Universidad de Valladolid

FACULTAD DE MEDICINA

INSTITUTO UNIVERSITARIO DE OFTALMOBIOLOGÍA APLICADA

TESIS DOCTORAL

THE ANTI-INFLAMMATORY COMPOUNDS RESVERATROL AND QUERCETIN AS THERAPEUTIC AGENTS FOR IMMUNE-BASED OCULAR SURFACE DISEASES

Compuestos antiinflamatorios Resveratrol y Quercitina como agentes terapéuticos para las enfermedades de base inmune de la superficie ocular

Presentada por **Antonio Abengózar Vela** para optar al grado de Doctor por la Universidad de Valladolid

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Impreso 2T

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Valladolid, 18 de noviembre de 2014

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Within the framework of a collaboration with the University of Valladolid (responsible supervisors María Jesús González-García, PhD and Amalia Enríquez-de-Salamanca, PhD), Mr. Antonio Abengózar-Vela visited my group in the Department of Ocular Biology and Therapeutics in London for a period of three months, working closely with Dr Virginia Calder. During his stay he worked in a joint project between UCL and the University of Valladolid to study the effect of two polyphenols, resveratrol and quercetin, on cord blood mast cells (CBMC) and peripheral blood mononuclear cells (PBMC). He performed proliferation and viability assays on PBMC and cytotoxicity and histamine release assays on CBMC, to study the immune-regulatory and anti-allergic properties of both polyphenols alone and in combination. He was a hard working person, full of enthusiasm and dedicated to the project. He obtained interesting results which will become part of his Thesis and a possible joint paper from both research groups.

Yours sincerely,

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A mi familia

Agradecimientos/Acknowledgments

"Cuando realmente deseas algo, todo el universo conspira para que lo consigas". Esta es una de las frases sacadas del libro "El alquimista" de Paulo Coelho que mejor podrían definir la culminación de esta tesis, ya que todo ese universo conspiratorio es la gente sin la cual este proyecto no hubiera podido salir adelante. Así, con estas líneas quiero expresar mi más profundo agradecimiento a todos ellos.

En primer lugar, a mis dos directoras, las doctoras María Jesús González y Amalia Enríquez de Salamanca. Gracias María porque desde el primer día, sin conocerme de nada, te preocupaste de que me sintiera como en casa, tanto fuera como dentro del IOBA (eso es algo que siempre recordaré); y por confiar en mi para realizar esa "idea que te rondaba la cabeza". Gracias Amalia porque siempre me has mostrado tu simpatía y amabilidad; por tu constante ayuda en cada problema experimental y en el trabajo diario de planificación de los experimentos. Agradeceros a las dos vuestra preocupación y cariño en lo personal durante este tiempo. En lo profesional, gracias por vuestros consejos y enseñanzas, apoyo y confianza en todas las etapas de esta tesis; porque habéis contado con mis ideas y humildes aportaciones, algo que un predoctoral siempre agradece de sus directores de tesis y que en pocas ocasiones sucede; y porque siempre habéis tenido la puerta de vuestros despachos abiertas para mi por cualquier motivo, tanto personal como profesional. Gracias por todas las oportunidades que me habéis ofrecido para que yo pudiera asistir a cursos y congresos, para la realización de mis estancias, por la posibilidad de publicar,… Por todo esto y más, ¡gracias!.

Por extensión, quisiera agradecer a "mi tercera directora", la doctora Margarita Calonge, su confianza en la realización de esta tesis y por permitirme formar parte del grupo de superficie ocular (GSO), de lo cual estoy muy orgulloso. Gracias Marga por tu apoyo desde el comienzo de este proyecto, tanto por hacerlo parte de AISWG como por tu implicación en mis estancias que tanto me han aportado personal y profesionalmente.

Gracias también a la doctora Yolanda Diebold por el voto de confianza en aquel estudiante que contactó con ella desde Valencia y al que puso en las manos de María. ¡Seguro que te extrañó ya que no dejabas de repetirme que "el doctorado no es a distancia"!.

Gracias al Profesor José Carlos Pastor porque sin su esfuerzo y dedicación durante tantos años para que el IOBA sea lo que hoy es, un centro de referencia, nada

de esto podría haberse realizado. El "chico resveratrol" quiere agradecerle el buen humor, el cariño y la confianza que me ha dado durante este tiempo…"y lo sabes".

I also would like to thank Dr. Michael E. Stern and Dr. Virginia Calder for the chance to do my stays in their laboratories, which were two of the best experiences of my life.

Dear Dr. Stern, thank you for your help and recommendations, for showing me how to "look for my lost wallet in a dark street". It has been an honor and privilege for me to have worked in your lab. I really appreciate your involvement in this project, giving me the opportunity to learn from you and your team during my stays in Allergan. Thank you very much for encouraging me to be more confident, not only in my research but also in my life.

Appreciation also goes out to all people who kindly helped me during my stays in Allergan: Chris Schaumburg, Jianping Gao, Kathy Held, Darren Oh, Betty Lee, Sveti Ugarte, Julio Nieves, Annie Ratanapinta, Reuben Sana, Amy Hopkin, Emily Santori, Anh Duong, Kelly Manley and Hsia Edward.

Dear Dr. Calder, thank you very much for your hospitality during my stay in London. I appreciate your advice, supervision and crucial contribution to this thesis…and your patience and efforts to understand my English! I also would like to thank you for opening your home to Gema and me.

I also thank Grazyna Galatowicz for her technical assistance in the lab during my stay at University College London.

I am grateful to Dr. Suzanne Hagan for reviewing the English language in this thesis.

También quisiera dar las gracias a los doctores Roberto Reinoso, Teresa Nieto y Alberto López por su ayuda y apoyo durante estos años. Y a la doctora Itziar Fernández por enseñarme "un poco" de estadística. Gracias a todos por vuestra paciencia infinita a la hora de resolverme dudas. Gracias también a los doctores Alfredo Corell y Carmen Martín por su ayuda y colaboración en los experimentos de citometría de flujo.

Por extensión, gracias a todo el GSO, porque vosotros habéis hecho sentirme en el IOBA como en casa desde el primer día que llegué a Valladolid. En especial, a los pre-doctorales (incluyendo los que ya han "crecido" y son doctores) y técnicos de laboratorio con los que he compartido muy buenos momentos y los que han hecho que cada día fuera especial. Así, quisiera comenzar por alguien que ha estado a mi lado incansablemente en el laboratorio durante todo este tiempo: Carmen García. Carmen,

gracias por todo el esfuerzo, la generosidad y entrega que has puesto en este trabajo; una parte de esta tesis también te pertenece. Gracias también a ti, Antonio López, porque no solo eres un gran profesional sino una grandísima persona. Al *Contact Team*, o lo que es lo mismo: José Pinto, Vicente Martín y Alberto López; porque hemos pasado muy buenos ratos juntos tanto en la biblioteca como en los congresos. ¡Qué buenas risas nos hemos echado!. A Sara Galindo, "Sarita", por tu simpatía y los "buenos" momentos que pasamos en Londrés. ¡Vaya experiencia!. Al *Nano Team*: Laura García, Laura Soriano y Mario Crespo; a Esther Rey, y por supuesto, a los que ya son doctores/doctoras: Laura Contreras, Maria Jesús Benito, Marisa Tesón, Ana de la Mata, Marina López, Isabel Arranz, Amar Kumar y, en especial, a Noelia García; agradeceros todos esos ratos tan fantásticos que hemos vivido. Y como no te iba a agradecer a ti, Mario Martino "Marino", los momentos de confidencias que hemos compartido y las horas que hemos pasado en el gimnasio arreglando el mundo a lo largo de todos estos años.

En general, quisiera dar las gracias a todos los miembros del IOBA con los que en algún momento he podido compartir mi tiempo. Especialmente quisiera agradecer a Ana Rodríguez, Mari Paz García y Lurdes Pérez su profesionalidad y amabilidad durante estos años. Y, por supuesto, no podría olvidarme de ti, Carlos Matesanz. "Charli", gracias por tu profesionalidad, amistad y cariño. Gracias por abrirme las puertas de tu casa y de tu fantástica familia durante estos años.

Tambien quiero dar las gracias a Cristina Raimundo por su ayuda para dibujar alguna de las figuras que en esta tesis se muestran (¡eres la mejor!).

Como felizmente ha pasado a lo largo de mi vida, mi familia ha estado a mi lado para apoyarme en todas las decisiones y proyectos que he llevado a cabo; por lo que mi gratitud y recuerdo van dirigidos a todos ellos. En especial, quiero agradecerte a ti, Gema Raimundo, el apoyo que me has ofrecido artruistamente durante estos años. Tu amor, cariño y persistente confianza en mi han sido mi aliento, fuerza y estímulo para seguir adelante y poder culminar este proyecto.

Pero, sobre todo, mi mayor agradecimiento es para mis padres, Antonio y Chelo, los que han sido siempre "mi universo conspiratorio" apoyándome y dándome su cariño y amor incondicional. Gracias papis por vuestro sacrificio y esfuerzo, que han hecho que yo pueda ser lo que soy hoy tanto profesionalmente como personalmente, y de lo cual me siento infinitamente agradecido. Quiero daros mi homenaje particular con estas líneas: si hoy estáis orgullos de mí, más aún lo estoy yo de vosotros. Os quiero.

Abstract

The aim of this thesis was to investigate the effect of two major natural compounds, resveratrol and quercetin, and their combination on cells involved in the inflammatory response at the ocular surface, as well as their potential therapeutic effects for dry eye disease.

For this purpose, the anti-inflammatory and anti-oxidant effects of resveratrol, quercetin and their combination were tested in two ocular surface (conjunctival and corneal) epithelial cell lines stimulated by the pro-inflammatory cytokine TNF-α and UV-B light. After that, their immunoregulatory effects were determined in activated peripheral blood mononuclear cells. Their anti-inflammatory effects (by topical application) were further studied in a murine model of dry eye induced by desiccating stress, followed by an adoptive transfer model using athymic mice. Additionally, their anti-allergic effects were determined in activated cord blood-derived mast cells.

Results showed that resveratrol, quercetin and their combination can decrease cytokine secretion (IL-6, IL-8/CXCL8 and IP-10/CXCL10) and COX-2 expression induced by TNF-α, and free radical production induced by UV-B light in conjunctival and corneal epithelial cells. The combination of both compounds modulated proliferation of peripheral blood mononuclear cells. In addition, both compounds (separately and combined) decreased cytokine production and corneal fluorescein staining in mice exposed to desiccating stress, as well as $CD4^+$ T cell infiltration in conjunctiva of athymic mice. Finally, resveratrol, quercetin and their combination inhibited histamine secretion and cytokine/chemokine production by activated mast cells.

In conclusion, resveratrol, quercetin and their combination can modulate the response of cells involved in inflammatory processes at the ocular surface such as dry eye and ocular allergy. In addition, topical application of resveratrol, quercetin and their combination in experimental dry eye leads to improved clinical signs and inflammation associated with the ocular surface, showing their potential therapeutic properties for treating dry eye disease.

Financial support

This research has been made possible thanks to the financial support of Allergan through the agreement: Allergan/IOBA Ocular Surface Inflammation Program, Art. 83 LOU.

I have also received the following support from Universidad de Valladolid:

- Beca de iniciación a la investigación en institutos LOU. 2009.
- Beca para la formación de personal investigador. 2010-2014.
- Ayudas para estancias breves en el desarrollo de tesis doctorales. 2011.
- Ayudas para estancias breves en el desarrollo de tesis doctorales. 2012.
- Ayudas para estancias breves en el desarrollo de tesis doctorales. 2013.

Publications related to this thesis

The following publications were a result of data from this doctoral thesis:

Patent:

Composición para su uso en el tratamiento y/o prevención de la inflamación, el estrés oxidativo y la neovascularización ocular. Número de publicación: P201230664. Asignado a: Universidad de Valladolid and Allergan, Inc. Inventores: María Jesús González García, Amalia Enríquez de Salamanca y Aladro, Margarita Calonge Cano, Michael E. Stern y Antonio Abengózar Vela.

Conference poster presentations:

Abengózar-Vela A, Schaumburg CS, Stern ME, Calder VL, Calonge M, Enriquez-De-Salamanca A, González-García MJ. *An in vivo study of anti-inflammatory effect of Quercetin and Resveratrol polyphenols in a desiccating stress mouse model of dry eye.* Annual Meeting of the Association for Research in Vision and Ophthalmology (ARVO). Orlando, Florida, USA. Invest Ophthalmol Vis Sci 2014;55: E-Abstract 3654.

Enriquez-De-Salamanca A, Abengózar-Vela A, García-Vázquez C, Schaumburg CS, Stern ME, Calder VL, Calonge M, González-García MJ. *An in vitro study of anti-inflammatory effect of Quercetin and Resveratrol polyphenols in ocular surface epithelial cells*. Annual Meeting of the Association for Research in Vision and Ophthalmology (ARVO). Orlando, Florida, USA. Invest Ophthalmol Vis Sci 2014;55: E-Abstract 1478.

“Let food be thy medicine and medicine be thy food”

Hippocrates of Cos

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Acronyms

ADDE	Aqueous tear-deficient dry eye
AKC	Atopic keratoconjunctivitis
ANOVA	Analysis of variance
AP-1	Activator protein 1
APC	Antigen presenting cell
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CAM	Cell adhesion molecule
CALT	Conjunctival-associated lymphoid tissue
CBMC	Cord blood-derived mast cell
CFS	Corneal fluorescein staining
CFSE	Carboxyfluorescein diacetate succinimidyl ester
CLN	Cervical lymph node
COX	Cyclo-oxygenase
CM	Complete medium
DC	Dendritic cell
DED	Dry eye disease
DEWS	International Dry Eye WorkShop
DMEM	Dulbecco's Modified Eagle's medium
DMSO	Dimethyl sulfoxide
DS	Desiccating stress
EALT	Eye-associated lymphoid tissue
EDE	Evaporative dry eye
EDTA	Ethylenediaminetetraacetic acid
EFA	Essential fatty acid
EGF	Epithelial growth factor
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal-regulated kinase
EtOH	Ethanol

FBS	Foetal bovine serum
FCS	Foetal calf serum
FDA	Food and Drug Administration
FS	Forward scatter
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GPC	Giant papillary conjunctivitis
GSH	Reduced glutathione
HCE	Human corneal epithelium
H_2DCF-DA	2',7'-dichlorodihydrofluorescein diacetate
HLA	Human leukocyte antigen
H_2O_2	Hydrogen peroxide
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
ICAM	Intercellular adhesion molecule
IEL	Intraepithelial lymphocytes
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IOBA-NHC	IOBA-normal human conjunctival epithelium
IP-10/CXCL10	Interferon gamma-induced protein 10
JNK	c-Jun N-terminal kinase
KIK13	Kallikrein 13
LDALT	Lacrimal drainage-associated lymphoid tissue
LFU	Lacrimal functional unit
LGALT	Lacrimal gland-associated lymphoid tissue
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MCP-1/CCL2	Monocyte chemoattractant protein-1
MCTC	Tryptase- and chymase-positive mast cell
MDA	Malondialdehyde

ME	Mercaptoethanol
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinase
MTC	Tryptase-positive mast cell
NaCl	Sodium chloride
$NaHCO_3$	Sodium bicarbonate
NF-κB	Nuclear transcription factor-kappa B
NK	Natural killer cell
NO	Nitric oxyde
O_2	Molecular oxygen
OCT	Optimal cutting temperature compound
PAC	Perennial allergic conjunctivitis
PAS	Periodic acid-Schiff
PAGE	Polyacrylamide gel electrophoresis
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PI	Propidium iodide
PMN	Polymorphonuclear leukocytes
PMSF	Phenylmethanesulfonyl fluoride
PHA	Phytohemagglutinin
QCT	Quercetin
RES	Resveratrol
RFU	Relative fluorescence units
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
SAC	Seasonal allergic conjunctivitis
SEM	Standard error of mean
SDS	Sodium dodecyl sulphate
SOD	Superoxide dismutase
SS	Side scatter
TBS	Tris-buffered saline

TCR	T cell receptor
TGF-β	Transforming growth factor beta
Th	T helper cell
TLR	Toll-like receptor
TP	Tear production
Treg	Regulatory T cell
TNF-α	Tumor necrosis factor alpha
UV	Ultraviolet light
VCAM	Vascular cell adhesion molecule
VKC	Vernal keratoconjunctivitis
WHO	World Health Organization
8-OHdG	8-hydroxydeoxyguanosine

I. Motivation and organisation

I.1. Motivation

Acute and chronic inflammatory ocular surface diseases affect millions of people around the world and the costs to treat these, for example dry eye, are substantial. In addition, quality of life in patients suffering dry eye has been likened to moderate-to-severe angina, even when clinical signs are minimal. The treatments of dry eye include artificial tears, cyclosporine A and corticosteroids. Artificial tears do not have any anti-inflammatory effect; and topical cyclosporine A and topical methylprednisolone corticosteroid have been associated with improvements in clinical signs and symptoms reported by dry eye patients, but those treatments either have side-effects (corticosteroids) or are only available in some countries (cyclosporine A). Thus, effective management strategies would be welcomed by clinicians and patients, because there is a gap between artificial tears and the anti-inflammatory therapies.

In addition, not only inflammation plays a key role in acute and chronic diseases. There is a close relationship between oxidative stress and inflammation because an increase in free radical production is able to activate the inflammatory process, and free radicals are also effectors of inflammation. As a consequence of oxidative stress, signal transduction cascades can stimulate the production of pro-inflammatory cytokines. The oxidative stress in the ocular surface has been evidenced in several diseases such as conjunctivochalasis,[1] ocular allergy[2] and dry eye,[3] demonstrating that oxidative stress and inflammation are intimately related.

This close relationship between oxidative stress and inflammation suggests that compounds with both antioxidant and anti-inflammatory properties may be a new approach for the treatment of ocular surface disorders, such as dry eye and ocular allergy.

Natural compounds, such as polyphenols, have been well described in the literature as therapeutic compounds by their unique biological properties. Resveratrol and quercetin, two major polyphenols, are promising natural anti-inflammatory and antioxidant agents because they have beneficial effects in many inflammatory conditions. Moreover, these compounds have various effects on different target cells involved in inflammation.

Thus, the aim of this thesis was to determine the potential effect of two polyphenols, resveratrol and quercetin, and their combination on maintenance of ocular surface health in inflammatory diseases, such as dry eye and ocular allergy.

I.2. Organisation

This Doctoral Thesis applies for the International-awarded Doctorate Degree. It has been performed at the University of Valladolid, under the regulations of the International Doctorate Committee. The joint requirements are as follows: the whole manuscript has been written in English and a general summary in Spanish, in which the objectives, methodology and summary of results are presented.

This Thesis report is organised in four sections according to each model used for testing polyphenols as follow: 1) in-vitro model of ocular surface inflammation, 2) in-vitro model of T cell proliferation, 3) murine model of dry eye disease and 4) in-vitro model of conjunctival mast cells. This thesis matches the requirements for the International-awarded Doctorate Degree with the sections V.2 and V.4, in which the experiments were performed during my stay in the research group of Dr. Virginia Calder at University College London (UCL). In addition, the patent from this thesis and achieved results from additional experiments carried out during my stay in the research group of Dr. Michael E. Stern at Allergan, Inc. (in which the section V.3 was performed) are included in the Appendices.

II. Introduction

II.1. Definition of the Ocular Surface and Lacrimal Functional Unit

The ocular surface is an important part of the eye not only by being a vital component of the refractive media of the eye but also by protecting the delicate inner structures of the eye. Unlike all other wet surface epithelia of the body, the ocular surface is exposed to the outside environment where it is especially subjected to pathogens, adverse environments and injury. As a result, several protective mechanisms are involved in the ocular surface to conserve corneal transparency and maintain a good refractive surface.

The ocular surface is an integrated unit comprising the cornea, the conjunctiva, the limbus and the overlying tear film. Moreover, it is also well known that all these components are interconnected through a continuous epithelium with no breaks between regions and neuroanatomically connected with the nervous, vascular, immune and endocrine systems. Based on this idea of interconnected tissues, Stern et al. described the term "Lacrimal Functional Unit" (LFU) in 1988.[4] They defined the LFU as an integrated system that connects the sensory tissues and the secretory glands to regulate tear secretion maintaining homeostasis of the ocular surface. This definition emphasises and highlights the close relationship between the lacrimal gland, the ocular surface and the nervous system. Thus, the LFU is composed of the ocular surface, the main and accessory lacrimal glands and the neural network that connects them[4] (Figure 1).

The main role of the LFU is to provide a normal and stable tear film leading to a smooth optical surface, so maintaining comfort, epithelial cell health and protection from environmental and microbial insults.[5] Nevertheless, dysfunction of, or injury to, some element of the LFU can alter the tear film structure and composition. This event may result in both corneal and conjunctival epithelia being directly exposed to the external environment, so losing the nourishment and lubrication provided by the tear film. As a result, inflammatory processes occur in the LFU, leading to the development of ocular surface disorders, such as dry eye disease (DED).

The treatment of the inflammatory process in the ocular surface mainly relies on topical drugs because of the easy access to the ocular surface epithelium. It focuses on initially treating the inflammatory process associated with the corneal and conjunctival epithelia. Therefore, it is necessary to understand the structure and immune response in both normal and pathological epithelia, in order to find therapeutic targets to treat ocular surface disorders.

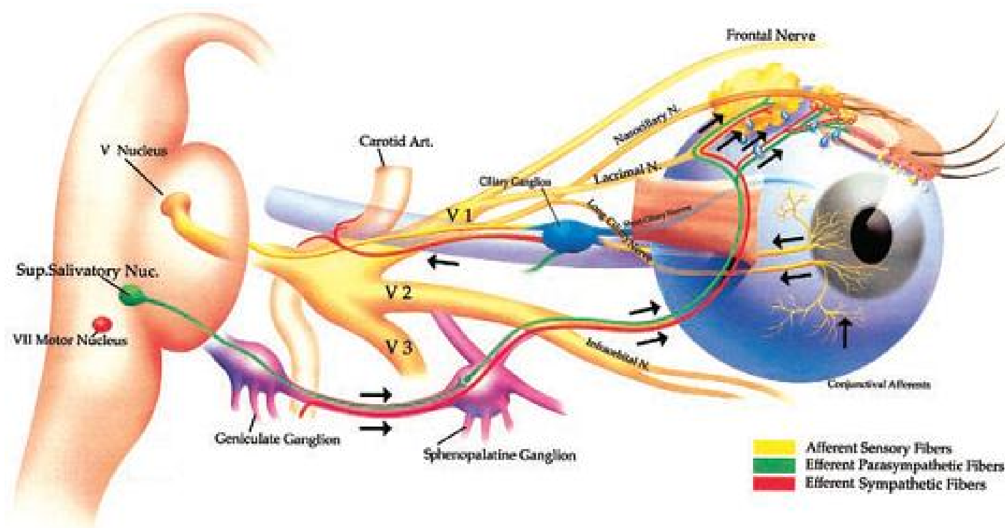

Figure 1. The lacrimal functional unit. Adapted from Pflugfelder et al. 2004.[6]

II.1.1. Tear film

As mentioned above, the tear film plays a key role in maintaining the homeostasis of the ocular surface. The traditional structure of the tear film comprises three layers: an outer lipid layer, an intermediate aqueous layer and an inner mucin layer. However, some studies have evidenced that there is no real clear-cut barrier between the layers.[7,8] Thus, it is currently proposed that a large portion of the tear film is a mucin/aqueous gel that decreases in density towards the external lipid layer[9,10] (Figure 2). Nevertheless, three components will be described separately here for simplicity.

The lipid layer is secreted by the meibomian glands. This layer is made of a mixture of lipids that prevent evaporation of the aqueous layer and provide some of the refractive qualities necessary for a good optical surface.

The aqueous layer is secreted by the lacrimal gland and accessory glands. This layer is an aqueous-mucin gel containing water, electrolytes and proteins that prevent corneal and conjunctival infections. The major locally-produced proteins are lysozyme, tear albumin, lipocalin, secretory immunoglobulin (Ig) A and lactoferrin,[11] which are increased during inflammation.[12,13] In addition, there are also proteins and peptides belonging to the immune system,[14] such as β-defensins,[15] which either have antimicrobial effects or serve to recruit and activate lymphoid cells.

