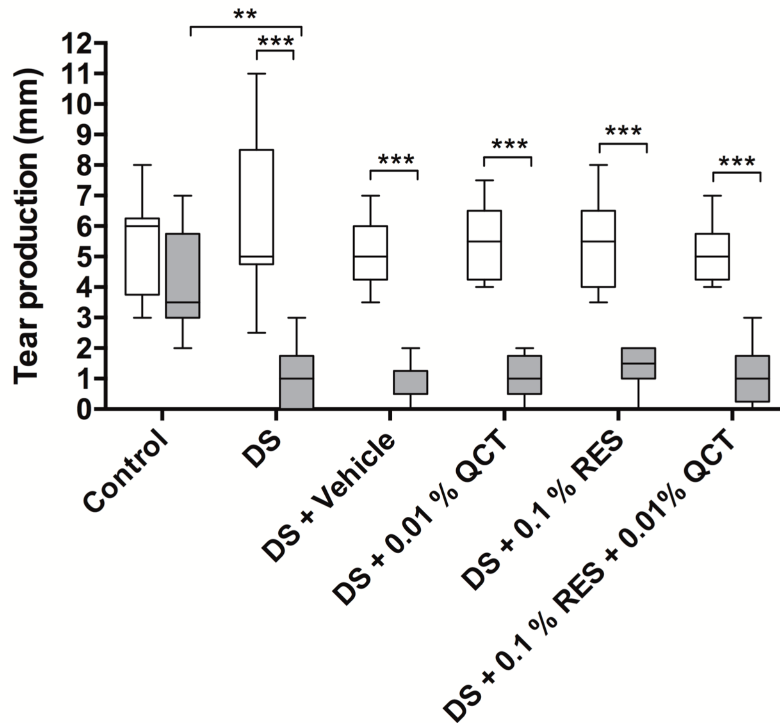

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543 **Figure 2. Effect of quercetin (QCT), resveratrol (RES) and QCT+RES on**
544 **tear production.** Tear production in mice at baseline (white boxes) and after
545 exposure to desiccating stress (DS) at day 9 (gray boxes). Data are expressed
546 as median ± interquartile range using Tukey's method for plotting the whiskers.
547 **P<0.01, ***P<0.001.

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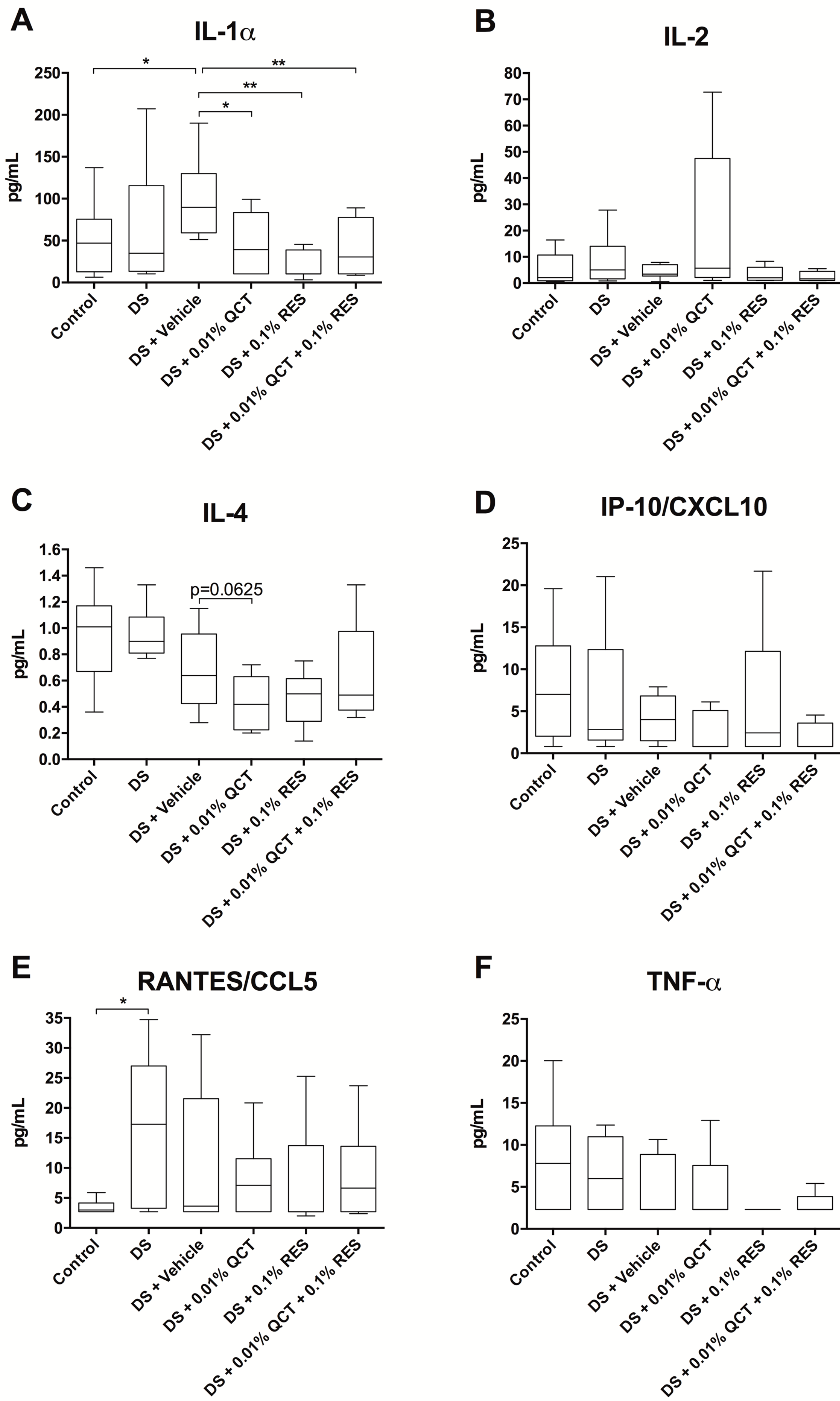