The mucin layer is secreted by goblet cells, along with conjunctival epithelial cells. It is a glycocalyx gel made of a heterogeneous glycoprotein group that promotes tear film attachment to the corneal epithelium. Mucins maintain lubrication and stabilise both lipid and aqueous components of the tear film, thus avoiding desiccating stress at

the ocular surface.[16] In addition, the mucin layer of the tear film also provides a physical barrier that restricts bacterial adherence.[17]

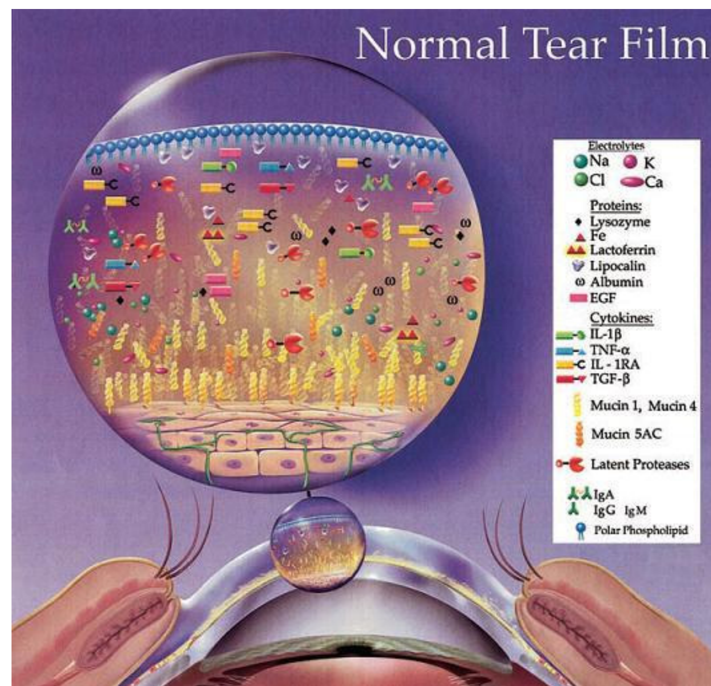

Figure 2. The normal tear film structure and components. Adapted from Stern et al. 2004.[5]

II.1.2. Conjunctiva

The conjunctiva is a thin and translucent mucous membrane which extends from the eyelid margin to the corneal limbus, including the nasal mucosal through the lacrimal puncta. Although the conjunctiva is a continuous membrane, it can be anatomically divided into three parts: palpebral, forniceal and bulbar conjunctiva (Figure 3).

The palpebral conjunctiva (also called the tarsal conjunctiva) starts on the lids at the mucocutaneous junction and ends at the forniceal conjunctiva. It is highly vascularised and firmly adhered to the tarsal plate.

The forniceal conjunctiva, also called the *cul de sac*, is a transition zone between the tarsal and bulbar conjunctiva. It is the loosest of the three conjunctival areas and is reflected into several folds. The ducts of the main lacrimal gland and Krause and Wolfring accessory glands open into the forniceal conjunctiva.

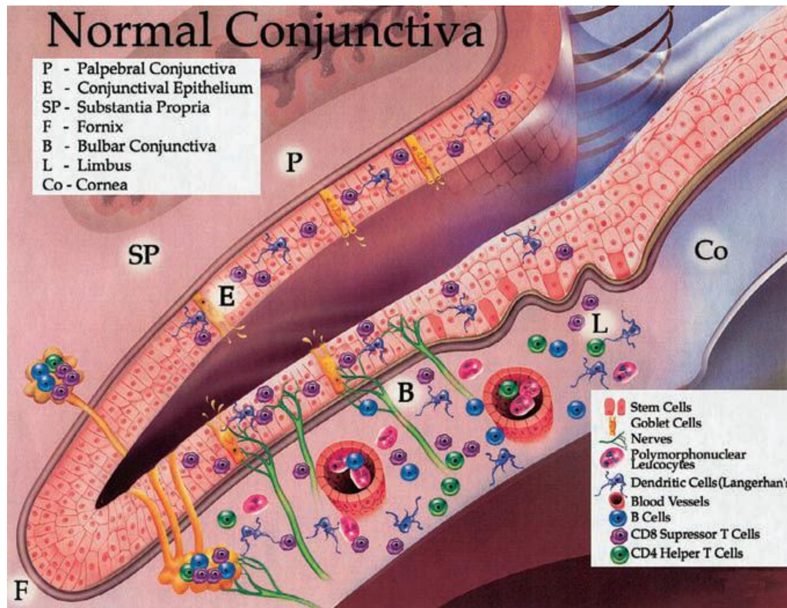

Figure 3. Main components of the normal conjunctiva. Adapted from Calonge et al. 2004.[18]

The bulbar conjunctiva envelops the anterior sclera. It can be further divided into two portions: scleral conjunctiva (from the fornix to the limbus) and limbal conjunctiva (a ring approximately 3 mm around the cornea in the transition zone between the corneal epithelium and conjunctival epithelium). The bulbar conjunctiva is tightly bound to the underlying Tenon´s capsule at the limbus but it is loosely attached to the globe.

Histologically, the conjunctiva consists of an epithelium and an underlying loose connective tissue, further subdivided into the *substantia propria* and a deeper fibrous layer. The conjunctival epithelium is a non-keratinised, stratified secretory layer and its structure varies depending on region. Epithelial cells are columnar in the tarsus, cuboidal in the bulbar conjunctiva, prismatic in the fornix, and squamous in the limbus and near the lids. Its thickness also varies regionally, from 2-3 layers in the tarsus, to 6-9 layers in the bulbar conjunctiva.[18] It contains mucous-secreting goblet cells, and cells involved in the defensive response, such as melanocytes, lymphocytes and antigen presenting cells (APCs) called Langerhans cells, as well as major histocompatibility complex (MHC) class II positive dendritic cells (DCs).

The superficial lymphoid layer is a connective tissue under the epithelium, which is not present at birth (it takes three months to develop), and it is loosely attached to the overlying epithelial base membrane. This tissue contains the immune system cells, such as lymphocytes, macrophages, natural killer (NK) cells, neutrophils and mast cells, which initiate and mediate the inflammatory response in the ocular surface.

The deeper fibrous layer is a thick layer, except for the tarsal part of the conjunctiva, which contains the majority of conjunctival blood vessels and nerves. The accessory lacrimal glands of Krause and Wolfring are also located within this fibrous layer.

II.1.3. Cornea

The cornea is the principal refractive component of the eye. The anterior surface of the cornea is bathed by the tear film and the posterior surface lines the aqueous humour-filled anterior chamber. The thickness of the cornea is approximately 500 µm,[19] increasing towards the peripheral limbus. Peripherally, the cornea lines the sclera at the region called the corneal sulcus. The cornea has the highest concentration of non-myelinated nerve fibres of the human body. These fibres are associated with numerous pain receptors that have a very low threshold, making the cornea highly sensitive to pain. As the cornea is an avascular tissue, both the tear film and aqueous humour (along with capillaries at the edge of the cornea) provide the necessary oxygen and nutrients.

Anatomically, the cornea is made up of 5 layers, each with distinct functions (Figure 4). The layers are from front to back: epithelium, Bowman's membrane, stroma, Descemet's membrane and endothelium. From all of these structures, the epithelium is the outer-most layer of the cornea. It is a non-keratinised, stratified squamous tissue that presents a physical barrier to prevent microorganisms from reaching the inner corneal layers. The central corneal epithelium is 50-55 µm thick with 5-6 cell layers, reaching 10 cell layers or more towards the limbus. The epithelium has 3 types of cells: two layers of flattened epithelial cells externally (squamous cells), followed by 2-3 layers of polygonal cells (wing cells), and a single internal basal layer of columnar cells (basal cells). The basal cells or stem cells located in the palisades of Vogt are responsible for mitosis. The new cell originates as another basal cell and migrates upwards, eventually becoming a wing cell. Upward movement continues towards the centre of the cornea, until it finally becomes an outer epithelial cell and is eventually shed. The external cell layer also has microvilli and microplicae that play a role in tear film adhesion and stabilisation to the corneal surface. Running between the epithelial cells are the ends of sensory nerve fibres.

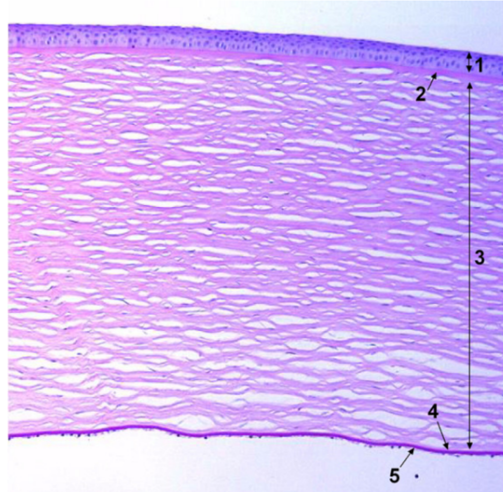

Figure 4. Cross section of the human cornea. Image courtesy of IOBA Ocular Pathology Lab. (1: Epithelium; 2: Bowman's membrane; 3: Stroma; 4: Descemet's membrane; 5: Endothelium).

II.2. Ocular Surface Inflammation

Most of the acute and chronic ocular surface disorders include a significant component of the inflammatory response. Therefore, it is very important to understand the cellular and molecular mechanisms involved in immune-based ocular surface diseases, in order to identify new therapeutic targets for treating them.

II.2.1. The Immune System at the Normal Ocular Surface

The ocular surface contains an immunological framework that limits host tissue damage when it is insulted by pathogens or environmental trauma. Conjunctiva-associated lymphoid tissue and the corneal epithelium are the two major components of this immunological framework at the ocular surface.

II.2.1.1. Conjunctiva-associated lymphoid tissue

As mentioned, there are several types of immune cells in the normal epithelium of the ocular surface (Table 1). The conjunctiva, along with the lacrimal gland and lacrimal drainage system, form a continuous lymphoid tissue called eye-associated lymphoid tissue (EALT).[20] The EALT consists of the lacrimal gland-associated lymphoid tissue (LGALT), the conjunctiva-associated lymphoid tissue (CALT) and the lacrimal drainage-associated lymphoid tissue (LDALT). The EALT is connected to the other

immune system tissues by the recirculation of lymphoid cells via specialised vessels. Therefore, lymphoid cells are resident at the normal conjunctival epithelium.

Focusing on the CALT, it has the typical characteristic distribution found in other lymphoid tissues in mucosal organs: diffuse lymphoid tissue and lymphoid follicles.[21]

Diffuse lymphoid tissue contains intraepithelial lymphocytes (IEL) in the basal layer of the epithelium and in *lamina propria*. IEL are predominantly $CD3^+$ T cells compared to $CD19^+$ B cells. T cells are predominantly $CD3^+CD8^+$ suppressor/cytotoxic T cells compared to $CD3^+CD4^+$ T helper (Th) cells, except for in the *lamina propria*, where a reverse distribution of T cells occurs.[22] Lymphocytes are complemented by bone marrow derived cells such as APCs, mast cells and macrophages, including IgA-secreting plasma cells.

Lymphoid follicles produce lymphoid effector cells and can be observed in the normal conjunctiva. They consist of B cells surrounded by T cells and associated high endothelial venules which support high levels of lymphocyte extravasation from the blood.

	Conjunctiva		**Cornea**
Cell type	*Epithelium*	*Substantia propria*	*Epithelium*
Basophils	-	-	-
Dendritic cells	+	+	+
Eosinophils	-	-	-
Lymphocytes			
B cells	-	+	Rare
T cells	+	+	+
α/β	+	+	?
γ/δ	+	+	?
T-helper	+	+	Rare
T-suppressor	+	+	Rare
Macrophages	-	+	-
Mast cells	-	+	-
Plasma cells	-	+	-
Polymorphonuclear Leucocytes	-	+	-

Table 1. Immune cells of the normal ocular surface. Adapted from Hamrah et al. 2004.[23]

II.2.1.2. Corneal epithelium

Although the corneal epithelium is a continuation of the conjunctival epithelium, it is specialised to exist over an avascular connective tissue. Lymphoid and blood

vessels end in the limbal zone and prevent an access of the vast majority of immunologically relevant cells into the cornea. This alymphatic and avascular characteristic leads to an "immune privilege" that retards trafficking of immune cells to the lymphoid tissues.

Nevertheless, some immune cells are located in the normal corneal epithelium. APCs are present in the corneal epithelium. There are different populations located in both central and peripheral epithelia;[24,25] declining in percentage from the limbus to centre of the corneal epithelium.[26,27] Peripheral APCs are mainly Langerhans cells, whilst central APCs are immature epithelial DCs[25] (Figure 5). In addition, B and T cells are also present in the limbic region.[28] To date, no other immune cell types have been described in the normal corneal epithelium.

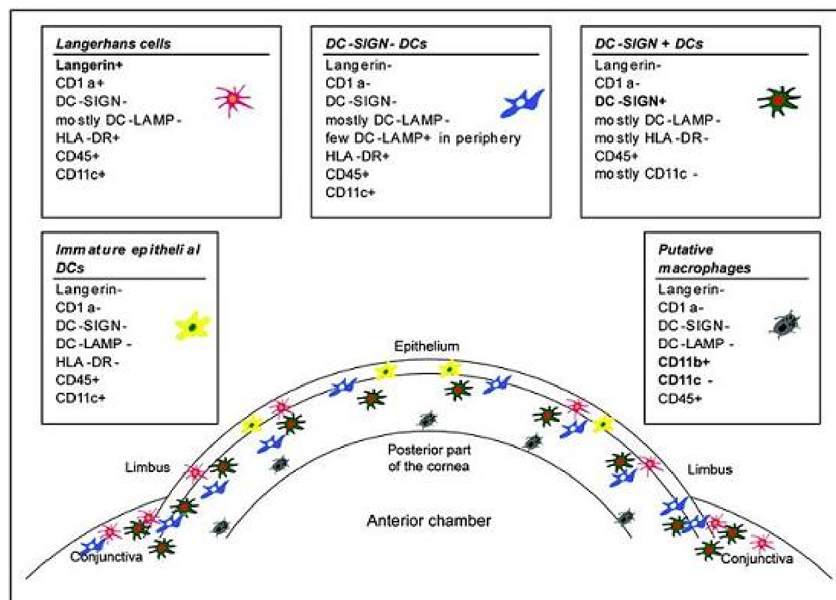

Figure 5. Distribution of antigen presenting cells within the central and peripheral cornea (DC: Dendritic cells). Adapted from Mayer et al. 2007.[25]

II.2.2. The Inflammatory Process at the Ocular Surface

The ocular surface has the same immune cell response as other mucosal epithelia in the human body. Therefore, both arms of the immune system, innate and adaptive systems, are the main features against infectious agents.

The innate response is the first line of defence against invading microbial pathogens. This immune system response occurs immediately, but in a non-specific manner, to invading pathogens because it recognises general classes of pathogens (i.e. virus, bacteria and fungi), but it cannot make fine distinction. It consists of the

physical and chemical barriers, macrophages, DCs, NK lymphocytes and circulating plasma proteins. The physical barriers of the ocular surface epithelia (i.e. corneal epithelial cells), along with mucous membranes (i.e. conjunctiva), secreted proteins (i.e. lysozyme and defensins) and mechanical barriers (i.e. eyelids) are just some features of the innate immune system at the ocular surface (Figure 6). The integrity of the ocular surface epithelium is provided by tight junctions between cells. In addition, epithelial cells can recognise pathogen-associated molecular pattern by Toll-like receptor (TLR) expression on their surfaces.

However, if pathogens manage to bypass, evade or overwhelm the innate defences, the adaptive immune response is necessary for effective elimination of pathogens. The adaptive or acquired immune system consists of two arms: humoral (specific antibodies secreted by B lymphocytes) and cellular (specific T lymphocytes).

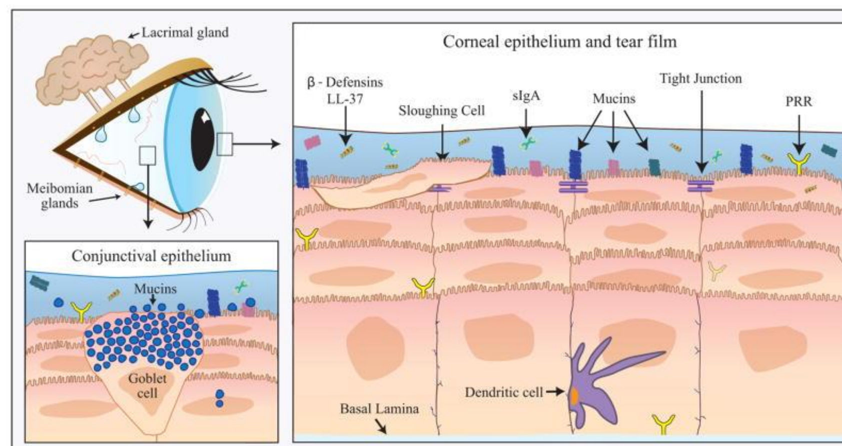

Figure 6. Innate immune system of the ocular surface (PRR: Pattern recognition receptors; LL-37: Cathelicidin; IgA: Immunoglobulin A). Adapted from Narayanan et al. 2013.[29]

Key links between the innate and adaptive immune systems are APCs (i.e. B cells, macrophages and DCs). Activated APCs migrate to draining lymphatic tissue (such as regional lymph nodes and spleen) via afferent lymphatic vessels. They have the function of priming naïve T cells in these lymphoid tissues, leading to differentiation and activation of effector T cells. Once the T cells are activated, the presence/absence of cytokines (signalling molecules that mediate intercellular communication like lymphocytes and epithelial cells), such as interleukin (IL)-12p70, IL-4, IL-6 and transforming growth factor (TGB)-β, determine the fate of naïve T cells as Th cells ($CD4^+$ T cells). They can differentiate into Th1, Th2, Th9, Th17 and Th22 cells, and regulatory T cells (Treg), all $CD4^+$ T cell subtypes. The T cell subsets promote different types of inflammatory responses depending on their effector cytokine profiles and

interactions with resident tissue cells. T cells can be distinguished from other lymphocytes such as B cells and NK cells by the presence of T cell receptor (TCR) on the cell surface.

Th1 cells secrete IL-2 and interferon (IFN)-γ. These cytokines play a role upregulating production of chemokines and cell adhesion molecules (CAMs) that further attract inflammatory cells, for example to the ocular surface, and also promote maturation of macrophages.[30] Therefore, Th1 lymphocytes are pro-inflammatory cells involved in the pathogenesis of some autoimmune diseases.[31]

Th2 cells produce inflammatory cytokines IL-4, IL-5 and IL-13. These cytokines are involved in the development of atopic diseases, including seasonal allergy, asthma and atopic dermatitis/keratoconjunctivitis. IL-4 and IL-13 promote IgE switching by B cells,[30,32] and IL-5 participates in the activation and chemotaxis of eosinophils.[33] Th2 cell activation occurs in allergic conjunctivitis.[30]

Th17 cells are involved in autoimmunity and tissue inflammation.[34] They particularly secrete IL-17,[31] which stimulates production of cytokines (i.e. tumor necrosis factor (TNF)-α and IL-6), chemokines (i.e. IL-8/CXCL8 and interferon gamma-induced protein (IP)-10/CXCL10) and matrix metalloproteinases (MMPs) on a broad range of cell types.[35] In the ocular surface, DED elicits Th1 and Th17 responses.[36]

Treg cells have a role in tolerance, rather than immunity and secrete IL-10 and TGF-β.[37,38] In addition, it has been described that Tregs play a key role as immunomodulatory T cells in experimental DED.[39-41]

Th9 cells secrete IL-9 and are closely related to Th2 cells.[42] Th22 cells produce IL-22.[43] Both cells may contribute to the development of chronic allergic inflammation and autoimmune disease.[44-46] The role of Th9 and Th22 in eye diseases is currently under investigation.[47]

II.3. Acute and Chronic Inflammatory Ocular Surface Diseases

Acute inflammation is a complex and essential early response, protecting tissue integrity and homeostasis after minor injuries like burns and cuts, as well as in major trauma. Acute inflammation is characterised by the rapid appearance of signs such as heat, swelling, pain, redness and loss of function.[48] One of the most common acute processes at the ocular surface is acute allergic conjunctivitis.

If acute inflammation is not resolved over time it can evolve into a chronic process. Chronic inflammation is characterised by prolonged duration (weeks or months, even years) caused by persistent infections, prolonged exposure to toxic reagents or autoimmune-mediated inflammatory disease. In autoimmune diseases, the

inflammatory response is triggered when there are no stimuli and the immune system attacks its own tissues. Some eye diseases such as vernal keratoconjunctivitis (VKC), atopic keratoconjunctivitis (AKC), DED and healing autoimmune conjunctivitis are chronic and/or severe conditions of the ocular surface. These latter types of chronic-severe conditions are characterised by the possibility of corneal damage, which may lead to loss of vision. Although recent studies have cast light on the biological mechanisms underlying acute and chronic ocular surface inflammation, identifying new potential therapeutic targets, there is still a lack of adequate treatment for more severe (chronic) ocular surface diseases.

II.3.1. Dry Eye Disease

DED is one of the most prevalent ocular surface disorders. According to the Dry Eye Workshop (DEWS), DED is “a multifactorial disease of the tears and ocular surface that results in symptoms of discomfort, visual disturbance, and tear film instability with potential damage to the ocular surface, accompanied by increased osmolarity of the tear film and inflammation of the ocular surface”.[49]

DED is recognised as a chronic eye disease due to inflammation and dysfunction of the LFU. These events cause abnormal tear film composition, leading to an unstable precorneal film layer. When these factors are combined, they produce ocular symptoms that can interfere with activities of normal daily life, such as reading or driving.[50] Patients with DED experience varying severity of symptoms and commonly refer to intermittent-to-constant eye irritation, pain, photophobia, redness, ocular burning and blurred vision.[49] Indeed, the impact of the severe form of DED on patients is in the range of a life-threatening cardiac condition.[51] There is no definitive therapy for DED and it remains one of the leading causes of patient visits to ophthalmologists.[52]

II.3.1.1. Prevalence and risk factors

The prevalence of DED ranges from 2 % to 14 % of the worldwide population, depending on the definition and the population studied.[53-56] The estimated prevalence of DED within the Spanish population is 11 %.[57] Although DED increases with age,[58] prevalence among women is greater than men in all age groups.[54,56]

Several risk factors for the development of DED have been identified repeatedly in epidemiological studies. DED occurs with greater frequency in women, particularly in postmenopausal women because sex hormones influence ocular surface conditions through their effects on tear secretion, meibomian gland function and goblet cell density.[59,60] Risk factors also involved in the development and impairment of DED

include contact lens wear,[61] refractive surgery[62] and cigarette smoking.[63] Extended visual tasking during computer use, television watching or prolonged reading can also provoke symptoms of DED.[64] Adverse environmental conditions like low relative humidity and aircraft cabins can cause and worsen DED.[65,66] Moreover, DED is also associated with systemic and autoimmune diseases such as diabetes mellitus[56] and rheumatoid arthritis,[67] respectively. Other factors that provoke and/or exacerbate DED include chronic use of preserved eye drops[68] and systemic drugs that inhibit tear production.[69]

II.3.1.2. Classification for dry eye disease

Based on aetiological factors that can influence LFU, DED is divided into aqueous tear-deficient dry eye (ADDE) and evaporative dry eye (EDE).[49]

ADDE is characterised by reduced tear secretion and volume due to a failure of the LFU. ADDE is subcategorised into non-Sjögren and Sjögren DED.

Non-Sjögren DED is due to lacrimal dysfunction without signs of systemic autoimmunity, whilst Sjögren DED is an autoimmune process that affects lacrimal, salivary and other exocrine glands in conjunction with other systemic diseases, such as rheumatoid arthritis.

EDE is a DED subset characterised by an excessive evaporation of the tear film from the ocular surface due to an unstable lipid layer, but tear secretion is normal. It is a result of a meibomian gland dysfunction, which is one of the most common causes, along with a low blink rate.

II.3.1.3. Pathophysiology of dry eye

Although the exact cellular and molecular mechanisms underlying DED are not completely understood, it has been shown that immune-mediated inflammation plays a key role in this disease.[70]

Inflammation in the LFU is the central feature of DED. Tear composition in DED is characterised by decreased levels of epithelial growth factor (EGF) and mucins,[71,72] and increased concentrations of pro-inflammatory cytokines and activated proteases.[73,74] Loss of tear film stability leads to disruption of the corneal epithelial barrier function resulting in abnormal signalling from the corneal nerves to the central nervous system, the lacrimal gland and other secretory glands. Corneal nerve abnormalities lead to further ocular surface damage and help perpetuate the vicious inflammatory cycle of DED (Figure 7).

Inflammation of both the lacrimal gland and conjunctiva has been described in Sjögren and non-Sjögren-associated DED patients.[75-78] Pro-inflammatory cytokines, for instance IL-1α, IL-1β, IL-6, TNF-α and TGF-β1, are increased in tears and/or in conjunctival epithelium of DED patients, compared to healthy subjects.[65,71,79-81] Several additional cytokines have been isolated from the ocular surface of patients with DED, including IL-2, IL-4, IL-5, IL-10 and IFN-γ.[82]

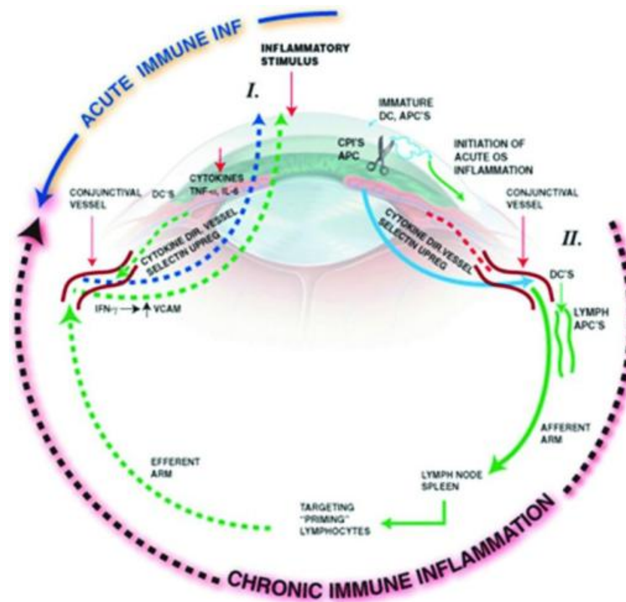

Figure 7. Inflammation circle of chronic DED. Adapted from Stern et al. 2013.[70]

Chemokines are cytokines that regulate the chemotaxis (directed migration) of immune cells. The chemokine IL-8/CXCL8 (a neutrophil chemoattractant involved in the innate immune response) has been identified in the tears and conjunctiva of patients with DED.[80] The closely related chemokines CXCL9, IP-10/CXCL10 and CXCL11 are also elevated in the tear film and ocular surface of patients with DED.[80,81] These latter chemokines are produced in response to IFN-γ, and function as T cell chemoattractants.

The release of these cytokines and chemokines from the diseased ocular surface epithelia indicate that lymphocytes play a key role in the immunopathogenesis of chronic DED. $CD4^+$ T cells infiltration into the conjunctiva and lacrimal glands have been found in patients with DED.[4,10,79] Moreover, activated $CD4^+$ T cells are also found in the ocular surface of DED;[77,78] along with $CD4^+$ T cell-derived cytokines such as IFN-γ, which can interfere with conjunctival epithelial differentiation and produce apoptosis in mucosal epithelia.[83] In addition, CAMs promote the infiltration of immune cells into the ocular surface of patients with DED. Elevated levels of intercellular adhesion

molecule (ICAM)-1 (that binds to lymphocyte function-associated antigen 1) and vascular CAM (VCAM)-1 (expressed by the vascular endothelium and binds to immune cell-expressed integrin $\alpha 4\beta 1$) have been identified in the conjunctiva and lacrimal gland of patients with DED.[84,85]

All of these events show that inflammation of the LFU is one the biological processes underlying DED.

II.3.1.4. Experimental murine model of dry eye

Animal models have contributed greatly to advanced understanding of DED and have helped to develop better therapeutic strategies. For studying DED signs, dogs, (less frequently) cats, and (rarely) horses may naturally develop DED, presenting decreased tear secretion and ocular surface changes. Indeed, immune-mediated destruction of the lacrimal gland tissues is a frequent cause of DED in dogs. There are also rabbit DED models, mostly focused on lacrimal gland inflammatory alterations. Nevertheless, murine models are the most commonly used to study autoimmune mechanisms.[86] One of the most important in-vivo models of DED is the so-called environmental model, first reported by Dursun et al.[87] (Figure 8). In this model, mice are exposed to desiccating stress (DS-constant airflow and low humidity), combined with the administration of the anticholinergic scopolamine in order to induce DED through different primary pathogenic mechanisms.[88] DS are produced using either an air blower or a controlled-environment chamber that allows regulation of environmental conditions,[89,90] similar to that used for human studies in which temperature, humidity and airflow can be controlled.[65,91] This in-vivo model has demonstrated that mice exposed to DS develop clinical and histopathological similarities to the human disease, such as lacrimal gland dysfunction, goblet cell loss, increased cytokine/chemokine production, and squamous metaplasia and apoptosis on the ocular surface, including a robust $CD4^+$ T cell infiltrate into the LFU.[87,92-96]

The most important evidence that confirmed DED is an immune-based disease was presented by Dr. Stern and colleagues, using an adoptive transfer model after the DS model.[94] In this adoptive transfer model, $CD4^+$ T cells are isolated from the cervical lymph nodes (CLNs) and/or spleen of mice exposed to DS, which are then transferred to athymic T-cell-deficient nude-recipient mice (Figure 8). As a result, $CD4^+$ T cells are localised within the ocular surface and lacrimal gland tissues of recipient mice where they mediate full-blown DED by increasing cytokine levels, and decreasing tear production, tear turnover and goblet cells density; even though recipient mice have never been exposed to DS. Furthermore, inflammation is restricted to the lacrimal

gland and ocular surface in recipient mice. This adoptive transfer model demonstrates that CD4^{+} T cells isolated from mice exposed to DS are sufficient to cause inflammatory disease in T-cell-deficient nude-recipient mice.

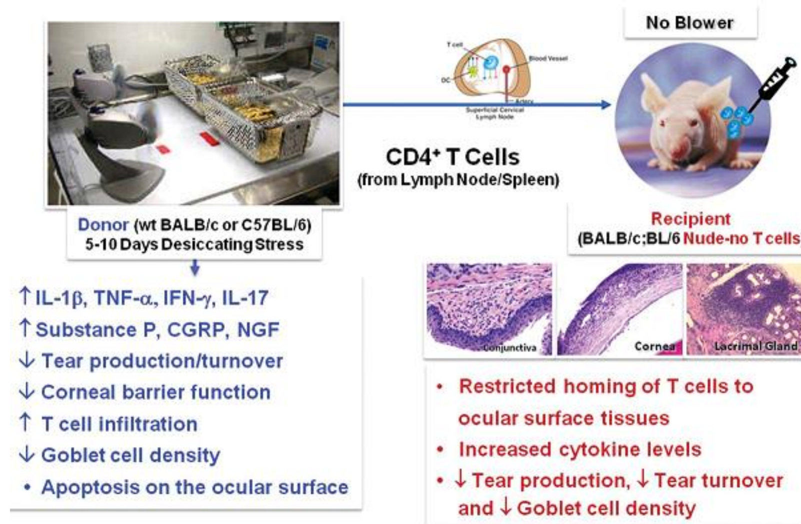

Figure 8. Experimental DED model and adoptive transfer model. Adapted from Stern et al. 2013.[70]

Recent evidence points towards the aetiology of DED being driven by autoantigens. Kallikrein 13 (Klk13), an autoantigen identified in a murine DED model,[97] is found in cornea, conjunctiva and lacrimal gland after DS.[98] Expression of this autoantigen corresponds with the development of autoantibodies specific for Klk13. Passive transfer of anti-Klk13 autoantibody to nude mice is sufficient to induce ocular surface inflammation through mechanisms such as a pro-inflammatory cytokine response (IL-6, IL-12, IL-17, TNF-α and IFN-γ) and infiltration of neutrophils within ocular surface tissue. These events suggest that autoantibodies are involved in the pathogenesis of experimental DED, but from the perspective of human disease the specific self-antigens are still widely unknown among organ-specific autoimmune diseases.[70]

In addition, APCs are necessary to naïve T cell activation and differentiation and it has been demonstrated that APCs play a role in regulating T cells in DS model. Accumulation of mature DCs correlates with CD4^{+} T cell activation in CLNs after exposure to DS.[99] APC depletion by liposome-encapsulated clodronate inhibits the generation of autoreactive CD4^{+} T cells and preserves goblet cells within the conjunctiva.[99] Moreover, lymphadenectomised mice exposed to DS also display less infiltrating CD4^{+} T cells and higher goblet cell density. These events corroborate the hypothesis that activation of autoreactive CD4^{+} T cells occurs in the draining CLNs via cell-to-cell contact with ocular surface-derived APCs.[99]

Taken together, all these studies indicate that a murine model of DED allows us to understand the pathophysiological mechanisms involved in the development of DED, being a suitable in-vivo model for testing new anti-inflammatory compounds.

II.3.1.5. Treatment of dry eye

Advances in the comprehension of the mechanisms involved in DED have led to developments of a wide range of therapeutic strategies. Traditionally, DED has been linked to tear evaporation and the aim has been to restore physiological tear function. Artificial tears are still the most common and widely used therapy for the treatment of DED patients. They are artificial lubricants containing electrolytes and surfactants, in an isotonic or hypotonic buffered solution. Although they temporally improve symptoms, such as eye irritation and blurred vision, they do not have any effect on the inflammatory cascade involved in DED. A major advance in DED therapy has been the incorporation of anti-inflammatory therapies. Currently, topical corticosteroids, oral tetracyclines, essential fatty acids (EFAs) and topical cyclosporine are routinely used in the management of the inflammatory process involved in DED.[100]

- **Topical corticosteroids**, such as methylprednisolone, are well known for their effects on the inflammatory cascade, specifically the blockade of cyclo-oxygenases (COX) and production of prostanoids from arachidonic acid. Corticosteroids have been shown to reduce inflammation and provide relief of symptoms in Sjögren DED patients.[101,102] However, chronic administration of corticosteroids has side-effects including increasing intraocular pressure and cataract formation.[103-105]
- **Tetracyclines** are antibiotics that interfere with protein synthesis of many bacteria, mycoplasma and chlamydia. These bacteria can release lipases that metabolise meibomian lipids into diglycerides and free fatty acids, which are highly toxic to the ocular surface epithelium in ADDE.[106] Although tetracyclines are considered as antibiotics, they also have several anti-inflammatory properties including inhibition of MMP synthesis and activity, and decrease of IL-1 in corneal epithelial cells.[107,108] Side-effects associated with tetracyclines include gastrointestinal upset (i.e. gastritis), yeast infections and photosensitivity.[109]
- **EFAs** are the precursors of eicosanoids (prostaglandins, prostacyclins, thromboxanes, and leukotrienes) that modulate immune response. Recent studies have shown significant improvement in ocular irritation symptoms and decreased ocular surface staining by EFAs such as linolenic acid and γ-linolenic acid, administered orally.[110] Evidence also suggests that supplementation with omega-3

and -6 EFAs may be beneficial in the treatment and prevention of DED.[111,112] However, more evidence is needed to identify the most efficacious forms and doses of EFAs, and their effects on the ocular surface.

- **Cyclosporine A**, a natural-occurring compound, is commonly used to treat autoimmune diseases and as an immunosuppressant to control transplant rejection. Topical cyclosporine A was approved by the Food and Drug Administration (FDA) in December 2002, and became commercially available in April 2003 as Restasis™ (0.05 % cyclosporine A, Allegan Inc., Irvine, California, USA) for treating inflammation in DED. Cyclosporine A relieves the symptoms and signs associated with DED. Patients suffering DED treated with topical cyclosporine A show a decreased in IL-6 levels,[113] reduced human leukocyte antigen (HLA)-DR and CD11a (a marker of activated T cells) in the conjunctival epithelium[77] and also show increased conjunctival goblet cell density after 6 months of treatment.[114,115] Not only does cyclosporine A decrease inflammation in the ocular surface, but it can also inhibit apoptosis involved in DED.[116] Some side-effects are related to cyclosporine A use. For example, oral and intravenous administration of cyclosporine A can produce serious side-effects (i.e. hypertension and nephrotoxicity), but topical cyclosporine A for DED treatment has low systemic absorption, and none of those side-effects have been reported. In addition, topical cyclosporine A takes several months to achieve its full effect. Activated lymphocytes live in the human body for about 110 days and cyclosporine A cannot "turn off" activated T cells like corticosteroids do, because it prevents further T cell activation. Therefore, it takes at least 110 days to reduce levels of all the activated lymphocytes in the inflamed tissues. As a result, several months of topical administration of cyclosporine A is necessary before reaching improvements in ocular surface disease.

II.3.2. Ocular Allergy

Ocular allergy is a localised allergic condition that is observed as the only (or dominant) presentation of an allergic sensitisation. Ocular allergies are encountered daily in the physician's office; being one of the most common ocular conditions observed in clinical practice with a prevalence of between 20 % and 40 %.[117] It is estimated that ocular symptoms are present in 40%-60% of allergic patients.[118] Allergic eye disorders primarily affect the conjunctiva, cornea and eyelids, and can be acute or chronic. Acute allergy is IgE-mediated mast cell degranulation, whilst chronic allergies are mediated by T cells, eosinophils and continuous activation of mast cells.[119,120] The acute form involves transient symptoms of itching, tearing and swelling, while chronic

allergies exhibit symptoms such as severe pain and visual disturbances. In recent years, new drug candidates have been developed from the understanding of the pathophysiology of ocular allergies. However, there is not yet an effective treatment for chronic conditions, which may lead to corneal opacity and vascularisation.

II.3.2.1. Allergic eye diseases

Allergic eye diseases can be divided into the following types: allergic conjunctivitis (seasonal-SAC- and perennial-PAC-), VKC, AKC and giant papillary conjunctivitis (GPC).[121] SAC and PAC display a mild presentation, whereas VKC, AKC and GPC are more severe.

SAC and PAC are the most common forms of ocular allergies, representing 15%-20% of the population,[122] and they affect the patient's quality of life, having a significant socioeconomic impact.[123,124] Comparing both conditions, the prevalence of PAC had been reported to be lower than that of SAC (3.5:10,000).[125] Clinical symptoms and signs in both diseases are the same: tearing, itching, redness, chemosis and swelling of the conjunctiva; whilst the cornea is unaffected in SAC and PAC. Symptoms of SAC are generally more severe in comparison to PAC. Both SAC and PAC involve IgE-mediated mast cell activation. They are often related to known allergens, such as grass and tree pollen. As allergens involved in SAC are seasonal, every symptom of SAC disappears at the end of season. However, allergens involved in PAC are continuous rather than seasonal, being a chronic condition. Eosinophils and neutrophils have been detected in the conjunctival tissues of patients with SAC or PAC. Additionally, T cells have also been detected in patients suffering PAC.[126]

VKC is a bilateral chronic condition. It is a persistent and severe form of ocular allergic, mainly affecting children and adolescents.[127] VKC is more common in warm climates: the estimated overall prevalence in Europe is 3.2/10000, with a higher prevalence observed in Italy (27.8/10000) where the climate is warmer; and a lower prevalence is found in Norway (1.9/10000).[128] There are three clinical forms of VKC: palpebral, limbal and mixed. Both the conjunctiva and cornea are involved in VKC. Symptoms include ocular redness, itching, tearing and swelling. Patients can also have photophobia. However, the most characteristic sign are giant papillae on the upper tarsal conjunctiva in which IL-4 and IL-13 are involved by inducing the proliferation of conjunctival fibroblasts.[119,129] Complications include conjunctival fibrosis and superficial punctate keratopathy. Although VCK is mainly mediated by Th2 lymphocytes, cells of innate immunity such as eosinophils, mast cells and neutrophils become activated, infiltrating the conjunctival subepithelial and stromal tissues.[130] In addition, IgE-

mediated sensitisation is found in 50 % of patients,[131] and high values of plasma histamine have been measured in VKC, but not in SAC, supporting the concept that VKC is a chronic disease.[132]

AKC is the most severe of chronic allergic eye diseases. It has been reported to affect between 25%-40% of patients suffering from atopic dermatitis, but its real prevalence is unknown because the prevalence of its dermatological counterpart, atopic dermatitis, seems to be growing.[133] Although there is no agreement about the diagnostic criteria for AKC, it may be defined as a chronic ocular surface non-infectious, inflammatory condition associated with other atopic conditions, occurring at any time point independently of its degree of severity, as well as corneal involvement at some time in the course of the disease.[133,134] Symptoms of AKC are characterised by itching, redness, blurred vision, photophobia and pain. Signs of AKC depend on the severity of this disease and may range from anterior blepharitis, conjunctival hyperaemia and superficial punctuate keratitis (in mild AKC) to anterior and posterior blepharitis, symblepharon and corneal neovascularisation (in severe AKC).[133] Regarding its molecular mechanism, $CD4^+$ T cells, eosinophils, and neutrophils are the predominant cell types that infiltrate the conjunctival tissue.[135] In addition, high levels of multiple cytokine and chemokine profiles have been detected in tears, suggesting that both Th1 and Th2 responses may be activated.[136-139]

GPC is an inflammatory condition characterised by papillary hypertrophy of the superior tarsal conjunctiva and its appearance is similar to vernal conjunctivitis, without corneal involvement.[140] GPC is most frequently caused by ocular prostheses, postoperative sutures and contact lenses.[141] The conjunctival papillary changes resolve when these irritating stimuli are removed.

II.3.2.2. Pathophysiology of ocular allergy

Several cells, such as mast cells, eosinophils, neutrophils or APCs are involved in allergic eye diseases. The clinical response in all forms of allergic eye disease is mainly due to mast cell activation; either via IgE cross-linkage or mediated by T cells, leading to release of inflammatory molecules and cytokines to the ocular surface.[68,120,142]

II.3.2.2.1. IgE mediated-hypersensitivity in ocular allergy

Ocular allergic inflammation is associated with IgE-mediated mast cell activation (type I hypersensitivity reaction) in conjunctival tissue. There are two phases in specific

IgE-mediated conjunctival mast cell activation, early and late, which involve different kinds of immune cells and inflammatory molecules.

The early phase clinically lasts between 20 and 30 minutes, as demonstrated by specific conjunctival allergen challenge.[143] This phase occurs when allergens bind to the high affinity IgE receptor (FcεRI) at the surface of mast cells, thus inducing cell degranulation. It leads to increased levels of histamine, tryptase and inflammatory mediators such as prostaglandin D2 and leukotrienes in tears,[142] which trigger the recruitment of eosinophils and basophils, producing clinical symptoms. These inflammatory factors initiate the late phase that occurs 4-6 h after the early phase response. It is a process that intensifies the allergic response and heightens the inflammatory process. This intensification is the major cause of ocular surface damage in ocular allergic diseases such as keratitis, limbal infiltration and corneal ulcers. The late phase is characterised by T lymphocyte activation, production of Th2-lymphocyte type cytokines and infiltration of inflammatory cells such as neutrophils, lymphocytes, basophils and eosinophils in the conjunctival mucosa.[119]

Mast cells are granulocytic cells located mainly in the *substantia propria* in SAC and PAC and in the normal conjunctiva, whilst mast cells in VKC and AKC are in both the *substantia propria* and epithelium. Mast cells have not been found in the cornea of normal eyes. There are two forms of conjunctival mast cells, characterised on the basis of their tryptase and chymase expressions: the tryptase-positive and chymase-positive mast cells (MCTC) and the tryptase-positive and chymase-negative mast cells (MCT). More than 95 % of mast cells found in the *substantia propria* of the normal human conjunctiva are MCTC.[144,145] Although MCTC are increased in subjects with vernal conjunctivitis and allergic conjunctivitis, MCT are only increased in the epithelium of the latter condition.[144]

Mast cell granules constitutively contain various enzymes and pro-inflammatory mediators. One of the most important mediators of anaphylactic reactions that is secreted by mast cells and basophils is histamine (2-[4-imidazolyl]ethylamine). Histamine is an amine that has a major role in the innate immune response in allergic inflammation because it produces vasodilatation (increasing vascular permeability), and it promotes DC migration[146] and maturation,[147] along with other biological effects, such as gastric acid secretion.[148] At the ocular surface, histamine is highly secreted by mast cells after IgE cross-linking in the conjunctiva, but is rapidly degraded by tear histaminase enzymes. It exerts its effects by binding to its four receptors (H1R, H2R, H3R and H4R), which all are present in the human normal conjunctiva. Clinically, tearing, itching, hyperaemia and lid swelling are related to histamine secretion.

Mast cells also release IL-4, IL-5, L-6, IL-8/CXCL8, IL-13 and TNF-α,[142,149] but both types of mast cells are heterogeneous with respect to cytokine storage.[150] For example, IL-4 and IL-13 preferentially localise to the MCTC, and IL-5 and IL-6 to the MCT phenotype.[150] This indicates that, depending on protease phenotype, mast cells have different effects. For example, IL-4 that is secreted by MCTC, plays a pivotal role in allergy promoting T cell growth, Th2 differentiation and IgE production;[150] whilst IL-6, a pro-inflammatory cytokine secreted by MCT, promotes Th2 differentiation and simultaneously inhibits Th1 polarisation through two independent molecular mechanisms.[151]

Not only do these molecules have effects on immune system cells but they also stimulate epithelial cell secretion. It is known that both corneal and conjunctival epithelial cells participate in the immune response via expression of surface antigens and adhesion molecules, and secretion of cytokines and chemokines.[152] For example, normal conjunctival tissue express several cytokines and chemokines such as IL-6, IL-8/CXCL8, RANTES/CCL5 and TNF-α, among others,[153] but do not express ICAM-1 and HLA-DR, whilst these latter two are found in patients suffering allergic conjunctivitis.[68,153] In addition, epithelial cytokines including IL-3, IL-8/CXCL8, granulocyte-macrophage colony-stimulating factor (GM-CSF) and RANTES/CCL5 are upregulated in allergic diseases.[153] Conjunctival and corneal epithelia are also stimulated by mast cell-secreted cytokines, leading to the production of more pro-inflammatory cytokines and chemokines. For example, TNF-α stimulates up-regulation of IL-6 and IL-8/CXCL8 by both conjunctival and corneal epithelial cells.[153,154] In addition, conjunctival fibroblasts contribute to cytokine and chemokine production. Stimulation of conjunctival fibroblasts with TNF-α, alone or in combination with either IL-4 or IL-13, results in upregulation of IL-6, IL-8/CXCL8, eotaxin, monocyte chemoattractant protein (MCP)-1/CCL2, RANTES/CCL5 and IP-10/CXCL10.[139] These studies therefore suggest both conjunctival epithelial cells and fibroblasts play a key role in allergic eye diseases.

II.3.2.2.2. T lymphocytes mediated-hypersensitivity in ocular allergy

T cells are involved in the type IV hypersensitivity response, also known as cell-mediated immunity, which occurs in chronic allergic conditions.[155] Type IV reactions involve two phases: sensitisation and elicitation. The sensitisation phase occurs when APC processes antigen-MHC class II complex to T cells, resulting in the differentiation of $CD4^+$ T cells into effector $CD4^+$ T cells and memory T cells. The elicitation phase occurs when memory T cells recognise the antigen peptide together with MHC class II

molecules on an APC. This interaction stimulates memory T cells to proliferate and release cytokines.

This hypersensitivity response is due to interaction of antigens with Th1 and Th2 lymphocyte subsets. Both Th1 and Th2 activation are involved in the immunological response of VKC and AKC, but VKC has a predominantly Th2 profile, whilst AKC has a shift towards a Th1-like profile.[120] Th17 and Treg lymphocyte subsets also have a role in the pathogenesis of conjunctivitis, but their roles are not completely understood.[156]

Recent findings suggest that innate T cells, such as NK cells and γ/δ T cells, also have an important role in allergic diseases.[157-159] NK cells can produce IFN-γ, and IL-4, IL5 and IL-13 cytokines, inhibiting or stimulating the allergic response, along with their known cytotoxic activity. In VKC, NK cell numbers decrease in the blood and increase in the conjunctiva.[157] This indicates a potential role for NK cells in the regulation of allergic reactions.[157]

II.3.2.3. Treatment for ocular allergy

Several treatments, both systemic and topical, are currently available to manage the signs and symptoms of allergic conjunctivitis. Topical lubricants, such as artificial tears, are usually applied for rinsing antigens from the eye. However, they only provide temporary relief of symptoms and do not have any effect on the inflammatory process associated with allergy.

Drugs for treating ocular allergies belong to different pharmacological classes: antihistamines, mast cell stabilisers, dual acting agents, vasoconstrictors, non-steroidal anti-inflammatory agents, corticoids and immunomodulatory agents.

- **Topical antihistamine drugs** are the most preferred treatment for ocular allergies. They are classified into first and second generation. The first generation H1 antagonists have a long safety record. Although they have rapid onset, their effects disappear very quickly. In addition, they are known for a burning sensation upon instillation and their limited potency.[160] In contrast, the second generation of H1 antagonists are non-sedating and have a longer duration of action (4-6 h), but they also have side-effects such as burning, redness and/or stinging upon instillation. Oral antihistamines are also available but when symptoms are primarily ocular, topical antihistamines should be used.
- **Mast cell stabilisers** inhibit degranulation resulting from the activation of IgE receptors.[161] Thus, they are effective in both acute and chronic allergic eye diseases. They have few side-effects including burning and stinging, but they all

require a preloading period and frequent instillation every day, which sometimes results in poor compliance.[162]

- **Dual-action agents**, which are mast cell stabilisers with antihistamine action, provide rapid relief from symptoms.[163] However, some transient and mild side-effects appear after instillation, such as stinging, burning and a transient bitter taste.[164]
- **Vasoconstrictors** are alpha-adrenergic agonists approved topically for relief of conjunctival redness, but have poor efficacy on other symptoms. They are also non-specific, without any effect on the allergic reaction. Adverse effects of topical vasoconstrictors include burning and stinging upon instillation, as well as mydriasis and rebound hyperaemia or conjunctivitis medicamentosa with long-term use.[165]
- **Non-steroidal anti-inflammatory drugs** partially inhibit events that follow degranulation, but they are not the first-line therapy for ocular allergies. They can cause discomfort upon instillation (stinging and burning sensation) and should be used with caution in patients with intolerance, because corneal melting and perforation have been described.[166]
- **Topical corticosteroids** do not have any effect on mast cells and further events, but they may modulate the mast cell response by inhibiting cytokine production, and activation/recruitment of inflammatory cells.[167] They have side-effects from chronic application, such as increasing intraocular pressure and cataract formation, delaying wound healing and increasing viral and bacterial infections.[168]

In addition, it is known that cyclosporine A reduces ocular surface inflammation and inhibits histamine release from mast cells and basophils.[169,170] Therefore, it has been suggested that topical cyclosporine A may be also a useful therapy for the treatment of severe VKC and AKC.[134]

II.4. Natural Compounds: Polyphenols

The medicinal use of compounds that come from natural sources, such as plants and fruit, has had a great contribution to human health care. Natural products have played a key role in medicine, being for several centuries the sole means to treat injuries and diseases. For example, from our earliest ancestors who relieve their pain chewing on herbs, or the ancient civilisations such as Greeks or Chinese that provided written evidence of using plants for the treatment of a wide variety of diseases,[171,172] to current anticancer drugs, such as paclitaxel or camptothecin.[173] The earliest known

record, written on clay tablets, is from Mesopotamia (2600 BC), describing the use of natural compounds for the treatment of coughs and colds.[174] Yet it was not until the 19th century, when active compounds from various medicinal plants were isolated and characterised,[175] that a series of natural products became very important clinical agents, that are still in use today. These compounds include quinine, morphine and codeine,[175,176] as well as antibacterial compounds from micro-organisms such as penicillin, cephalosporin and streptomycin.[172] In addition, new technologies have provided new tools to purify and determine natural product structures, allowing development of natural compound-based drugs.