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551 **Figure 3. Effect of topical quercetin (QCT), resveratrol (RES) and**
552 **QCT+RES on tear cytokine/chemokine levels in mice exposed to**
553 **desiccating stress (DS).** Graphs represent the concentration at day 6 of IL-1α
554 (A), IL-2 (B), IL-4 (C), IP-10/CXCL10 (D), RANTES/CCL5 (E) and TNF-α (F) in
555 tears of mice treated topically with QCT, RES or QCT+RES and exposed to DS.
556 Data are expressed as median ± interquartile range using Tukey's method for
557 plotting the whiskers. *P<0.05, **P<0.01.

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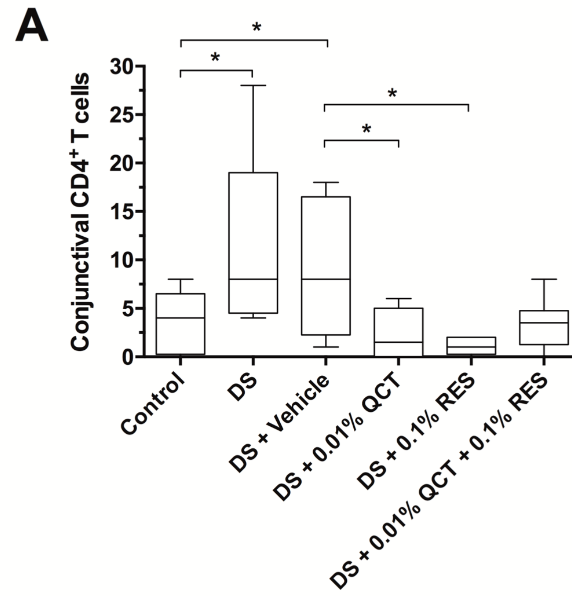
A
Conjunctival CD4+ T cells
30
25
20
15
10
5
0
*
*
*
*
Control
DS
DS + Vehicle
DS + 0.01% QCT
DS + 0.1% RES
DS + 0.01% QCT + 0.1% RES

560

561 **Figure 4. Effect of quercetin (QCT), resveratrol (RES) and QCT+RES on**
562 **$CD4^+$ T cell infiltration in the conjunctivas of recipient mice.** (A) $CD4^+$ T cell
563 numbers in the conjunctiva of the recipient mice 72 h after transferring
564 adoptively $CD4^+$ T cells, isolated from cervical lymph nodes and spleens of mice
565 exposed to desiccating stress (DS) for 10 days. Data are expressed as median
566 ± interquartile range using Tukey's method for plotting the whiskers. *P<0.05.
567 Representative digital pictures of immunohistochemical staining from each
568 group of recipients of DS-exposed mice are shown: control (B), DS (C), DS +
569 vehicle (D), DS + 0.01 % QCT (E), DS + 0.1 % RES (F), DS + 0.01 % QCT +
570 0.1 % RES (G), and isotype control (H). Solid black arrowheads indicate areas
571 of $CD4^+$ T cell infiltration. Images captured at 10X magnification.