The World Health Organisation (WHO) pointed out that up to 80 % of people in developing countries use traditional, complementary or alternative medicine as part of primary health care.[177] According to Newman, 60 % of currently available drugs have been either directly or indirectly derived from natural compounds.[178] About 25 % of prescribed drugs worldwide come from plants and 121 such active compounds are in use.[179] Moreover, 11 % of the drugs considered essential medicines by the WHO are exclusively from plant origin or natural compound-based synthetic drugs.[180] Therefore it is a matter of great scientific, economic and medical interest to understand and analyse how natural products are beneficial for human health.

All natural products can be classified according to their source: marine, plant, animal and the microbial world. Focusing on plant sources, polyphenols are probably the most investigated naturally-occurring compounds.

Polyphenols are normally produced by plants for their antibiotic and antifungicidal features.[181] Structurally, polyphenols can be classified into different groups according to the number of phenol rings that they contain, and to the structural elements that bind these rings to one another. Distinctions are thus made between lignans, phenolic acids, stilbenes and flavonoids[182] (Figure 9). Among all these polyphenol groups, interest focuses on stilbenes and flavonoids.

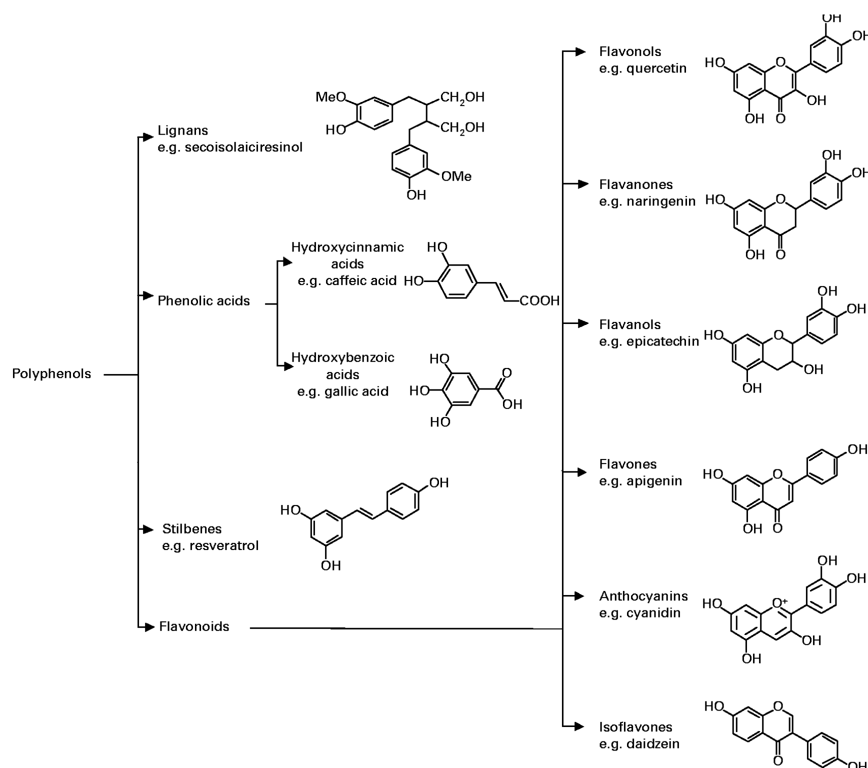

Figure 9. Classification and chemical structure of major classes of polyphenols. Adapted from Spencer et al. 2008.[182]

II.4.1. Stilbenes

Stilbenes are a small family of molecules found in a wide range of plants and fruits such as berries and grapes, but they are found in low quantities in the human diet. The name for stilbenes was derived from the Greek word *stilbos* that means “shining”. Stilbenes are often referred to as phytoalexins, due to their protective action upon secretion.[183] These metabolites act as protective agents to defend the plant against microbial attack, excessive ultraviolet (UV) light exposure and diseases.[184] Upon environmental threat, the plant host activates the phenylpropanoid pathway and as a consequence stilbene structures are secreted.

Stilbenes are molecules characterised by a 1,2-diphenylethylene backbone (two phenolic rings connected by a two-carbon methylene bridge) in their chemical structures. These compounds have generated much scientific research in their potential clinical applications in the treatment of diseases, because they have shown potent anti-inflammatory, anti-cancer and chemoprotective effects, among others.[185-187] One of the most popular and well known stilbene is resveratrol (RES).

II.4.1.1. Resveratrol

RES (5-[2-(4-hydroxyphenyl)ethenyl]benzene-1,3-diol; molecular weight = 228.25 g/mol; CAS 501-30-0; Figure 10) is a stilbene-based compound that was first isolated in 1940 by Michio Takaoka from the roots of white hellebore (*veratrum grandiflorum)*.[188] It is produced by plants to protect them against fungal infections but RES is also produced under environmental stress, such as UV radiation.[189] Although RES is not widely distributed throughout the plant kingdom, it is mainly found in berries and in the skin and seeds of grapes, including red wine.

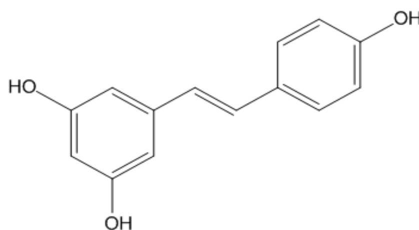

Figure 10. Chemical structure of trans-resveratrol

RES consists of two aromatic rings bridged by ethylene and three hydroxyl groups are attached to the carbon atoms of aromatic rings. There are two isoforms of RES, *trans* and *cis* diastereoisomers, due to a double bond in its chemical structure. Although *cis*-RES is also a natural product found in plants, the name of "resveratrol" commonly refers to *trans*-RES. Both diastereoisomers are commercially available as c*is*-isomerisation occurs when the *trans*-isoform is exposed to UV light and high pH,[190,191] and because *trans*-isoform is an extremely photosensitive compound.[190] Nevertheless, the *cis*-isomer is less stable in the solid form and less biologically effective[192] than the *trans*-isomer, probably due to its non-planar conformation.[193] In this thesis, the term RES will always refer to the *trans*-isomer.

RES is a white powder that is soluble in ethanol (EtOH; 50 mg/mL) and dimethyl sulfoxide (DMSO; 16 mg/mL), but is poorly soluble in water (~0.07 mg/mL). Nevertheless, RES is a lipophilic molecule and it has high membrane permeability. According to the biopharmaceutical classification system guidance by the FDA, which classifies compounds based on their solubility and permeability,[194] RES may be considered as a class-II compound (high permeability, low solubility).

Several studies have been carried out to elucidate the pharmacokinetics of RES. For example, Amri et al. summarised the in-vivo fate of RES following oral administration from data obtained in in-vitro cell cultures, ex-vivo cell isolation and in-vivo experiments, in animals and humans.[195] When RES is orally administered, intestine absorbs 70 % RES as a result of a rapid passive diffusion process. RES is

metabolised by enterocytes forming glucuroconjugates and sulfoconjugates that lead to a maximum plasma concentration between 30 min and 1.5 h after intake, depending on the dose administered.[196,197]

The popularity of RES comes from the so-called "French paradox", an epidemiological observation that confirms an inverse correlation between high-fat diet and low incidence of coronary heart disease in some southern regions of France, due to red wine consumption,[198] meaning RES has cardioprotective properties. However, it was not until 1997, when Jang et al.[199] reported for the first time the anticancer potential of RES, that the scientific community became more interested in this compound. Since then, there has been an exponential growth of literature about its biological properties related to the number of entries (articles) in PubMed (Medline) published (see Figure 11).

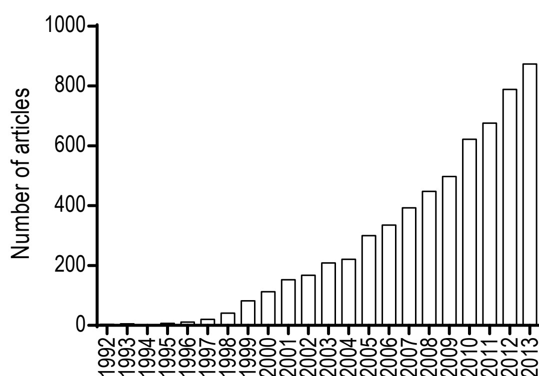

Figure 11. The number of entries (articles) in PubMed published every year using "resveratrol" as keyword.

II.4.1.1.1. Biological properties

RES has several biological properties as a neuroprotective, cardioprotective and anticarcinogenic compound,[199-202] but it is also well known as an antioxidant. RES is an effective scavenger of free radicals (reactive oxygen species -ROS- and reactive nitrogen species -RNS-)[203] that leads to reduced lipid peroxidation and DNA damage by ROS.[204] For example, RES is able to inhibit ROS production by polymorphonuclear leukocytes (PMN) stimulated by formyl-methionyl-leucyl-phenyalanine.[205] In addition, RES protects human lymphocytes activated with hydrogen peroxide (H_2O_2) against oxidative DNA damage, by increasing the levels of several antioxidant enzymes, such as glutathione peroxidase and glutathione reductase.[206] RES also extends its antioxidant effect on epithelial cells. Cerqueira et al. found that RES up-regulates glutathione peroxidase and reduces intracellular ROS generation, as well as ICAM-1

and human beta-defensin-2 expression in lung epithelial cells infected with *Pseudomona aeruginosa*.[207] Moreover, Kode et al. demonstrated that RES restores glutathione levels and also quenches ROS in human primary small lung epithelial cells exposed to cigarette smoke.[208]

Although RES has several biological effects, mainly as an antioxidant, the main scientific interest focuses on its anti-inflammatory properties.

II.4.1.1.1.1. Anti-inflammatory properties

Regarding its anti-inflammatory effects, in-vitro and in-vivo studies have evidenced that RES downregulates the inflammatory response associated with inhibition of activator protein (AP)-1 and the transcription factor nuclear factor (NF)-κB, which is required for the expression of many inflammatory proteins such as GM-CSF, IL-8/CXCL8 and COX-2.[209,210] Therefore, inhibition of NF-κB may reduce the expression of inflammatory molecules. For example, Manna et al. studied the effect of RES on myeloid cells, lymphoid cells and epithelial cells. They found that RES blocks TNF-α-induced activation of NF-κB in a dose- and time-dependent manner, and suppresses TNF-α-induced phosphorylation and nuclear translocation of the p65 subunit of NF-kB and AP-1 in all cell types studied. RES also inhibits the TNF-α-induced activation of mitogen-activated protein kinases (MAPKs) and abrogates TNF-α-induced cytotoxicity and caspase activation.[211] Similar results were reported by Kundu et al. in mouse skin stimulated with a prototype tumour promoter 12-O-tetradecanoylphorbol-13-acetate. Their study revealed that topical application of RES inhibits activation of NF-κB, and phosphorylation of extracellular signal-regulated kinase (ERK) and p38 MAPK in 12-O-tetradecanoylphorbol-13-acetate-stimulated mouse skin.[212,213]

RES has an anti-inflammatory effect upon immune system cells. Sharma et al. found that RES suppresses the activity of T and B cells, decreasing the expression of CD28 and CD80; it also inhibits the secretion of IFN-γ, IL-1, IL-4, IL-6 and TNF-α by macrophages, while IL-10 production is increased.[214] In addition, RES also have effect on DCs. Svajger et al. demonstrated that human DCs treated with RES lose their ability to produce IL-12(p70), but increase IL-10 production after activation. In addition, DCs treated with RES are poor stimulators of allogeneic T cells and decrease their ability to induce $CD4^+$ T cell migration.[215] Moreover, Buttari et al. found that RES can prevent activation of human monocyte-derived DCs by glucose-treated albumin, exerting an inhibitory effect on DC surface maturation marker and cytokine expression.[216]

RES has inhibitory effects on the expression of cell adhesion molecules and attenuates expression of ICAM-1 in bovine aortic endothelial cells stimulated with IL-6 and TNF-α.[217] RES also blocks the expression of adhesion molecules, ICAM-1 and VCAM-1, on lipopolysaccharide (LPS)-induced endothelial dysfunction in human microvascular endothelial cells, by inhibiting NF-kB activation.[218]

The anti-inflammatory effect of RES has also been studied in epithelial cells. Zaidi et al. found RES inhibits secretion of IL-8/CXCL8 and suppresses ROS production in human gastric epithelial cells infected by *Helicobacter pylori* (*H. pylori*). Moreover, *H. pylori*-initiated morphological changes are markedly blocked by RES.[219] More recently, Houser et al. found that RES inhibits MCP-1/CCL2 (a chemokine that regulates migration and infiltration of monocytes/macrophages) in human airway cells stimulated by TNF-α.[220]

Some studies have suggested the use of RES as potent anti-inflammatory compound in allergic diseases like asthma. Donnelly et al. demonstrated that RES inhibits GM-CSF, NF-kB and COX-2 expressions, and IL-8/CXCL8 release in human primary airway epithelial cells.[221] Meeyoung et al. found that RES inhibits increases in T-helper-2-type cytokines (IL-4 and IL-5) in plasma and bronchoalveolar lavage fluid, and also effectively suppresses airway hyperresponsiveness, eosinophilia and mucus hypersecretion in an ovalbumin-induced allergic mouse model of asthma. The efficacy of RES is therefore found to be similar to that of dexamethasone.[222] More recently, Okada et al. described that RES reduces serum IgE production, anaphylactic reaction, and IL-13 and IFN-α production from the mesenteric lymph nodes and spleens of mice sensitised by intragastric administration of ovalbumin plus cholera toxin, and treated with RES.[223]

II.4.1.1.2. Resveratrol and ocular disorders

RES has also shown anti-inflammatory and antioxidant properties when applied as a treatment for some ocular disorders. Kubota et al. demonstrated that RES can prevent ocular inflammation and oxidative stress in a mouse model of uveitis induced by LPS.[224] They found that orally-administered RES inhibits retinal leukocyte adhesion and reduces protein levels of MCP-1/CCL2 and ICAM-1 (inflammation-related molecules involved in leukocyte recruitment,[225] and adhesion[226]) in the retina and retinal pigment epithelium-choroid of mice. They also found that LPS augmented 8-hydroxydeoxyguanosine (8-OHdG, a marker of oxidative stress in DNA) and RES was able to reduce 8-OHdG generation at 3 h after inducing uveitis.

Other studies have shown that RES also has an antioxidant effect on the lens. Doganay et al. studied the effect of RES on a sodium selenite-induced experimental cataract in rats.[227] They found that selenite induces cataract formation in rats and increases malondialdehyde (MDA) concentration, a marker of lipid peroxidation, following low level of reduced glutathione (GSH, a biological antioxidant). RES administered intraperitoneally decreases MDA levels and cataract formation, and increases GSH in lens of treated rats, compared to untreated rats. Li et al. reported similar results in an in-vitro experiment using porcine and human primary lens epithelial cells, under both chronic hyperoxic oxygen condition and acute oxidative stress.[228] They demonstrated that RES increases human lens epithelial cell survival and Forkhead box-O expression (a family of transcription factors involved in DNA repair and apoptosis). Zheng et al. described similar results in a human lens epithelial cell line exposed to H_2O_2.[229] They found that RES is able to reduce intracellular ROS production and p38 and c-Jun N-terminal kinase (JNK) phosphorylation, and to increase the expression levels of antioxidant enzymes such as superoxide dismutase (SOD)-1 and catalase. The anti-inflammatory effect of RES has also been demonstrated in primary porcine trabecular meshwork cells. Chronic treatment of trabecular meshwork cells with RES exposed to a chronic oxidative stimulus (40 % O_2) prevents the increase of intracellular ROS, and IL-1β, IL-6 and IL-8/CXCL8 cytokine secretion, along with antiapoptotic effects.[230]

RES exerts its anti-inflammatory effects not only on cytokine production and cells-mediated inflammation, but also on bacterial-induced inflammation of the ocular surface. Marino et al. exposed ex-vivo rabbit corneas to *Staphylococcus aureus (S. aureus)*.[231] They found *S. aureus* increases the expression of TLR -2 and upregulates IL-8/CXCL8 gene expression on corneal epithelial cells. However, RES decreases cell surface TLR-2 and downregulate IL-8/CXCL8 gene expression in cells exposed to *S. aureus* and treated with RES.

The eye, particularly the cornea, are continuously exposed to UV radiation which can provoke inflammation and oxidative stress.[232-234] Due to its chemical structure, RES has also been shown to have a protective effect on the eye under UV-B exposure. Chou et al. showed that RES inhibits protein kinase B (or AKT) and p38 MAPK phosphorylation, when retinal pigment epithelial cells are irradiated with UV-B.[235] In addition, Kubota et al. found that RES protects mouse retina from white light exposure by reducing the number of TUNEL-positive photoreceptor cells and AP-1, the major transcription factor that regulates cellular cycle, differentiation and apoptosis.[236]

II.4.2. Flavonoids

The name flavonoid refers to the Latin word "*flavus*" meaning yellow, and includes more than 6500 natural compounds. These molecules are secondary metabolites of plants involved in defence against pathogens or UV radiation. Flavonoids are found in several natural sources such as onions, broccoli and leeks, among others. Although they are also part of the human diet, they are not considered as nutrients. Flavonoids have shown several biological effects including antibacterial,[237] antiviral,[238] and anticancer[239] properties. Yet, similarly to stilbenes, there is greater interest in their anti-inflammatory properties.[240]

The basic flavonoid structure is the flavan nucleus, which consists of 15 carbon atoms arranged in three rings (C6-C3-C6). The various classes of flavonoids differ in the level of oxidation and pattern of substitution of the rings (see Figure 9). Therefore, flavonoids can be subdivided into six groups on the basis of their molecular structure: anthocyanins, flavanols, flavanones, flavones, flavonols and isoflavones.[182] Flavonols are the most ubiquitous flavonoids in food, and one of the most important flavonols is quercetin (QCT).

II.4.2.1. Quercetin

QCT (3,3',4',5,7-pentahydroxyflavone, molecular weight = 302.24 g/mol; CAS 117-39-5; Figure 12) was first discovered, along with other flavonoids, by Nobel Prize laureate Albert Szent-Gyorgyi in the 1930s. It is chemically characterised by a phenyl-benzo(γ)pyrone-derived structure consisting of two benzene rings linked through a heterocyclic pyran ring. The three rings are planar with hydroxyl groups attached. The molecule is quite polarised. QCT is a yellow powder entirely insoluble in cold water, poorly soluble in hot water, but quite soluble in lipids, alcohol (2 mg/mL) and DMSO (30 mg/mL). In addition, QCT is a lipophilic molecule and has high membrane permeability. Therefore, according to the biopharmaceutical classification system, it could also be considered as a class-II compound, like RES.[194]

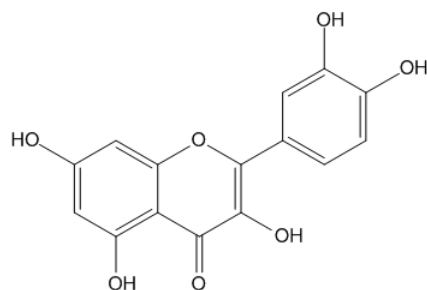

Figure 12. Chemical structure of quercetin

QCT is the most abundant flavonol in food, representing 75 % of the total intake.[241] It is found in a variety of foods including apples, berries, onions and tea, as well as many seeds. QCT is also found in some medical botanical plants and trees, such as *Ginkgo biloba*. The amount of QCT intake in a normal diet is 5-40 mg/day, but this can reach 200-500 mg/day when consumption of fruits and vegetables containing QCT is high.[242] QCT is metabolised in various organs such as the small intestine, colon liver and kidney. Concentrations of QCT in plasma are in the nanomolar range and depend on dosage intake.[243] QCT is generally well tolerated; doses up to 1 g/day for several months do not produce side-effects in humans.[244]

QCT, like RES, has both anti-inflammatory and antioxidant properties that have also led to an exponential growth of the scientific literature regarding its biological properties (Figure 13).

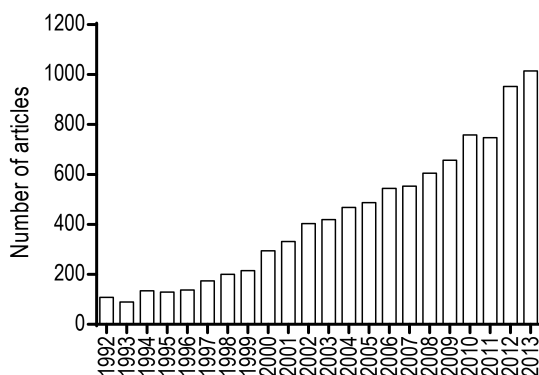

Figure 13. The number of articles in PubMed published every year using "quercetin" as keyword.

II.4.2.1.1. Biological properties

QCT, like other flavonoids, has anticancer, antiviral, cardioprotective and neuroprotective effects.[245,246] Among these effects, QCT is also well-known for its antioxidant properties, being one of the most effective free radical scavengers.[247] In-

vivo studies support the antioxidant effect of QCT. For example, Kamaraj et al. showed that QCT supplementation (25 mg/kg body weight) in Swiss albino mice decreases lipid peroxidation and tumour marker enzymes (aryl hydrocarbon hydroxylase, 5'-nucleotidase and lactate dehydrogenase) and increases antioxidant enzymes (SOD, catalase, glutathione peroxidase and glutathione reductase) in benzo(a)pyrene-induced carcinogenesis of mouse lung.[248] The antioxidant effect of QCT has been shown, not only in in-vitro and in-vivo animal research, but also in humans. A daily dose of QCT for 6 weeks was shown to decrease plasma concentration of artherogenic oxidised low density lipoprotein in overweight and obese subjects with metabolic syndromes.[249,250] In addition, QCT decreases serum MDA (an indicator of lipid peroxidation) levels in long distance runners, after 6 weeks of supplementation.[251]

Nevertheless, despite its numerous biological effects, this thesis will focus on the anti-inflammatory properties of QCT.

II.4.2.1.1.1. Anti-inflammatory properties

QCT has several anti-inflammatory effects, targeting multiple intracellular signalling pathways such as signal transducer and activator of transcription 1 (STAT1), NF-κB activation and MAPK family phosphorylation.[252,253] For example, Comalada et al. reported the in-vitro and in-vivo effects of QCT in bone marrow-derived macrophages and in an experimental model of rat colitis induced by dextran sulfate sodium, respectively. They found, both in-vitro and in-vivo, that QCT is able to down-regulate the inflammatory response of inhibiting cytokines (TNF-α and IL-1β) and inducible nitric oxide synthase (iNOS, an enzyme that generates the high reactive nitric oxide-NO-) expression through inhibition of the NF-κB pathway, without modification of JNK activity.[254]

Several in-vitro studies using different cell lines and animal models have shown that QCT can inhibit LPS-induced cytokine production. For instance, in-vitro data have shown that QCT inhibits LPS-induced TNF-α and IL-6 secretion in macrophages,[255,256] and LPS-induced IL-8/CXCL8 production in lung cells.[257] Moreover, it was shown that QCT can inhibit LPS-induced mRNA levels of TNF-α and IL-1α in glial cells.[258] Similar results were described by Qureshi et al. in peritoneal macrophages from mice stimulated with LPS. These results demonstrated that QCT inhibits LPS-induced expression of several pro-inflammatory genes (IL-1α, IL-1β, IL-6, TNF-α, IL-12, VCAM-1, ICAM-1 and COX-2).[259] Moreover, in macrophages QCT strongly reduces activation of phosphorylated ERK and p38 MAPK, and inhibits NF-κB activation through stabilisation of the NF-κB/IκB complex and IκB degradation.[260,261] Similar results were

reported in Chang liver cells where QCT produces a significant decrease of iNOS and COX-2, as well as inhibition of mRNA level of iNOS and COX-2.[262] In addition, QCT inhibits NF-κB activation and protein concentration of the phosphorylated form of the inhibitor IκBα.[262]

QCT also exerts its anti-inflammatory effect upon epithelial cells. For example, Nanua et al. demonstrated that QCT reduces TNF-α-induced IL-8/CXCL8 and MCP-1/CCL2 expression in cultured human airway epithelial cells.[263] QCT also inhibits TNF-α-induced phosphatidylinositol 3-kinase activity and AKT phosphorylation (enzymes involved in cellular functions, such as cell growth and proliferation), intracellular H_2O_2 production, NF-κB activation and IL-8/CXCL8 promoter activity.[263] Similar anti-inflammatory effects were shown in alveolar basal epithelial cells, where QCT inhibits IL-8/CXCL8 and GM-CSF release, as well as NF-κB and AP-1 and cAMP response element binding protein-dependent transcription to a greater extent than dexamethasone.[221] These results have suggested that QCT has the ability to inhibit TNF-α transcription by inhibiting phosphorylation and activation of JNK. Therefore, QCT can suppress activation of the transcription factor AP-1, ERK1/2 and p38 MAPK activities, which are important in the post-transcriptional regulation of TNF-α mRNA.[264] In-vivo experiments also support the anti-inflammatory properties of QCT. Muthigan et al. showed that QCT ameliorates experimental allergic encephalomyelitis in mice (a Th1 cell-mediated autoimmune disease) by blocking IL-12 signalling and Th1 differentiation.[265] QCT also ameliorates autoimmune myocarditis induced in rats by decreasing TNF-α and IL-17, and by increasing IL-10 cytokine secretions, both in serum and culture supernatants from lymph node cells.[266]

Similar combined anti-inflammatory and anti-allergy properties of QCT have been found in animal models of allergic airway disease. Joskova et al. found that QCT causes bronchodilation in guinea pigs stimulated by ovalbumin after 21 days, reducing hyper-reactivity of airways.[267] In the same in-vivo model, Jung et al. described that QCT reduces recruitment of leukocytes, particularly neutrophils and eosinophils, during the late-phase response of allergic response.[268] Indeed, QCT inhalation has the same effect as oral administration, showing similar anti-asthmatic activity to cromolyn sodium and dexamethasone.[269] In a murine model of allergic airway inflammation, Rogerio et al. showed that QCT inhibits NF-κB activation and reduces both IL-4 and IL-5 levels in plasma,[270] and eosinophil and neutrophil counts/infiltration in lung tissue.[271] In addition, using the same in-vivo model, Park et al. found that QCT regulates Th1/Th2 balance, inhibiting asthmatic reactions.[272] Moreover, QCT suppresses the IgE response by reducing plasma histamine in Wistar rats experimentally sensitised to have an anaphylactic reaction to peanut.[273]

II.4.2.1.2. Quercetin and ocular disorders

The antioxidant and anti-inflammatory properties of QCT have been investigated, not only in cell lines from major organs, but also in some types of ocular tissues and cell lines. One of the first biomedical effects described for QCT in the eye, more that 30 years ago, was its inhibitory action on lens aldose reductase, which can provoke lens opacity.[274] Oxidative stress is an initiating factor in the development of maturity-onset cataract and Cornish et al. showed that QCT exerts its effect on lens transparency following oxidative stress induced by H_2O_2.[275] Moreover, Cao et al. demonstrated that QCT can protect human lens epithelial cells from DMSO-induced apoptosis, by upregulating Bax expression.[276]

QCT was also investigated for its effects on suppression of retinal S antigen-induced intraocular inflammation in Lewis rats by Romero et al.[277] QCT administered intraperitoneally reduces uveal and retinal inflammation, vasculitis and perivasculitis. Morphometric analysis revealed that QCT also reduces choroidal thickness, when compared with control animals. These results clearly show the anti-inflammatory effect of QCT in experimental uveitis.

QCT, like RES, has a protective effect on the eye under UV exposure. Kaidzu et al. studied the antiapoptotic and antioxidant effects of QCT on light-exposed rat retina. They found that QCT alleviates histological damage, as shown by decreasing numbers of TUNEL positive cells and reduced expression of oxidative markers such as 8-OHdG, in rat retina exposed to light.[278] Focusing on the lens, UV radiation is related to cataract formation. Jiang et al. studied the effect of QCT in human lens epithelial cells exposed to both UV radiation and H_2O_2.[279] They found that both UV and H_2O_2 induce a decrease in collagen type I and activates JNK and its downstream component, c-Jun, in both a time- and dose-dependent manner. By contrast, QCT protects against UV- and H_2O_2-induced decrease of collagen type I and inhibits both UV- and H_2O_2-induced JNK and c-Jun activation.

III. Hypothesis

The development of this thesis study was dependent upon the following hypothesis:

The topical application of quercetin, resveratrol and their combination has an anti-inflammatory effect on ocular surface diseases, such as dry eye and ocular allergy, which is due to modulation of epithelial cells and immune cell response.

IV. Objectives

In order to corroborate the hypothesis, a general objective and further specific objectives were established:

General objective:

To study the potential effect of quercetin, resveratrol and their combination on cells involved in the inflammatory process of immune-based ocular surface diseases such as dry eye disease and ocular allergy.

Specific objectives:

- To determine the anti-inflammatory effect of quercetin, resveratrol and their combination on conjunctival and corneal cell lines stimulated by TNF-α.
- To determine the antioxidant effect of quercetin, resveratrol and their combination on conjunctival and corneal cell lines irradiated by UV-B light.
- To determine the immunomodulatory activity of quercetin, resveratrol and their combination in peripheral blood mononuclear cells.
- To determine the therapeutic effect of topical quercetin, resveratrol and their combination in a murine model of dry eye disease.
- To determine the effect of quercetin, resveratrol and their combination on mast cell degranulation.

V. Material and methods

This section describes the methodology followed in this thesis and it has been divided into four sections, according to the established objectives in section IV. Firstly, the effect of QCT, RES and their combination was studied on ocular surface epithelial cells stimulated with either TNF-α or UV-B light. Secondly, the immunoregulatory effect of polyphenols was determined in peripheral blood mononuclear cells. After that, the anti-inflammatory effect of QCT, RES and their combination was confirmed in an in-vivo model of DED. And finally, the anti-allergic effect of QCT, RES and their combination was studied in cord blood-derived mast cells.

VI.1. In-vitro model of inflammation and oxidative stress in human conjunctival and corneal epithelial cells

The anti-inflammatory and antioxidant effects of QCT, RES and their combination (QCT+RES) were tested in-vitro using two established ocular surface cell lines, one derived from human conjunctival epithelium (IOBA-NHC) and other derived from human corneal epithelium (HCE), under two different stimuli: 1) TNF-α-induced inflammation[154] and 2) UV light-induced oxidative stress.[280]

The cytotoxicity of polyphenols on epithelial cells was firstly assessed, in order to determine the non-toxic concentrations for further experiments. Following this, the effect of QCT, RES and their combination on cytokine/chemokine secretion, COX-2 expression and ROS production were assessed.

VI.1.1. Reagents

All reagents and antibodies (Table 3) used in this part of the thesis have been grouped based on the manufacturer:

- *Invitrogen* (Inchinnan, UK): Dulbecco's Modified Eagle Medium/Nutrient Mixture F12 (DMEM/F12), alamarBlue® cell viability assay, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES).
- *Nunc* (Roskilde, Denmark): Plastic culture dishes and flasks.
- *Panreac* (Barcelona, Spain): EtOH and D-glucose.
- *PeproTech EC* (London, UK): Cytokine TNF-α.
- *Sigma-Aldrich* (St Louis, MO, USA): RES, QCT, DMEM (culture medium without sodium bicarbonate ($NaHCO_3$), sodium pyruvate and phenol red), 2’,7’-

dichlorodihydrofluorescein diacetate (H_2DCF-DA), L-glutamine, foetal bovine serum (FBS), cholera toxin, human epithelial growth factor (EGF), bovine insulin, penicillin, streptomycin, fungizone and hydrocortisone, DMSO, phosphate buffered saline (PBS), tris-hydrochloride (Tris-HCl), sodium chloride (NaCl), deoxycholic acid, Triton X-100, sodium dodecyl sulphate (SDS), ethylenediaminetetraacetic acid (EDTA), phenylmethanesulfonyl fluoride (PMSF), aprotinin, sodium orthovanadate (Na_3VO_4), glycerol, 2-mercaptoethanol (2-ME), bromophenol blue, bovine serum albumin (BSA), Tween 20, tris-buffered saline (TBS).

- *Thermo Fisher Scientific* (Rockford, IL, USA): Bicinchoninic acid (BCA) assay.

Antibody	Dilution	Manufacturer	Reference
Rabbit anti-COX-2	1/100	Novus Biologicals (Littleton, CO, USA)	NB100-689
*Mouse anti-g*lyceraldehyde-3-phosphate dehydrogenase *(GAPDH)*	1/500	Santa Cruz Biotechnology (Heidelberg, Germany)	SC-166545
HRP-conjugated human anti-IgG	1/2000		SC-2004
HRP-conjugated mouse anti-IgG	1/5000	Jackson Laboratory (Bar Harbor, ME, USA)	715-035-150

Table 3. Antibodies used in this part of the thesis experiments (section V.1.8).

VI.1.2. Preparation of polyphenol solutions

QCT and RES were dissolved in EtOH. Fresh stock solutions of QCT and RES were prepared for each experiment, after which serial dilutions were carried out to achieve final concentrations, ranging from 0.5-25 µM QCT and 0.5-300 µM RES. Concentrations tested for each assay are detailed in Table 4.

Combinations of QCT and RES were prepared by mixing QCT and RES solutions, in order to reach the final concentrations for each experiment (as indicated in Table 4).

All solutions were prepared in such a way that the final concentration of vehicle, 0.5 % EtOH, was non-toxic and was the same in all samples when polyphenols were added into each well. All solutions were kept away from light because light degrades polyphenols.

In order to clarify the rationale for the selected concentrations of polyphenols, the cytotoxicity of both compounds was firstly analysed for a range of concentrations

(0.5-25 μM QCT and 0.5-300 μM RES). Once non-toxic concentrations of each compound for both cell lines were determined, non-toxic concentrations (0.5-25 μM QCT and 0.5-50 μM RES) were selected for dose-response studies in the two in-vitro models (TNF-α-induced inflammation and UV-B-induced oxidative stress). Finally, one concentration of each compound (0.5 μM QCT and 5 μM RES), which did not significantly decrease cytokine/chemokine secretion and ROS production in the dose-response studies, was selected in order to analyse the effect of both compounds in combination. Regarding COX-2 assay, as the dose-response curve was not obtained, the highest concentrations of each compound tested on dose-response curves (25 μM QCT and 50 μM RES) and the combination of both compounds tested previously (0.5 μM QCT and 5 μM RES) were used in this experiment in order to maintain the same concentrations tested.

<table>
<tr><th rowspan="3">Measurement</th><th rowspan="3">Assay</th><th rowspan="3">Time point</th><th colspan="4">Concentration of polyphenol (μM)</th></tr>
<tr><th rowspan="2"><u>QCT</u></th><th rowspan="2"><u>RES</u></th><th colspan="2"><u>Combination</u></th></tr>
<tr><th>QCT</th><th>RES</th></tr>
<tr><td rowspan="3">Cytotoxicity</td><td rowspan="3">AlamarBlue®
(absorbance)</td><td rowspan="3">24 h</td><td rowspan="3">1, 5, 10, 15, 20 & 25</td><td rowspan="3">1, 5, 10, 25, 50, 100, 150 & 300</td><td>15</td><td>25, 50 & 100</td></tr>
<tr><td>20</td><td>25, 50 & 100</td></tr>
<tr><td>25</td><td>25, 50 & 100</td></tr>
<tr><td>Cytokine/chemokine secretion (IL-6, IL-8/CXCL8, IP-10/CXCL10 & VEGF)</td><td>Multiplex-bead based array X-MAP technology (Luminex)</td><td>24 h</td><td>0.5, 1, 5, 10, 15, 20 & 25</td><td>0.5, 1, 5, 10, 25 & 50</td><td rowspan="3">0.5</td><td rowspan="3">5</td></tr>
<tr><td>COX-2 expression</td><td>Western blot
(chemilumiscence)</td><td>24 h</td><td>0.5 & 25</td><td>5 & 50</td></tr>
<tr><td>Intracellular ROS production</td><td>H_2DCF-DA dye
(fluorescence)</td><td>1 h</td><td>0.5, 1, 5, 10 & 25</td><td>1, 5, 10, 25 & 50</td></tr>
</table>

Table 4. Summary of assays and concentrations of polyphenols tested in IOBA-NHC and HCE cell lines. (QCT: quercetin; RES: resveratrol; H_2DCF-DA: 2',7'-dichlorodihydrofluorescein diacetate).

VI.1.3. Cell lines and culture conditions

Two different ocular surface epithelial cell lines, IOBA-NHC and HCE, were used for these experiments.

A) The IOBA-NHC is a non-transfected, spontaneously immortalised epithelial cell line derived from normal human conjunctiva.[281] It was used from passage 62. IOBA-NHC cells were cultured in DMEM/F-12 L-glutamine supplemented with 10 % FBS, 0.1 µg/mL cholera toxin, 2 ng/mL EGF, 1 µg/mL bovine insulin, 5000 U/mL penicillin, 5 mg/mL streptomycin, 2.5 µg/mL fungizone and 0.5 µg/mL hydrocortisone.

B) The HCE is an SV40-immortalised human corneal epithelial cell line[282] kindly gifted by Professor Arto Urti (University of Helsinki, Finland). It was used from passage 45. HCE cells were cultured in DMEM/F-12 L-glutamine supplemented with 15 % FBS, 0.5 % DMSO, 0.1 µg/mL cholera toxin, 10 ng/mL EGF, 5 µg/mL insulin, 100 U/mL penicillin and 0.1 mg/mL streptomycin.

Both cell lines were cultured in an incubator at 37 ºC in a humidified atmosphere of 5 % CO_2. Media were changed every other day and cells were observed every day by phase contrast microscopy.

All experiments were carried out in DMEM culture medium without $NaHCO_3$, sodium pyruvate and phenol red, and supplemented with 3.15 g/L D-glucose, 2 mM L-glutamine and 25 mM HEPES, hereinafter referred to as complete medium (CM).

VI.1.4. Cytotoxicity assay

The toxicity of QCT, RES and QCT+RES on epithelial cells was assessed by the resazurin reduction test. AlamarBlue®, or resazurin, is a non-toxic and non-fluorescent compound that is reduced continuously by cells to resorufin, a fluorescence compound. This conversion occurs intracellularly by mitochondrial, microsomal and cytosolic oxidoreductases.[283] AlamarBlue® can distinguish metabolically active cells because non-viable cells have lower innate metabolic activity and hence they generate a proportionally lower signal than healthy cells. The fluorescence intensity of alamarBlue® reagent is directly proportional to cell number.

IOBA-NHC and HCE cells were seeded in 96-well plates at density of 10^4 cells per well and grown for 3 days. After that, culture medium was replaced with serum-free, non-supplemented medium and cells were maintained in it for 24 h. Then, the serum-free medium was replaced with CM and cells were treated with different

concentrations of QCT, RES or QCT+RES (see Table 4 for details of polyphenol concentrations) and incubated for 24 h at 37 ºC. Control cells were treated with vehicle (0.5 % EtOH). Following incubation, supernatants were discarded and 10 % alamarBlue®, prepared in supplemented DMEM/F12 cultured medium, was added. Cells were incubated for 4 h at 37 ºC. Finally, medium from each sample was collected and fluorescence was measured at 560 nm_{ex} / 590 nm_{em} by UV/Vis spectrophotometry (SpectraMax® M5, Molecular Devices, Sunnyvale, CA, USA). Three independent experiments were performed and measurements were from 8 replicates for each condition studied.

VI.1.5. Cell cytokine stimulation and polyphenol treatments

IOBA-NHC and HCE cells were seeded in 24-well plates at densities of $45x10^3$ and $60x10^3$ cells per well ($23x10^3$ and $32x10^3$ cell/cm^2) respectively, and grown for 3 days. For the COX-2 assay, cells were seeded and grown in 25 cm^2 flasks at density of $60x10^3$ cells per flasks until confluence. Culture medium was then replaced with serum-free, non-supplemented medium and cells were maintained in this for 24 h at 37 ºC. Subsequently, serum-free medium was discarded and cells were pre-treated with QCT, RES, QCT+RES (see Table 4 for concentration details) or vehicle, in CM for 2 h at 37 ºC. After that, pre-treatments were removed, cells were stimulated with 25 ng/mL TNF-α in the presence of QCT, RES, QCT+RES or vehicle, and incubated for 24 h in CM. Unstimulated cells treated with polyphenols but without TNF-α were used as control. After the treatment period, the conditioned media were collected and centrifuged at 59 x g for 5 min. Supernatants, and plates and flasks with adherent cells were stored at -80 ºC until use. Three independent experiments were performed in duplicate, unless otherwise stated.

VI.1.6. Cellular lysis and total protein assay

Total protein content was measured in plates/flasks with adherent cells. Firstly, cells were lysed. The adherent cells were washed with cold PBS and disrupted with ice-cold radioimmunoprecipitation assay (RIPA) buffer [10 mM tris-HCl (pH 7.4), 150 mM NaCl, 1 % deoxycholic acid, 1 % Triton X-100, 0.1 % SDS, and 1 mM EDTA] supplemented with two proteases inhibitors (0.1 mg/mL PMSF and 60 µg/mL aprotinin), and a tyrosine phosphatase inhibitor (0.1 µM Na_3VO_4). Samples were incubated on ice for 30 min. Then, samples were centrifuged at 18,000 x g for 30 min at 4 ºC. Finally, supernatants were collected and stored at -80 ºC until use.

Total protein was assessed using the BCA assay. This method combines the Biuret reaction (reduction of Cu^{+2} to Cu^{+1} by protein in an alkaline medium) with the highly sensitive colourimetric detection of Cu^{+1} using BCA.[284] The purple-coloured reaction product of this assay is formed by the chelation of two molecules of BCA with one Cu^{+1}. This complex exhibits a strong absorbance that is nearly linear with increasing protein concentrations. The BCA assay was carried out on cell lysates according to the manufacturer's instructions. Briefly, 25 µL of standards and cell lysate samples were treated with BCA working reagent (BCA in 0.1 mM sodium hydroxide and 4 % cupric sulphate solution) and incubated for 30 min at 37 ºC. Then, samples were cooled at room temperature and the absorbance was read at 562 nm using the SpectraMax® M5 UV/Vis spectrophotometer (Molecular Devices). Data were analysed using the software SoftMax® Pro (Molecular Devices).

VI.1.7. Measurement of cytokine/chemokine secretion

Cytokine/chemokine secretion was assessed by a multiplex-bead based array, using Luminex™ x-MAP® multiplexing bead technology.[285,286] This assay consists of a series of microspheres (beads) containing fluorochromes of differing intensity embedded within the bead, giving each group of beads with a specific molecules attached a unique signal. The multiplex system enables the detection and quantification of multiple analytes (proteins and peptides, or nucleic acids) in a single sample volume.

IL-6, IL-8/CXCL8, IP-10/CXCL10 and VEGF levels were determined in cell supernatants with a commercial Milliplex 4-plex human cytokine/chemokine immunobead-based assay (HCYTO, Millipore, Watford, UK), according to the manufacturer's instructions. Briefly, 25 µL of cell supernatant from each sample were incubated in 96-well plates with antibody-immobilised beads overnight at 4 ºC. Then, beads were washed and incubated with biotinylated cytokine/chemokine antibody solution for 1 h at room temperature, followed by incubation with streptavidin-phycoerythrin for 30 min at room temperature. Finally, beads were washed and read on a Luminex™ 100-IS instrument (Luminex Corporation, Austin, Tx, USA). Standard curves of known concentrations of recombinant human cytokines/chemokines were used to convert fluorescent units to cytokine/chemokine concentration units (pg/mL). The minimum detectable level for each cytokine/chemokine, based on the manufacturer specifications, was: 0.3 pg/mL for IL-6; 0.2 pg/mL for IL-8/CXCL8; 1.2 pg/mL for IP-10/CXCL10; and 5.8 pg/mL for VEGF. When a cytokine level was not

detectable, the minimum detectable level was used in the analysis. Data were analysed with the BeadView™ Software (Upstate, UK) and normalised to total protein content for each sample.

VI.1.8. Cyclo-oxygenase-2 expression

The expression levels of COX-2 in cells were assessed by SDS-polyacrylamide gel electrophoresis (PAGE) and Western blotting, according to the Laemmli method.[287] Cell homogenates from flasks were mixed with an equal volume of 2X Laemmli [4 % SDS, 20 % glycerol, 10 % 2-ME, 0.004 % bromphenol blue and 0.125 mM Tris-HCl, pH~6.8].

Proteins in samples were denatured and reduced by boiling them for 5 min at 110 ºC. Then, 20 µg of total protein from each sample were separated by SDS-PAGE on 10 % acrylamide gels. After SDS-PAGE, proteins in the gel were transferred to 0.2 µm pore size nitrocellulose membranes, blocked with 5 % non-fat milk, 3 % BSA and 0.05 % Tween 20 in TBS for 1 h under agitation, at room temperature. Membranes were then incubated with rabbit anti-COX-2 antibody (1/100) overnight under agitation at 4 ºC. Subsequently, membranes were washed and incubated with HRP-conjugated human anti-IgG antibody (1/2000) diluted in blocking buffer for 1 h at room temperature. Membranes were washed and immunoreactive bands of COX-2 protein were detected by incubation of membranes in enhanced chemiluminiscent solution (Santa Cruz) for 1 min. Images were captured by a charge-coupled device (CCD) camera in a ChemiDoc XRS (Bio-Rad, Inc., Hercules, CA, USA). GAPDH band was used as loading control to normalise the levels of COX-2 protein detected. Briefly, membranes were washed and incubated with Restore™ western blot stripping buffer (Thermo Fisher Scientific) for 1 h at 60 ºC. Subsequently, membranes were washed, blocked and further incubated with primary mouse anti-GAPDH (1/500) antibody and secondary HRP-conjugated mouse anti-IgG antibody (1/5000), as described above. Quantification of protein immunoreactive bands was assessed by volumetric densitometry using Quantity One® software (Bio-Rad). Two experiments were performed in duplicate. Band intensity data were expressed as percentage of band intensity.

VI.1.9. Measurement of reactive oxygen species induced by UV-B radiation

The generation of intracellular ROS by UV-B exposure of epithelial cells was assessed using H_2DCF-DA, which is a non-fluorescent dye that passively diffuses into cells, where it is cleaved and deacetylated to H_2DCF by intracellular esterases. Non-fluorescent H_2DCF is rapidly oxidised to fluorescent DCF by intracellular ROS.

IOBA-NHC and HCE cells were cultured in supplemented medium in 24 well UV transparent plates for 72 h. Then, culture medium was discarded, serum-free non-supplemented medium was added in which cells were maintained for 24 h. After that, culture medium was replaced and cells were pre-treated with QCT, RES, QCT+RES (see table 4 for doses used) or vehicle for 1 h at 37 ºC in CM. At that point, pre-treatments were discarded, and cells were loaded with H_2DCF-DA adding 500 µL of 10 µM H_2DCF-DA solution in CM and incubated for 30 min. H_2DCF-DA medium was then aspirated; cells were treated with QCT, RES, QCT+RES or vehicle at the same concentrations used before, and exposed to 8W UV-B lamps (with an excitation peak of 302 nm), located 3 cm below cells. At that distance the UV-B radiation power density was 7.15 mW/cm^2, according to the manufacturer (Bio-Rad). Cells were irradiated for 15 s from the bottom of the well plate to avoid UV-B absorption by polyphenols in the culture media. After 15 s, UV-B radiant exposure was 107.25 mJ/cm^2, as calculated with the following formula: $H=E \cdot t$, where H is the radiant exposure (J/cm^2), E is the irradiance (W/cm^2) and t is the exposure time (s). Control cells were not irradiated. After UV-B exposure, cells were cultured for 1 h and then intracellular fluorescence was measured at 488 nm$_{ex}$ / 522 nm$_{em}$ using the SpectraMax® M5 UV/Vis spectrophotometer (Molecular Devices). Fluorescence data from each sample were normalised to the corresponding total protein content, determined previously in adherent cells by the BCA protein assay kit. Three different experiments were carried out and samples were performed in duplicate.

VI.2. In-vitro proliferative response of human T cells

The immunosuppressive effect of QCT, RES and their combination QCT+RES was tested on human peripheral blood mononuclear cells (PBMCs). Cytotoxicity, and cell proliferation and viability were studied.

These experiments were carried out at University College London, under the supervision of Dr. Virginia Calder. The doctoral candidate's stay was supported by "ayudas para estancias breves en el desarrollo de tesis doctorales" by University of Valladolid in 2012. Additionally, some experiments were carried out at IOBA, University of Valladolid.

VI.2.1. Reagents

All reagents were purchased from Sigma-Aldrich, unless otherwise specified. All assays were performed in Roswell Park Memorial Institute (RPMI)-1640 HEPES Modification medium supplemented with 2 mM L-glutamine, 10 % FBS, 50 U/mL penicillin, 50 µg/mL streptomycin, non-essential amino acids (glycine, L-alanine, L-asparagine, L-aspartic acid, L-glutamic acid, L-proline and L-serine), 1 mM sodium pyruvate and 2.5 µM 2-ME. Propidium iodide (PI) was also obtained from Sigma.

Anti-CD3 (clone HIT3a) and anti-CD28 (clone 28.2) were purchased from Becton Dickinson Biosciences (BD, Oxford, UK). Carboxyfluorescein diacetate succinimidyl ester (CFSE) and Ficoll-Hypaque were purchased from Invitrogen. Foetal calf serum (FCS) was purchased from Labtech International (Ringmer, UK).

VI.2.2. Preparation of polyphenol solutions

QCT, RES and QCT+RES solutions were prepared as previously described (see Section V.1.2), with some modifications.

Stock solutions of QCT and RES were prepared in EtOH, after which serial dilutions were carried out to achieve final concentrations ranging from 0.5-25 µM QCT and 0.5-100 µM RES. Aliquots of each solution were kept at -20 ºC. Concentrations tested for each assay are detailed in Table 5. The final concentration of vehicle in cells treated with QCT or RES alone was a non-toxic concentration of 0.5 % EtOH in the culture medium.

Final concentrations tested of QCT+RES were achieved by adding QCT solutions and RES solutions to each sample in order to reach the final concentrations

in cultured medium as indicated in Table 5. The final concentration of vehicle in cells treated with QCT+RES was a non-toxic concentration of 1 % EtOH in the culture medium.

In order to clarify the rationale for the selected concentrations of polyphenols, the cytotoxicity of both compounds was firstly analysed for a range of concentrations (0.5-25 μM QCT and 0.5-100 μM RES, alone and in combination). After that, low concentrations of QCT (0.5, 1, 5 and 10 μM) and RES (5 and 10 μM) were selected in order to study their effect on PBMC proliferation. These latter concentrations were selected based on the previous experiment, although some of them were toxic for PBMCs. This issue is discussed in Section VII.2 because both experiments were not comparable.

Measurement	Assay	Time point	Concentration of polyphenol (µM)			
			QCT	*RES*	*Combination*	
					QCT	*RES*
Cytotoxicity	PI (flow cytometry)	96 h	0.5, 1, 5, 10, 15, 20 & 25	0.5, 1, 5, 10, 25, 50 & 100	0.5, 1, 5, 10, 15, 20 & 25	0.5, 1, 5, 10, 25, 50 & 100
Proliferation and viability	CFSE (flow cytometry)	120 h	0.5, 1, 5 & 10	5 & 10	0.5, 1, 5 & 10	5 & 10

Table 5. Summary of assays and concentrations of polyphenols tested in PBMCs. (QCT: quercetin; RES: resveratrol; PBMCs: peripheral blood mononuclear cells; PI: propidium iodide; CFSE: carboxyfluorescein diacetate succinimidyl ester).

VI.2.3. Donors

Peripheral blood was obtained from 3 anonymous healthy donors with informed consent. Exclusion criteria included a history of autoimmune, inflammatory or allergic disease, haematological disorder or current usage of systemic medication. The protocols used in this study were reviewed and approved by the Local Ethics Committee ("UCL committee" and "Comité Ético de la Universidad de Valladolid", the latter included in appendix III). All studies involving human subjects were conducted according to the tenets of the Declaration of Helsinki.

VI.2.4. Peripheral blood mononuclear cell isolation

PBMCs were isolated by Ficoll-Hypaque density gradient centrifugation. Peripheral blood collected using heparinised tubes was diluted with an equal volume of culture medium. Diluted blood was slowly layered over a Ficoll-Hypaque solution and centrifuged at 624 x g for 40 min at room temperature, with no brake. Mononuclear cells were then collected, washed by adding excess of culture medium and centrifuged at 193 x g for 10 min at room temperature. After washing, PBMCs were resuspended in RPMI culture medium for further experiments.

VI.2.5. Cytotoxicity assay

The cytotoxicity of polyphenols alone and in combination on PBMCs was assayed by PI staining by means of flow cytometry. PI is a membrane impermeant fluorescence dye that binds to DNA and RNA. Late stages of apoptosis are characterised by loss of membrane permeability and DNA fragmentation and, consequently, loss of nuclear DNA content. Hence, PI penetrates into cells undergoing apoptosis and binds to fragmented DNA, identifying dead cells.

PBMCs were seeded in 96-well plates at density of $2x10^5$ cells per well and treated with different concentrations of QCT, RES, QCT+RES (see Table 5 for concentration details) or vehicle. After that, PBMCs were stimulated with 50 ng/mL anti-CD3 and 10 µg/mL anti-CD28, and incubated in 5 % CO_2 for 96 h at 37 ºC. Control cells were stimulated in presence/absence of vehicle. At the end of incubation, 5 µg/mL PI were added and cells were acquired using a FACSCalibur™ flow cytometer (BD). At least 15,000 events were analysed using CellQuest™ Pro software (BD). Samples were previously gated on forward scatter (FS) *versus* side scatter (SS) to exclude debris and clumps. All cells were further gated to assess level of viable cells (PI-

negative). Three different experiments were carried out and samples were performed in single samples.

VI.2.6. Proliferation and viability assay

The effect of polyphenols on PBMC proliferation and viability was assessed by means of the CFSE dye.[288] CFSE is a colourless and non-fluorescent dye that passively diffuses into cells. CFSE becomes highly fluorescent when acetate groups are cleaved by intracellular esterases. When a cell divides the daughter cells each comprise half of the parental cell. Hence CFSE passes equally on to the daughter cells upon cell division and it is able to trace the division of cells by the dilution of CFSE dye during the first and subsequent cell divisions.

Cells were incubated with 5 µM CFSE in serum-free non-supplemented medium for 10 min at 37 °C in a water bath. After that, 1 mL of cold stop buffer (10 % FCS in RPMI) was added to cells and were then incubated for 30 min at room temperature, washed once with RPMI, and further resuspended to reach a concentration of 2×10^5 cells per well. PBMCs were then treated with different concentrations of QCT, RES, QCT+RES (see Table 5 for more details) or vehicle, stimulated with 50 ng/mL anti-CD3 and 10 µg/mL anti-CD28, and incubated in 5 % CO_2 for 120 h at 37 ºC. At the end of incubation period, cells were counterstained with PI (5 µg/mL). Samples were acquired using FACSCalibur flow cytometer, and listmode data were generated using CellQuest Pro software. Data were further analysed using WinList (Verity Software House, Topsham, ME, USA). Samples were gated on FS *versus* SS to exclude debris and clumps. For proliferation, cells were gated in a CFSE/PI dot plot to track the divisions of CFSE-labelled cells enabling identification of the percentage of divided (proliferated) cells. Percentage of divided cells was obtained by the formula:

$$Cell\ division\ (\%) = \left({}^{Divided\ cells}/_{Divided\ cells + Undivided\ cell} \right) \times 100$$

where "divided cells" were PI-negative/CFSE-negative, and "undivided cells" were PI-negative/CFSE-positive.

For the viability assay, all lymphocytes were gated to assess the levels of PI-negative cells (live cells). Gates for CFSE-positive and PI-negative cells were set on viable lymphocytes at day 0.

In all cases, treatments were performed in triplicate for each experiment and three different experiments were carried out.

VI.3. Experimental murine model of dry eye

The anti-inflammatory effect of QCT, RES and their combination (QCT+RES) was tested in an experimental murine model of DED, followed by an adoptive transfer model.[87,94] Corneal fluorescein staining (CFS), aqueous tear production (TP), goblet cell count, cytokine levels in tears and CD4^{+} T cell infiltration in conjunctiva were assessed (Figure 14).

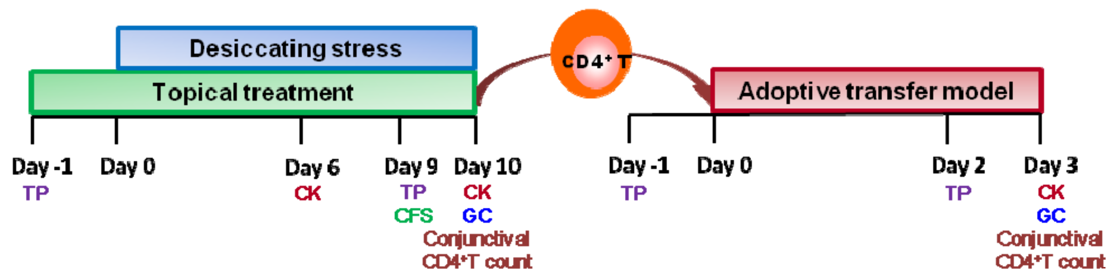

Figure 14. Timeline for experimental DED and adoptive transfer model. (TP: tear production; CK: cytokine/chemokine production; CFS: corneal fluorescein staining; GC: goblet cell count).

These experiments were carried out in Allergan headquarters (Irvine, CA, USA) under the supervision of Dr. Michael E. Stern, Vice-President of the Inflammation Research Program. The doctoral candidate's stay was supported by “ayudas para estancias breves en el desarrollo de tesis doctorales” by University of Valladolid in 2011 and 2013.

VI.3.1. Animals

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

VI.3.2. Desiccating stress-induced dry eye and topical treatments

Experimental DED was induced in female C57BL/6 mice (hereinafter referred to as donor mice) as previously described.[87,94] Up to five mice were placed in a cage with a perforated plastic screens on two sides of the cage. Mice were exposed to DS by

placing them in a controlled-environment chamber with airflow from fans (two fans on each side of the cage) for 10 h a day, and room relative humidity at 20 % and temperature maintained at 23 °C. A continuous dose of 0.1 mg/day scopolamine hydrobromide (Sigma-Aldrich) was administered by subcutaneously implanted osmotic pump (Alzet®, Cupertino, CA, USA). DS was carried out for 10 days. Control mice were kept in a non-stressed environment between 50 % and 80 % relative humidity and temperature of 21-23 °C, without exposure to forced airflow.

Mice were randomly divided into 6 groups, each group with 9 mice:

1) Control group (no DS and no topical treatment).
2) DS (no topical treatment).
3) DS + vehicle (vehicle was confidentially developed by Allergan).
4) DS + 0.01 % QCT.
5) DS + 0.1 % RES.
6) DS + 0.01 % QCT + 0.1 % RES.

The treatments started one day before DS was set up and they were administered topically in both eyes (5 µL/eye), three times a day. At the end of the experiment, mice were euthanised with CO_2 and left eyeballs (with attached lids) were immediately embedded in optimal cutting temperature compound (OCT; VWR, Suwanee, GA, USA) and flash frozen. Right eyeballs with attached lids were also excised and kept in 4 % paraformaldehyde at 4 ºC, until use.

VI.3.2.1. Corneal staining in donor mice

CFS was used to evaluate corneal epithelial damage caused by DS in donor mice after 9 days. A dose of 5 µL of 0.125 % fluorescein sodium salt (Sigma) was applied into the lateral conjunctival sac of the mouse and 1 min later both corneas were examined, using a stereo microscope under cobalt blue light. Superficial punctate staining was recorded in a masked fashion using the National Eye Institute grading system, scoring 0 to 3 (Grade 0 = no punctate staining to less than 5. Grade 1 = 5-20 punctates; Grade 2 = 20-50 punctates; Grade 3 > 50 punctates) for each of 5 areas of both corneas: central, superior, inferior, nasal and temporal.[289]

VI.3.2.2. Aqueous tear production in donor mice

TP was performed the day before DS induction and after 9 days of DS in donor mice, using phenol red-impregnated cotton threads (Zone-Quick; Lacrimedics, Eastsound, WA, USA). The thread was held with sterilised jeweller forceps and placed

in the lateral cantus of the right eye for 30 s. Threads turn from yellow to red on absorption of tears. Wetting of the thread was measured using the millimeter scale on the cotton thread package.

VI.3.2.3. Tear collection in donor mice

Tear samples were collected on day 6 and 10 in donor mice. A volume of 1.5 μL of cytokine assay buffer (Millipore) was instilled into each eye, and immediately 2 μL (1 μL/eye) of tear fluid and buffer were collected from the tear meniscus in the lateral cantus with a glass capillarity tube and a microcap (Drummond Scientific, Broomall, PA, USA). Then, the tear sample pooled from both eyes was diluted in 8 μL of Beadlyte assay buffer and stored at -80 °C until the time of assay.

VI.3.2.4. Measurement of cytokine and chemokine levels in donor mouse tears

The level of selected cytokines and chemokines in the tears was evaluated using a multiplex bead analysis with x-MAP® technology (as previously described in Section V.1.7). Cytokine/chemokine tear levels were assessed using a Milliplex 15-plex (IFN-γ, IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12(p70), MMP-9, IL-13, IL-17, IP-10/CXCL10, MCP-1/CCL2, RANTES/CCL5 and TNF-α) mouse cytokine/chemokine immunobead-based assay (Millipore) and analysed on a Luminex 200 instrument (Luminex Corporation). The minimum detectable level for each cytokine/chemokine, based on the manufacturer specifications, was: 1.1 pg/mL for IFN-γ, 10.3 pg/mL for IL-1α, 5.4 pg/mL for IL-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-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 pg/mL for IL-17, 0.8 pg/mL for IP-10/CXCL10, 6.7 pg/mL for MCP-1/CCL2, 2.7 pg/mL for RANTES/CCL5 and 2.3 pg/mL for TNF-α. When a cytokine level was not detectable, the minimum detectable level was used in the analysis. Data were analysed using Milliplex analyst software.

VI.3.2.5. Goblet cell count in donor mice

Right eyeballs with attached lids from donor mice fixed in 4 % paraformaldehyde were further embedded in paraffin. Eight-micrometer sections were stained with periodic acid-Schiff (PAS) reagent. Sections were viewed by light microscopy using an Eclipse E400 (Nikon, Melville, NY, USA) with a 20x objective. For quantification of goblet cells, a midline section of each eye was counted following the morphometric guideline of the entire superior and inferior conjunctiva, starting at the

limbus and spanning the entire length to the tarsal conjunctiva, including the conjunctival epithelium and stroma to a depth of 75 μM below the basement membrane. Goblet cells were counted in a masked fashion.

VI.3.2.6. Immunohistochemistry in donor mice

CD4+ T cell immunostaining and counting were performed as previously described.[39,94,290,291] OCT-embedded left eyeballs (with attached lids) from donor 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 °C overnight and fixed in -20 °C acetone for 10 min at room temperature. Endogenous peroxidases were then quenched with H_2O_2 for 10 min, slides were rinsed with PBS and air dried for 1 h. After that, non-specific sites were blocked with 20 % normal rabbit serum (Dako, Carpinteria, CA; USA), and rat anti-mouse CD4 primary antibody (BD) was added to each slide and incubated for 1 h at room temperature. After slides were extensively washed, sections were incubated with biotinylated polyclonal anti-rat IgG secondary antibody (BD) for 30 min. Then, Vectastain Elite ABC reagent (Vector laboratories, Burlingame, CA, USA) was added for antigen localisation, following manufacturer's instructions. Finally, samples were incubated with NovaRED (Vector Laboratories) peroxidase substrate to give a red stain, and subsequently counterstained with haematoxylin (Invitrogen). In addition, rat anti-mouse IgG isotype (BD) was used as negative control. CD4+ T cells in the conjunctiva were countered in a masked fashion by light microscopy, using an Eclipse E400 (Nikon). For quantification of CD4+ T cells, a midline section of each eye was counted following the morphometric guideline of the entire superior and inferior conjunctiva, starting at the limbus and spanning the entire length to the tarsal conjunctiva, including the conjunctival epithelium and stroma to a depth of 75 μM below the basement membrane. CD4+ T cells were counted in a masked fashion.

VI.3.3. Adoptive transfer model

CD4+ T cells from each group of donor mice (DS and control mice) were isolated from spleens and cervical lymph nodes, and enriched using the CD4+ T cell isolation kit II (MACS System, Miltenyi Biotec, Auburn, CA, USA), according to the manufacturer's instructions. Then, one donor-equivalent of cell suspension in PBS was transferred intraperitoneally into each T cell deficient nude C57BL/6 mouse (hereinafter referred to as recipient mouse), as previously described.[39,94] One donor-equivalent is

defined as the number of cells remaining after the respective in-vitro manipulation (i.e. $CD4^+$ T cells) of a single set of lymph nodes or spleen (approximately $5x10^6$ $CD4^+$ T cells). Eight recipient mice per donor group were used. Recipient mice were sacrificed 72 h after adoptively transferred $CD4^+$ T cells. Immediately, left eyeballs with attached lids were embedded in OCT and flash frozen, and right eyeballs with attached lids were also excised and kept in 4 % paraformaldehyde at 4 ºC until use.

VI.3.3.1. Aqueous tear production in recipient mice

TP was performed in recipient mice one day before and 48 h after transferring $CD4^+$ T cells. TP was measured following the same procedure described in Section V.3.2.2.

VI.3.3.2. Tear collection in recipient mice

Tear samples were collected on day 3 in recipient mice following the same procedure described in Section V.3.2.3.

VI.3.3.3. Measurement of cytokine and chemokine levels in recipient mouse tears

The level of selected cytokines and chemokines (IFN-γ, IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12(p70), MMP-9, IL-13, IL-17, IP-10/CXCL10, MCP-1/CCL2, RANTES/CCL5 and TNF-α) in the tears of recipient mice was evaluated using a multiplex bead analysis with x-MAP® technology, as previously described in Sections V.1.7 and V.3.2.4. The minimum detectable level for each cytokine/chemokine, based on the manufacturer specifications, was: 1.1 pg/mL for IFN-γ, 10.3 pg/mL for IL-1α, 5.4 pg/mL for IL-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-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 pg/mL for IL-17, 0.8 pg/mL for IP-10/CXCL10, 6.7 pg/mL for MCP-1/CCL2, 2.7 pg/mL for RANTES/CCL5 and 2.3 pg/mL for TNF-α. When a cytokine level was not detectable, the minimum detectable level was used in the analysis. Data were analysed using Milliplex analyst software.

VI.3.3.4. Goblet cell count in recipient mice

In order to count goblet cell numbers in the conjunctiva, right eyeballs from recipient mice (with attached lids) and previously fixed in 4 % paraformaldehyde, were embedded in paraffin, sectioned and stained as described in Section V.3.2.5.

VI.3.3.5. Immunohistochemistry in recipient mice

OCT-embedded left eyeballs, with attached lids, from recipient mice were sectioned and analysed as described in Section V.3.2.6 for counting $CD4^+$ T cell infiltration in conjunctiva.

VI.4. In-vitro model of conjunctival mast cell response

The anti-allergic effect of QCT, RES and their combination (QCT+RES) was tested on human cord blood-derived mast cells (CBMCs).[292] Cell viability, and histamine and cytokine secretions were assessed.

These experiments were carried out in at University College London, under the supervision of Dr. Virginia Calder. The doctoral candidate's stay was supported by “ayudas para estancias breves en el desarrollo de tesis doctorales” by University of Valladolid in 2012.

VI.4.1. Reagents

Stemspan™ serum-free medium was purchased from StemCell Technologies (Grenoble, France).

Stem cell factor (SCF) and cytokines IL-3 and IL-6 were purchased from Peprotech. FCS was purchased from Labtech. Human IgE was obtained from Abcam (Cambridge, MA, USA). PI and anti-human IgE (ε-chain specific) antibody was obtained from Sigma.

VI.4.2. Preparation of polyphenol solutions

QCT, RES and QCT+RES solutions were prepared as previously described in Section V.1.2.

Stock solutions of QCT and RES were prepared in EtOH, after which serial dilutions were carried out to achieve final concentrations ranging from 0.5-25 µM QCT and 0.5-100 µM RES. Cells treated with each polyphenol alone had a final concentration of 0.5 % EtOH for dose-response curves. Aliquots of each solution were kept at -20 ºC. Concentrations tested for each assay are detailed in Table 6.

Final concentration testing of QCT+RES for preliminary studies (two experiments performed in single samples) were achieved by adding QCT solutions and RES solutions to each sample in order to reach the final concentrations in cultured medium, as indicated in Table 6. The final concentration of vehicle was a non-toxic concentration of 1 % EtOH in the culture medium.

A combined QCT and RES solution for a final experiment, performed in triplicate, was prepared by mixing a QCT solution and a RES solution in order to reach the final concentrations of 10 μM QCT + 25 μM RES, and 0.5 % EtOH as vehicle.

In order to clarify the rationale for the selected concentrations of polyphenols, the cytotoxicity of both compounds was firstly analysed for a range of concentrations (0.5-25 μM QCT and 0.5-100 μM RES, alone and in combination). Once non-toxic concentrations were determined, dose-response curves to QCT (0.5-25 μM) and RES (0.5-100 μM) for histamine release were determined (the same concentrations of QCT and RES were also selected in order to study their effect on cytokine release in a further study). Finally, one concentration of each compound (10 μM QCT and 25 μM RES), which did not significantly decrease histamine release in the dose-response study, was selected in order to analyse the effect of both compounds in combination on histamine release and cytokine secretion.

Measurement	Assay	Time point	Concentration of polyphenol (µM)			
			QCT	RES	Combination	
					QCT	RES
Cytotoxicity	PI (flow cytometry)	24 h	0.5, 1, 5, 10, 15, 20 & 25	0.5, 1, 5, 10, 25, 50 & 100	0.5, 1, 5, 10, 15, 20 & 25	0.5, 1, 5, 10, 25, 50 & 100
Histamine secretion	ELISA kit (absorbance)	1 h	0.5, 1, 5, 10, 15, 20 & 25	0.5, 1, 5, 10, 25, 50 & 100	0.5, 1, 5, 10, 15, 20 & 25	0.5, 1, 5, 10, 25, 50 & 100
Cytokine secretion **(IL-1β, IL-4, IL-5, IL-8/CXCL8, IL-9, IL-10, IL-13, TNF-α & IFN-γ)**	Multiplex-bead based array X-MAP technology (Luminex)	24 h	0.5, 1, 5, 10, 15, 20 & 25	0.5, 1, 5, 10, 25, 50 & 100	10	25

Table 6. Summary of assays and concentrations of polyphenols tested in CBMCs.
(QCT: quercetin; RES: resveratrol; CBMCs: cord blood-derived mast cells; PI: propidium iodide).

VI.4.3. Cord blood-derived mast cells

Commercially available cord blood CD34$^+$ stem cells (Lonza, Wokingham, UK) were cultured as previously described[293] in Stemspan serum-free medium, supplemented with 100 ng/mL SCF and 50 ng/mL IL-6. Additionally, 1 ng/mL IL-3 was added to cultured medium during the first 14 days of culture. From week 8 to 11, cell culture medium was further supplemented with 10 % FCS. After 11 weeks of culture, cells were characterised [CD117$^+$ (c-kit); > 90 % tryptase-positive, > 90 % chymase-positive] using a FACSCalibur flow cytometer (BD) and used for experiments between 11 and 16 weeks of culture.

VI.4.4. Sensitisation and activation of mast cells

CBMCs were activated by cross-linking surface IgE with an anti-IgE antibody, as previously described.[292,293] CBMCs were seeded in 96-well plates at a density of $2x10^5$ cells per well in 200 µL of Stemspan medium supplemented with 100 ng/mL SCF. Cells were then pre-sensitised by adding human IgE (4 µg/mL) and were incubated for 18 h at 37 °C. Cells not exposed to IgE were used as a control. After that, anti-human IgE antibody (50 µg/mL) was added to IgE-sensitised cells and further incubated for 24 h at 37 ºC.

VI.4.5. Polyphenol treatments

After 18 h of pre-sensitisation with IgE, QCT, RES, QCT+RES or vehicle were added to samples and cells were then incubated for 30 min prior to add anti-IgE. Cell-free supernatants were collected at 1 h and 24 h after anti-IgE activation and stored at -80 °C for subsequent analysis (Table 6).

VI.4.6. Cytotoxicity assay

In order to determine the non-toxic concentration of polyphenols on CBMCs, cell toxicity after exposure to different concentrations of QCT, RES or QCT+RES (see Table 6 for more details) and vehicle were assessed 24 h after IgE/anti-IgE activation by PI staining using flow cytometry, as previously described in Section V.2.5. Briefly, 24 h after sensitisation/activation of CBMCs and polyphenol treatments, 5 µg/mL PI was added to cells and immediately afterwards cells were acquired using a FACSCalibur flow cytometer (BD). At least 15,000 events were analysed using CellQuest Pro software (BD). Samples were previously gated on FS versus SS to exclude debris and

clumps. All cells were further gated to assess level of viable cells (PI-negative). One experiment was carried out and samples were performed in triplicate for testing polyphenols individually and in single samples for testing polyphenol mixtures.

VI.4.7. Histamine release

The concentration of histamine was measured in cell-free supernatants after 1 h of IgE/anti-IgE activation, using a commercial enzyme-linked immunosorbent assay (ELISA) kit (IBL, Hamburg, Germany). The assay was conducted following the manufacturer's instructions. First, 50 µL of supernatants and standards were acylated and diluted before being placed into plate. Then, peroxidase-conjugated histamine and histamine antiserum were added to each sample. The plate was incubated for 3 h, washed and developed with tetramethylbenzidine solution. Finally, absorbance was measured at 450 nm using a microplate reader (Titertek™, Vienna, Austria). Dose-response curves were obtained from two experiments performed in single samples. A further experiment were performed in triplicate for testing the effect of QCT and RES combination (10 µM QCT + 25 µM RES) on histamine secretion.

VI.4.8. Cytokine secretion

IL-1β, IL-4, IL-5, IL-8/CXCL8, IL-9, IL-10, IL-13, TNF-α and IFN-γ levels were determined in cell supernatants after 24 h using Luminex bead technology with a commercial Milliplex 9-plex human cytokine/chemokine immunobead-based assay (HCYTOMAG, Millipore), as previously described in Section V.1.7. The minimum detectable levels for each cytokine/chemokine were 0.8 pg/mL for IL-1β, 4.5 pg/mL for IL-4, 0.5 pg/mL for IL-5, 0.4 pg/mL for IL-8/CXCL8, 1.2 pg/mL for IL-9, 1.1 pg/mL for IL-10, 1.3 pg/mL for IL-13, 0.7 pg/mL for TNF-α and 0.8 pg/mL for IFN-γ. When a cytokine level was not detectable, the minimum detectable level was used in the analysis. Dose-response curves were obtained from two experiments performed in single samples. A further experiment were performed in triplicate for testing the effect of QCT and RES combination (10 µM QCT + 25 µM RES) on cytokine secretion.

VI.5. Statistical Analysis

For in-vitro experiments, all data were expressed as mean ± standard error of the mean (SEM). Statistics were analysed using the SPSS software package (SPSS version 15.0 for Windows, SPSS Inc., Chicago, IL, USA). Homogeneity of variances was analysed using Levene's test. Cytotoxicity, cytokine secretion, COX-2 expression and intracellular ROS production data from IOBA-NHC and HCE cells were analysed using the *t*-test or *t*-test with Welch correction for comparison of unstimulated cells versus stimulated cells, and one-way analysis of variance (ANOVA) with Dunnett's post-hoc test or Games-Howell test for intergroup comparisons. Cytotoxicity, and proliferation and viability data from PBMCs were analysed using the *t*-test or *t*-test with Welch correction. Cytotoxicity, histamine secretion and cytokine/chemokine production from CBMCs were analysed using the *t*-test or *t*-test with Welch correction.

For the in-vivo model of DED and adoptive transfer model, data were expressed as median ± interquartile range (the difference between the 25th and 75th percentiles) using Tukey's method for plotting the whiskers and outliers. The Mann-Whitney test was used for statistical comparisons (outliers obtained from Tukey´s method were included in the statistical analysis).

Two-sided p-values equal to or less than 0.05 were considered statistically significant.

VI. Results

Results have been divided into four sections following the same scheme of the chapter “material and methods” chapter:

1. In-vitro model of inflammation and oxidative stress in human conjunctival and corneal epithelial cells.
2. In-vitro proliferative response of human T cells.
3. Experimental murine model of DED.
4. In-vitro model of conjunctival mast cell response.

VI.1. In-vitro model of inflammation and oxidative stress in human conjunctival and corneal epithelial cells

This section summarises all data obtained from testing the cytotoxicity, anti-inflammatory and antioxidant effects of polyphenols on conjunctival and corneal epithelial cells.

VI.1.1. Cytotoxicity of polyphenols

The cytotoxicity of QCT, RES and QCT+RES in conjunctival and corneal epithelial cells after 24 h exposure was tested using the alamarBlue® assay.

Prior to testing polyphenols in both epithelial cell lines, it was verified that the vehicle used for polyphenol solubilisation (0.5 % EtOH) did not have any effect on conjunctival and corneal epithelial cells (as shown in Figure 15).

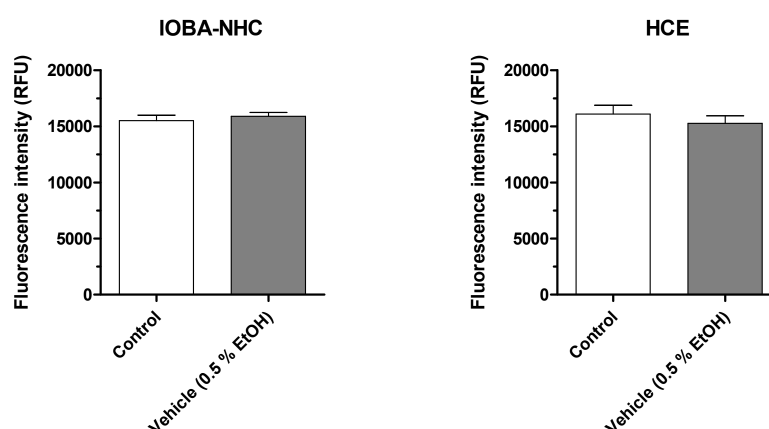

Figure 15. Effect of 0.5 % ethanol (EtOH) on both IOBA-NHC and HCE cells.
N=3.

VI.1.1.1. Cytotoxicity in IOBA-NHC cells

Figure 16 (A-C) shows the fluorescence intensity, in relative fluorescence units (RFU), for IOBA-NHC cells treated with QCT, RES and QCT+RES. QCT did not decrease IOBA-NHC cell viability at any concentration tested (1, 5 10, 15, 20 and 25 µM) compared to vehicle (Figure 16A). Moreover, a significant increase in fluorescence intensity was found at 20 µM QCT ($p<0.05$). Regarding RES concentrations tested (1, 5, 10, 25, 50, 100 and 300 µM), only 300 µM RES provoked a significant decrease of cell viability ($p<0.05$), compared to vehicle (Figure 16B). IOBA-NHC cells were also treated with 15, 20 and 25 µM QCT in combination with each 25, 50 and 100 µM RES concentrations. There was a significant decrease in fluorescence intensity when HCE

cells were treated with 25 μM QCT + 25 μM RES ($p<0.01$), 20 μM QCT + 50 μM RES ($p<0.05$), 15 μM QCT + 100 μM RES ($p<0.05$) and 20 μM QCT + 100 μM RES ($p<0.05$; Figure 16C).

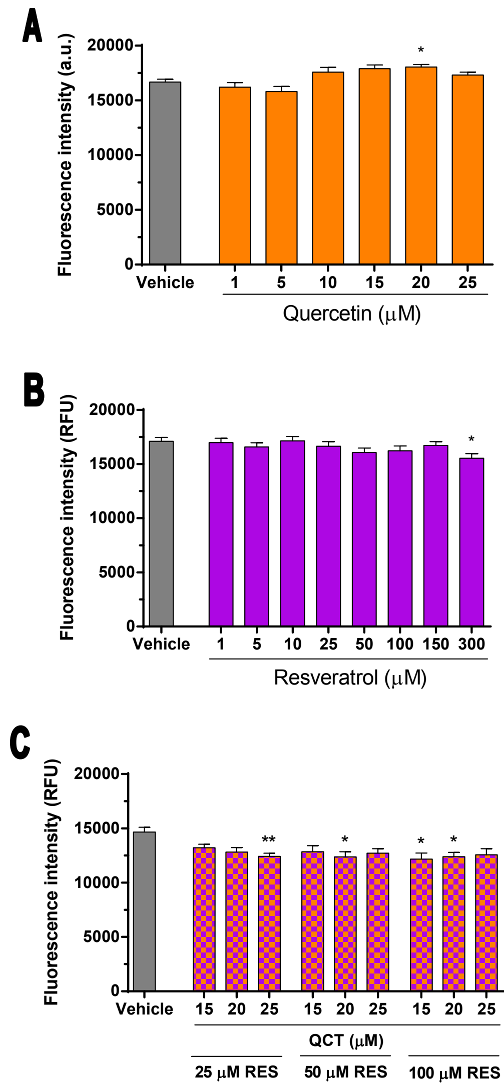

Figure 16. Cytotoxicity effect of quercetin (QCT) (A), resveratrol (RES) (B) and their combinations (C) on IOBA-NHC cell line. *$p<0.05$, **$p<0.01$, compared to vehicle (0.5 % ethanol). N=3.

VI.1.1.2. Cytotoxicity in HCE cells

Figure 17 (A-C) shows the fluorescence intensity for HCE cells treated with QCT, RES and QCT+RES. QCT did not affect HCE cell viability at any concentration tested (1, 5 10, 15, 20 and 25 μM), compared to vehicle (Figure 17A). When HCE cells were treated with RES (1, 5, 10, 25, 50, 100 and 300 μM), cell viability only decreased at 300 μM RES ($p<0.001$), compared to vehicle (Figure 17B). No combination of 15, 20 and 25 μM QCT with each of 25, 50 and 100 μM RES was toxic for HCE cells (Figure 17C).

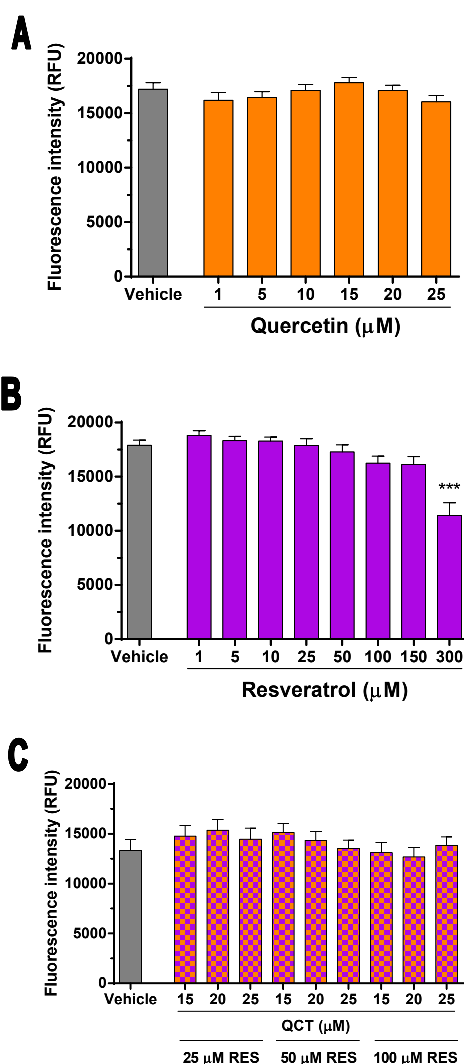

Figure 17. Cytotoxicity effect of quercetin (QCT) (A), resveratrol (RES) (B) and their combinations (C) on HCE cell line. ***p<0.001, compared to vehicle (0.5 % ethanol). N=3.

VI.1.2. Effect of Quercetin and Resveratrol on cytokine/chemokine secretion induced by TNF-α

After determining the non-toxic concentrations of QCT, RES and their combinations, the anti-inflammatory effect of polyphenols were studied. For this purpose, secreted IL-6, IL-8/CXCL8, IP-10/CXCL10 and VEGF dose-response curves were obtained for QCT (0.5, 1, 5, 10, 15, 20 and 25 µM) and RES (0.5, 1, 5, 10, 25 and 50 µM) in conjunctival and corneal epithelial cells stimulated with 25 ng/mL TNF-α.

After that, two concentrations of polyphenols (0.5 µM QCT and 5 µM RES), which did not have a significant effect on cytokine/chemokine secretion in dose-response curves, were chosen in order to determine the effect of both compounds together. Cytokine/chemokine secretion by both cell lines was determined using a multiplex-bead based array.

For a better comprehension of all data presented herein, results from polyphenols-treated IOBA-NHC cells are firstly presented, followed thereafter by the results from polyphenols-treated HCE cells. For each cell type, results are also subdivided into four sections, according to the cytokines under study: I) IL-6, II) IL-8/CXCL8, III) IP-10/CXCL10 and IV) VEGF.

VI.1.2.1. Cytokine/chemokine secretion by IOBA-NHC cells

VI.1.2.1.1. IL-6 secretion

Figure 18 (A-C) shows the effect of QCT, RES and their combination on IL-6 secretion by TNF-α-stimulated IOBA-NHC cells. Stimulation of IOBA-NHC cells with TNF-α induced a significant increase of IL-6 ($p<0.01$), compared to control cells. This TNF-α-induced IL-6 secretion decreased in a dose-dependent manner when IOBA-NHC cells were treated with 15, 20 and 25 µM QCT ($p<0.05$; $p<0.01$ and $p<0.01$, respectively; Figure 18A), reducing IL-6 to almost basal levels. There was also a decrease in TNF-α-induced IL-6 secretion when IOBA-NHC cells were treated with 25 and 50 µM RES ($p<0.05$ and $p<0.01$, respectively, Figure 18B). When 0.5 µM QCT and 5 µM RES were mixed and tested in IOBA-NHC cells, there was an inhibitory effect of 0.5 µM QCT + 5 µM RES ($p<0.01$), which was stronger than that observed for 0.5 µM QCT ($p<0.05$) and 5 µM RES ($p>0.05$) individually (Figure 18C).

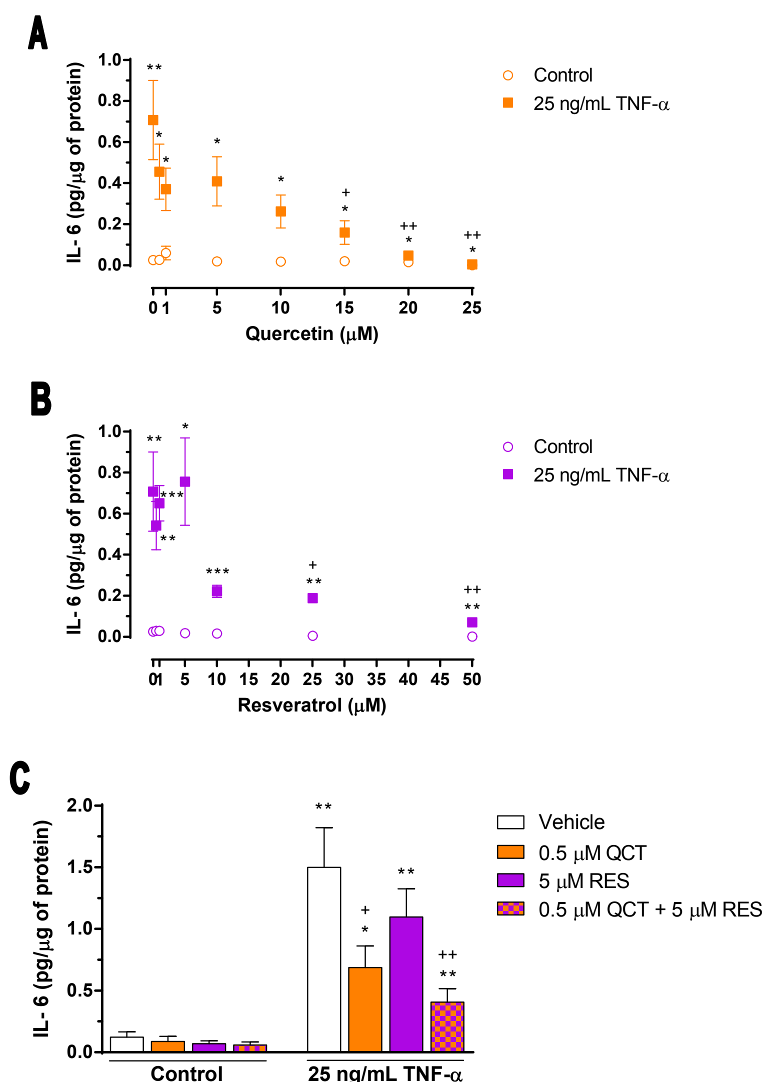

Figure 18. Effect of quercetin (QCT) (A), resveratrol (RES) (B) and their combination (C) on IL-6 secretion by IOBA-NHC cells. *p<0.05, **p<0.01, ***p<0.001, compared to control cells; +p<0.05, ++p<0.01, compared to vehicle-treated stimulated cells (0.5 % ethanol). N=3.

VI.1.2.1.2. IL-8/CXCL8 secretion

Figure 19 (A-C) shows the effect of QCT, RES and their combination on IL-8/CXCL8 secretion by TNF-α-stimulated IOBA-NHC cells. Stimulation of IOBA-NHC cells with TNF-α induced a significant increase of IL-8/CXCL8 (p<0.001), compared to control cells. When cells were treated with polyphenols, this stimulated secretion of IL-8/CXCL8 decreased at 20 and 25 µM QCT (p<0.05 and p<0.01, respectively, Figure 19A), whilst RES treatment decreased TNF-α-induced IL-8/CXCL8 secretion only at 50 µM (p<0.05; Figure 19B). The mix of both compounds also significantly decreased IL-

8/CXCL8 production (p<0.05), but this decrease was similar to that found in cells treated with 0.5 µM QCT (p<0.05; Figure 19C).

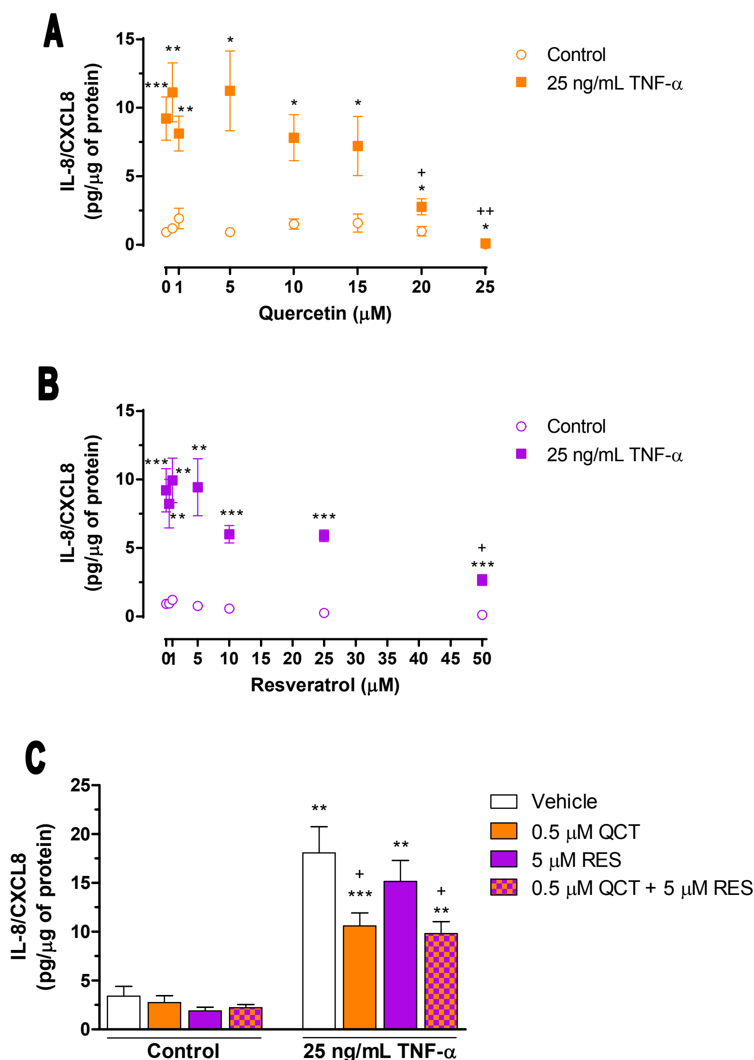

Figure 19. Effect of quercetin (QCT) (A), resveratrol (RES) (B) and their combination (C) on IL-8/CXCL8 secretion by IOBA-NHC cells. *p<0.05, **p<0.01, ***p<0.001, compared to control cells; $^{+}$p<0.05, $^{++}$p<0.01, compared to vehicle-treated stimulated cells (0.5 % ethanol). N=3.

VI.1.2.1.3. IP-10/CXCL10 secretion

The effect of QCT, RES and QCT+RES on IP-10/CXCL10 secreted by TNF-α-treated IOBA-NHC cells is shown in Figure 20 (A-C). TNF-α provoked an increase of IP-10/CXCL10 secretion (p<0.01). As shown in Figure 20A, this increase of IP-10/CXCL10 secretion was attenuated by 10 µM QCT (p<0.01), and decreased to basal levels at 15, 20 and 25 µM QCT (all p<0.01, compared to TNF-α-stimulated cells). RES

decreased IP-10/CXCL10 at concentrations of 10, 25 and 50 μM (all $p<0.05$) compared to stimulated cells (Figure 20B), but not down to basal levels. No significant differences in IP-10/CXCL10 levels were found when IOBA-NHC cells were treated with 0.5 μM QCT + 5 μM RES with respect to stimulated cells (Figure 20C).

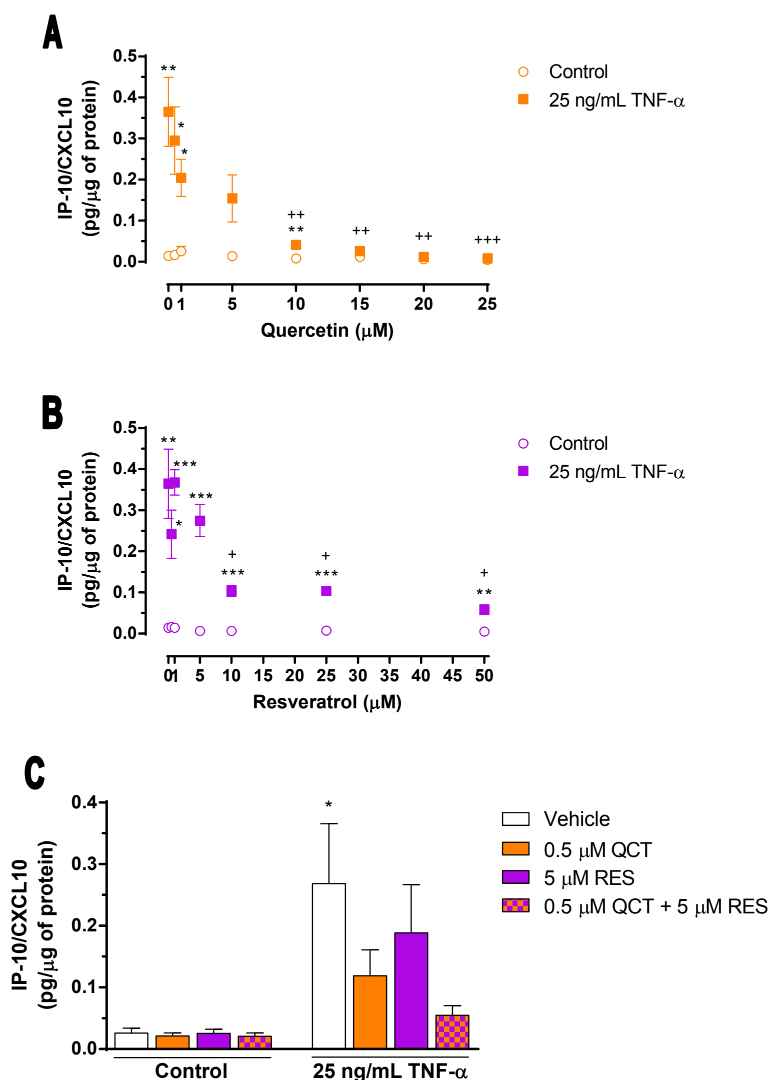

Figure 20. Effect of quercetin (QCT) (A), resveratrol (RES) (B) and their combination (C) on IP-10/CXCL10 secretion by IOBA-NHC cells. *$p<0.05$, **$p<0.01$, ***$p<0.001$, compared to control cells; $^{+}p<0.05$, $^{++}p<0.01$, $^{+++}p<0.001$, compared to vehicle-treated stimulated cells (0.5 % ethanol). N=3.

VI.1.2.1.4. VEGF secretion

Figure 21 (A-C) shows the effect of QCT, RES and 0.5 μM QCT + 5 μM RES on VEGF secretion by IOBA-NHC cells. TNF-α did not induce VEGF production by IOBA-NHC cells. None of the QCT and RES concentrations had a significant effect on VEGF secretion in dose-response curves (Figure 21 A-B). Although 0.5 μM QCT showed a lower level of VEGF secretion (but not significant compared to control, Figure 21A), it was significant ($p<0.01$) when the QCT+RES combination was further tested, as shown in Figure 21C. In addition, the combination 0.5 μM QCT + 5 μM RES decreased VEGF secretion in TNF-α-stimulated conjunctival epithelial cells ($p<0.01$), compared to vehicle-treated stimulated cells (Figure 21C).

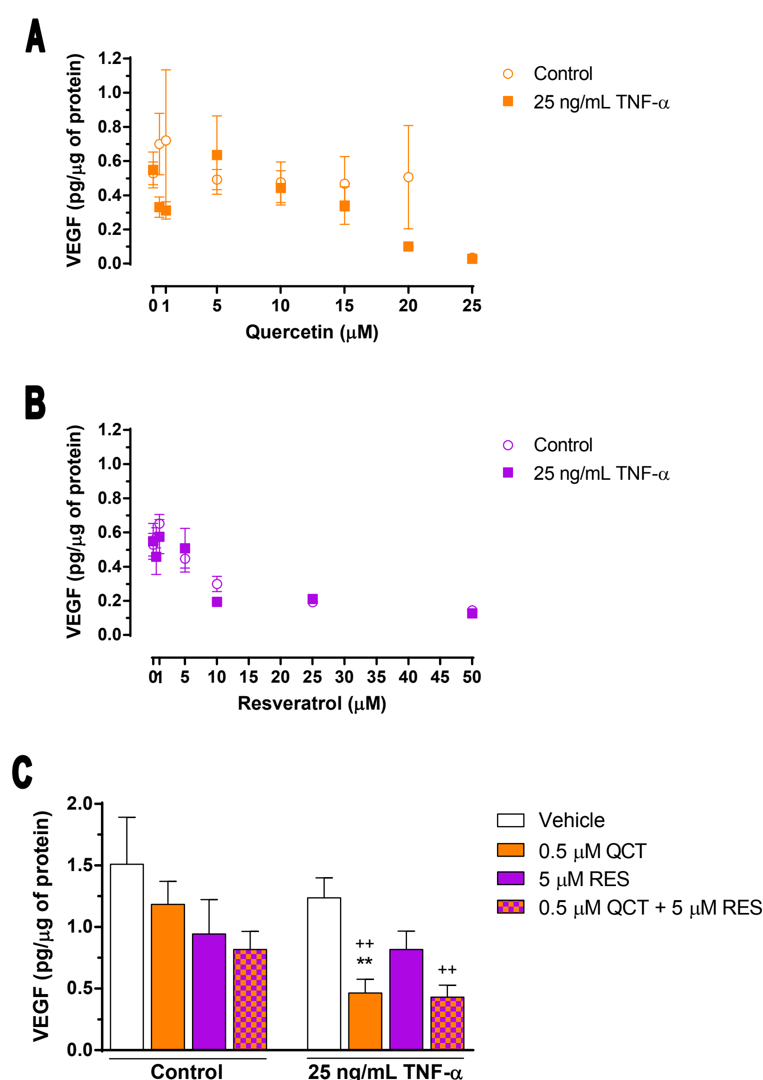

Figure 21. Effect of quercetin (QCT) (A), resveratrol (RES) (B) and their combination (C) on VEGF secretion by IOBA-NHC cells. $^{**}p<0.01$, compared to control cells; $^{++}p<0.01$, compared to vehicle-treated stimulated cells (0.5 % ethanol). N=3.

VI.1.2.2. Cytokine/chemokine secretion by HCE cells

VI.1.2.2.1. IL-6 secretion

The effects of QCT, RES and their combination on IL-6 secretion by HCE cells are shown in Figure 22 (A-C). In these cells, TNF-α induced a significant increase of IL-6 secretion ($p<0.001$). When TNF-α-stimulated cells were treated with 1 µM QCT, IL-6 secretion was significantly decreased ($p<0.01$). Moreover, concentrations of 5, 10, 15, 20 and 25 µM QCT strongly reduced IL-6 production, with all concentrations showing similar inhibitory effects (all $p<0.001$; Figure 22A). There was a decrease of IL-6 secretion in a dose-dependent manner when HCE cells were treated with 10, 25 and 50 µM RES (all $p<0.001$; Figure 22B). However, the combination of QCT and RES did not have a significant effect on TNF-α-induced IL-6 secretion (Figure 22C).

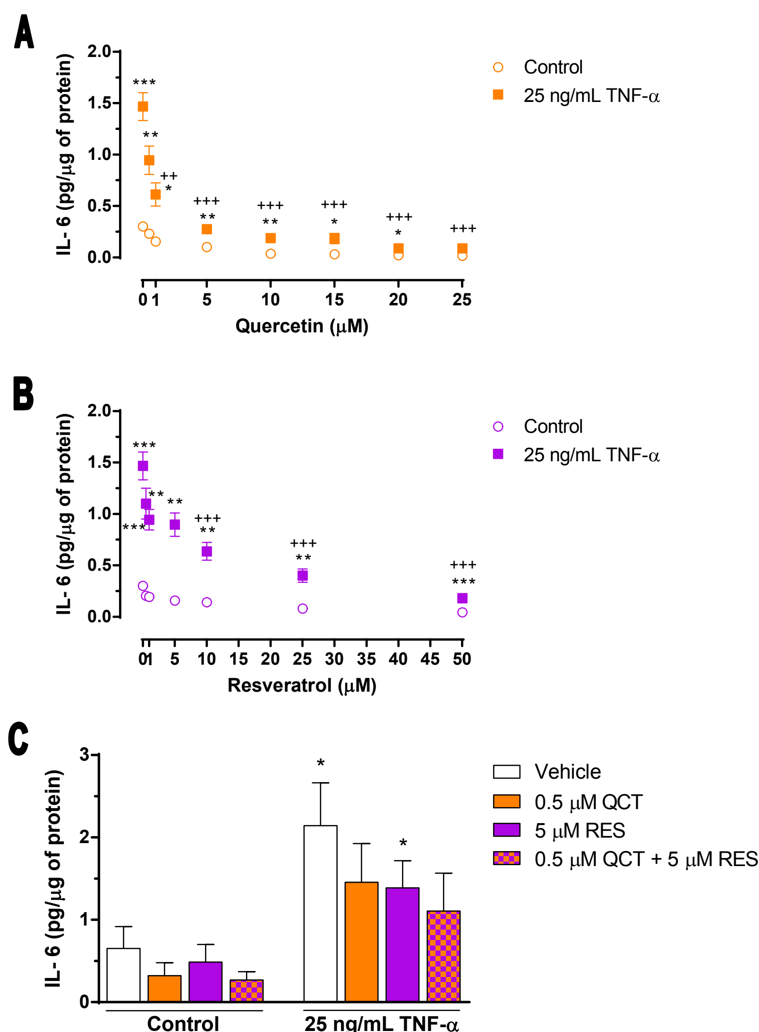

Figure 22. Effect of quercetin (QCT) (A), resveratrol (RES) (B) and their combination (C) on IL-6 secretion by HCE cells. *$p<0.05$, **$p<0.01$, ***$p<0.001$, compared to control cells; ++$p<0.01$, +++$p<0.001$, compared to vehicle-treated stimulated cells (0.5 % ethanol). N=3.

VI.1.2.2.2. IL-8/CXCL8 secretion

The effect of QCT, RES and their combination on IL-8/CXCL8 secretion by TNF-α-stimulated HCE cells is shown in Figure 23 (A-C). Stimulation of HCE cells with TNF-α induced a significant increase of IL-8/CXCL8 chemokine ($p<0.001$). When HCE cells were treated with polyphenols, TNF-α-stimulated IL-8/CXCL8 secretion was significantly decreased at 5 µM QCT ($p<0.05$). This effect was found to be stronger at 10, 15, 20 and 25 µM QCT ($p<0.01$; Figure 23A). Different results were found for RES treatments, because only the highest concentration used (50 µM) significantly decreased IL-8/CXCL8 secretion ($p<0.05$; Figure 23B). The mix of both compounds did not have any effect on TNF-α-stimulated IL-8/CXCL8 secretion (Figure 23C).

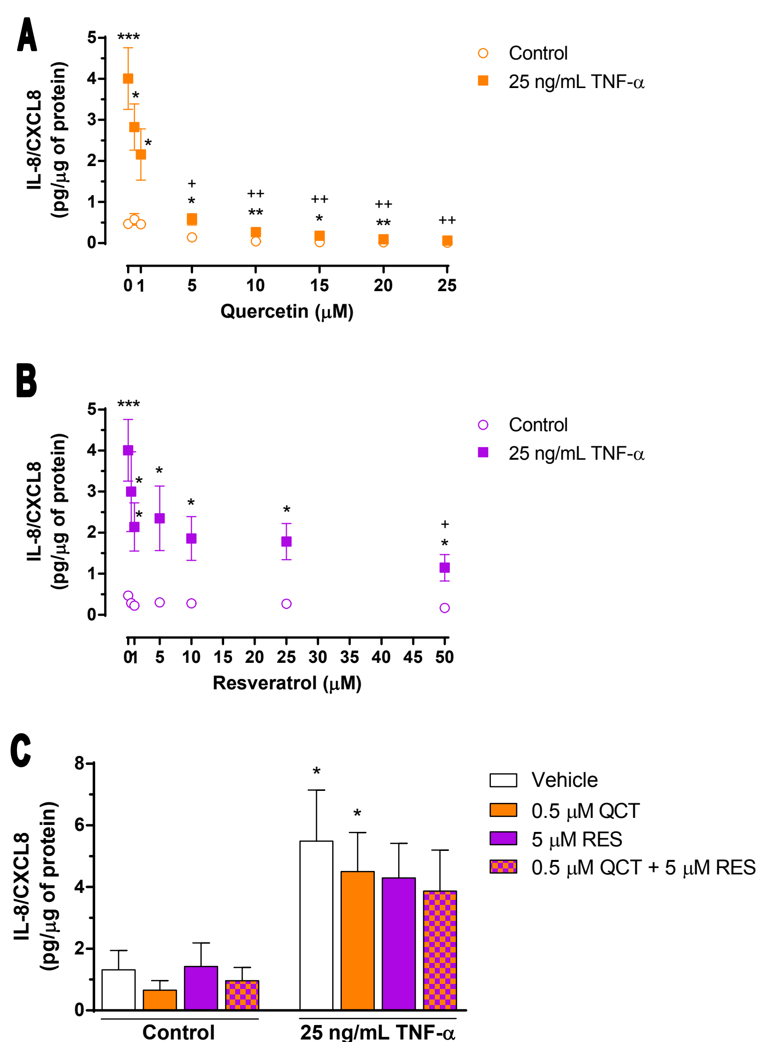

Figure 23. Effect of quercetin (QCT) (A), resveratrol (RES) (B) and their combination (C) on IL-8/CXCL8 secretion by HCE cells. *$p<0.05$, **$p<0.01$, ***$p<0.001$, compared to control cells; $^{+}p<0.05$, $^{++}p<0.01$, compared to vehicle-treated stimulated cells (0.5 % ethanol). N=3.

VI.1.2.2.3. IP-10/CXCL10 secretion

Figure 24 (A-C) shows the effect of QCT, RES and their combination on TNF-α-induced IP-10/CXCL10 secretion by HCE cells. TNF-α provoked an increase of IP-10/CXCL10 secretion (p<0.01). As shown in Figure 24A, IP-10/CXCL10 secretion by TNF-α-stimulated HCE cells was attenuated by QCT at 1 μM (p<0.05), and by 5, 10, 15, 20 and 25 μM QCT, which significantly (all p<0.001) reduced chemokine secretion down to unstimulated levels. RES decreased IP-10/CXCL10 secretion stimulated by TNF-α at all concentrations tested: 0.5 and 1 μM (p<0.05), and 5, 10, 25 and 50 μM (p<0.01); however only 50 μM RES decreased IP-10/CXCL10 to basal levels (Figure 24B). No significant differences in IP-10/CXCL10 levels were found when IOBA-NHC cells were treated with 0.5 μM QCT + 5 μM RES (Figure 24C).

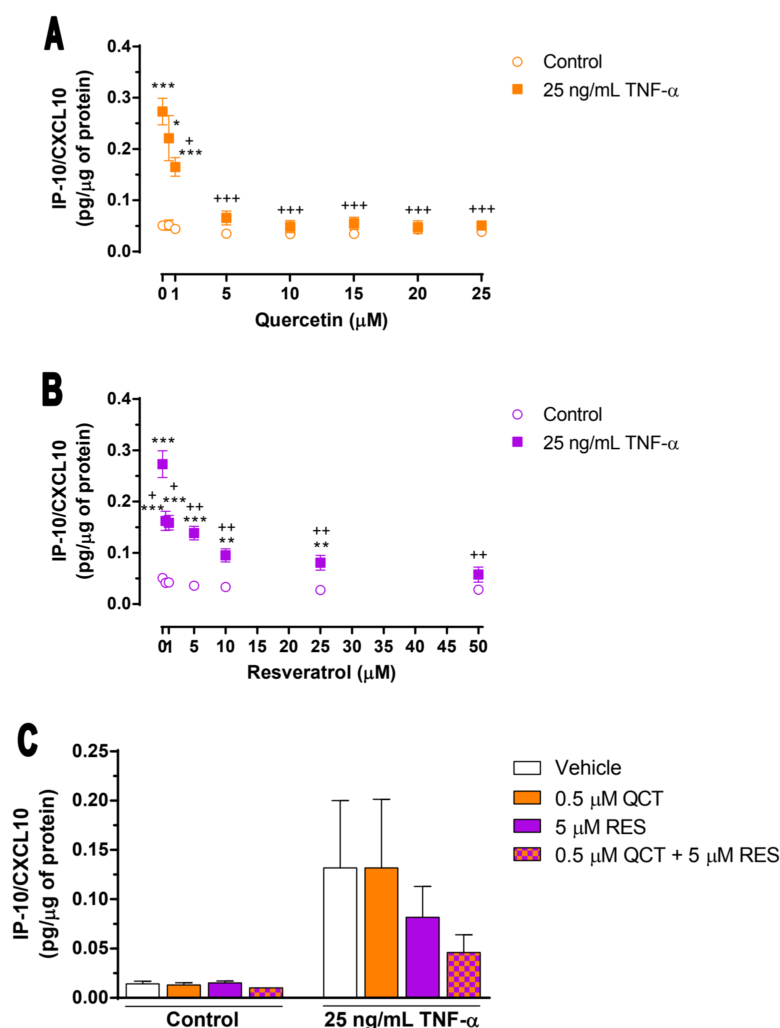

Figure 24. Effect of quercetin (QCT) (A), resveratrol (RES) (B) and their combination (C) on IP-10/CXCL10 secretion by HCE cells. *p<0.05, **p<0.01, ***p<0.001, compared to control cells; $^{+}$p<0.05, $^{++}$p<0.01, $^{+++}$p<0.001, compared to vehicle-treated stimulated cells (0.5 % ethanol). N=3.

VI.1.2.2.4. VEGF secretion

Figure 25 (A-C) shows the effect of QCT, RES and 0.5 μM QCT + 5 μM RES on VEGF secretion by HCE cells. TNF-α did not induce VEGF production in HCE cells. Neither QCT, nor RES, nor their combination had an effect on VEGF secretion by corneal epithelial cells (Figures 25 A, B and C).

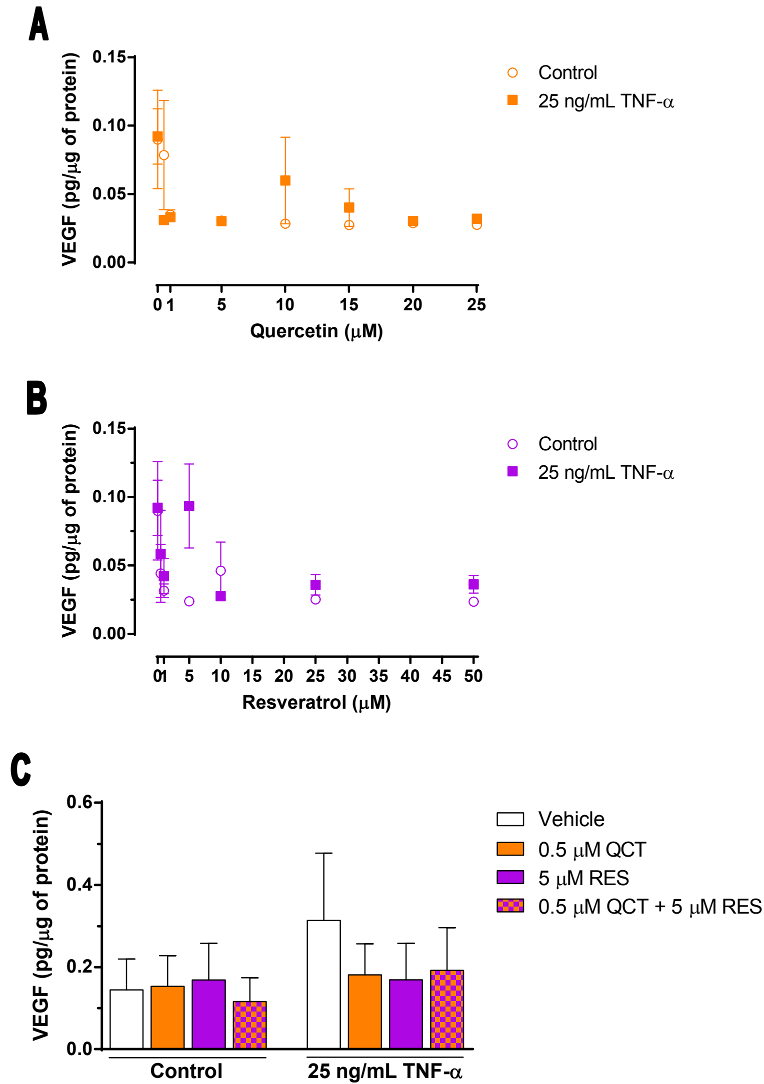

Figure 25. Effect of quercetin (QCT) (A), resveratrol (RES) (B) and their combination (C) on VEGF secretion by HCE cells. N=3.

VI.1.3. Effect of Quercetin and Resveratrol on COX-2 expression

The effect of QCT and RES on COX-2 expression was examined in order to investigate their underlying mechanisms on TNF-α-induced inflammation in both epithelial cell lines. In the present study, both IOBA-NHC and HCE cells were stimulated with 25 ng/mL TNF-α and treated with 0.5 and 25 μM QCT, 5 and 50 μM RES, and the polyphenol mixture (0.5 μM QCT + 5 μM RES). COX-2 expression was analysed by SDS-PAGE Western blotting. All data were normalised to GAPDH expression.

VI.1.3.1. COX-2 expression by IOBA-NHC cells

Figure 26A shows the effect of polyphenols on TNF-α-induced COX-2 expression by IOBA-NHC cells, normalised to GAPDH expression. TNF-α caused an increase of COX-2 expression ($p<0.001$), compared to unstimulated cells. None of the polyphenol treatments had any effect on COX-2 expression, but 25 μM QCT produced an increase of COX-2 expression in both unstimulated and stimulated cells. Data are also presented as fold-change (Figure 26B). TNF-α produced a 5-fold increase of COX-2 expression. Both concentrations of RES (5 and 50 μM) produced a 4-fold decrease of COX-2 expression, whereas 0.5 and 25 μM QCT induced a 3-fold and 2.5-fold decrease, respectively. The polyphenol mixture (0.5 μM QCT + 5 μM RES) demonstrated a stronger decrease in COX-2 expression (2.3-fold) than both polyphenols alone.

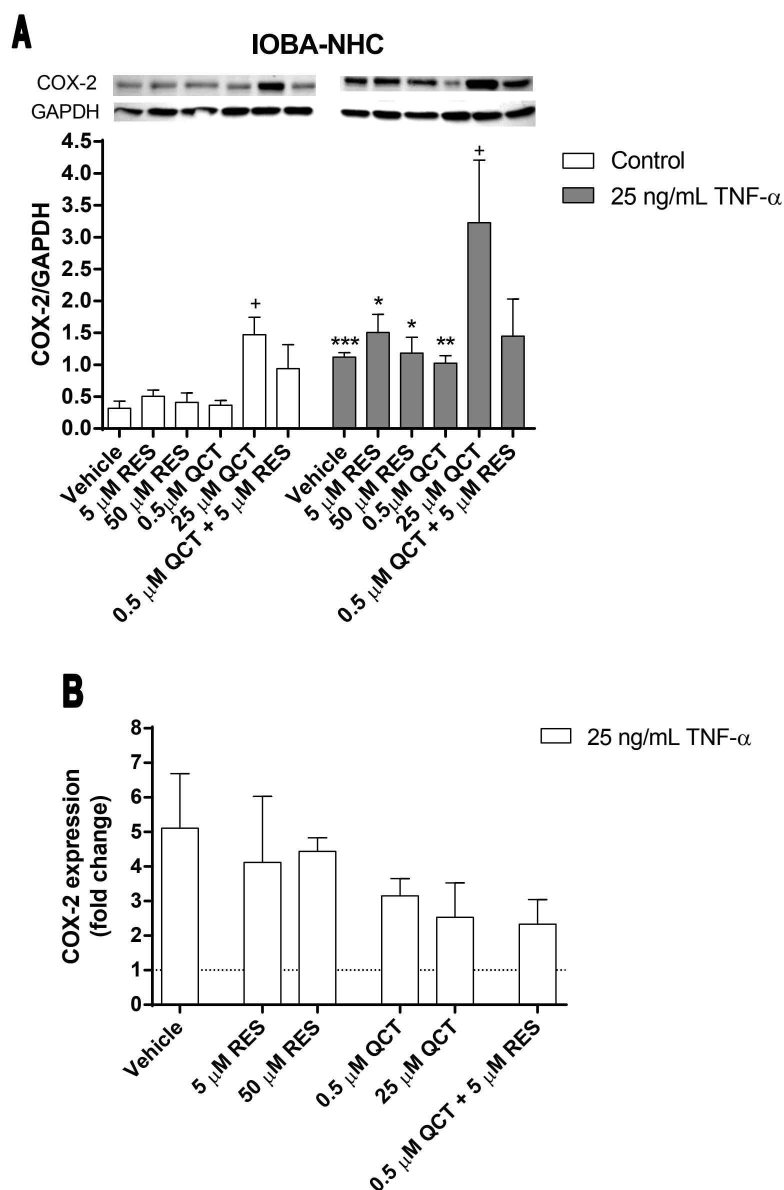

Figure 26. Effect of quercetin (QCT), resveratrol (RES) and their combination on COX-2 secretion by IOBA-NHC cells. *p<0.05, **p<0.01, ***p<0.001, compared to control cells; +p<0.05, compared to cells treated with vehicle (0.5 % ethanol). Dashed line represents control cells. A representative Western blot image is shown in Figure A. N=2.

VI.1.3.2. COX-2 expression by HCE cells

Figure 27A shows the effect of polyphenols on TNF-α-induced COX-2 expression by HCE cells, normalised to GAPDH expression. TNF-α did not produce an increase of COX-2 expression. Neither RES treatments nor 0.5 μM QCT produced a significant decrease of COX-2 expression. Although 25 μM QCT induced a significant decrease of COX-2 (p<0.05), compared to vehicle-treated stimulated cells. Treatment with 0.5 μM QCT + 5 μM RES did not affect significantly COX-2 expression. Data

presented as fold-changes are shown in Figure 27B. TNF-α (vehicle) produced a 2-fold increase of COX-2 expression. Both RES treatments (5 and 50 μM) produced a 1-fold decrease. The 0.5 μM QCT concentration produced a 1.5-fold decrease, whilst 25 μM QCT induced a 0.2-fold decrease of COX-2 expression. The mix of polyphenols (0.5 μM QCT + 5 μM RES) decreased COX-2 expression up to 0.4-fold (Figure 27B).

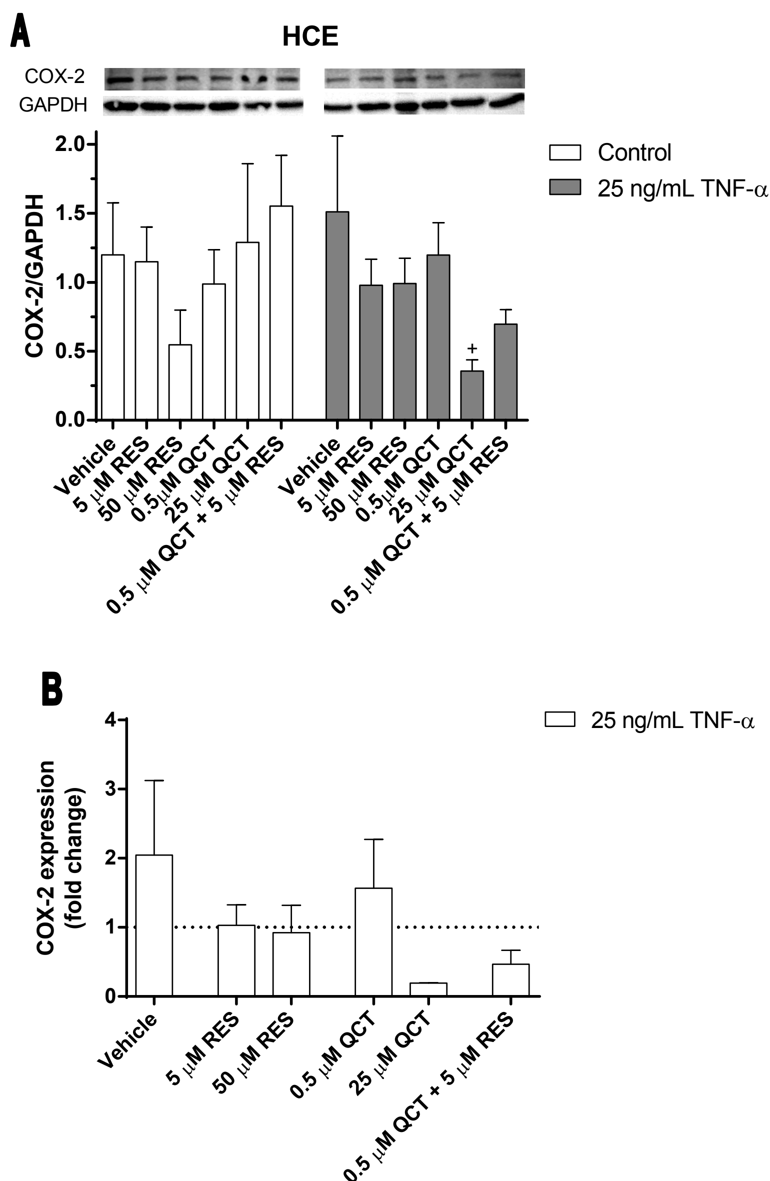

Figure 27. Effect of quercetin (QCT), resveratrol (RES) and their combination on COX-2 secretion by HCE cells. [+]$p<0.05$, compared to cells treated with vehicle (0.5 % ethanol). Dashed line represents control cells. A representative Western blot image is shown in Figure A. N=2.

VI.1.4. Reactive oxygen species induced by ultraviolet B radiation

UV-B is the most common cause of radiation damage to the eye and is a contributor to oxidative damage in cells. As mentioned previously, QCT and RES are well known antioxidants. Moreover, both polyphenols are UV-absorbing compounds. Thus, dose-response curves were firstly obtained to study the effect of QCT and RES on UV-B-induced intracellular ROS, and a combination of low doses of both compounds was further analysed. As in previous sections within this “results chapter”, data from IOBA-NHC cells are firstly presented, followed by results from HCE cells.

VI.1.4.1. Intracellular ROS production by IOBA-NHC cells

UV-B radiation produced a significant increase of intracellular ROS on IOBA-NHC cells after 1 h ($p<0.01$; Figure 28). None of the concentrations tested of QCT (0.5, 1, 5, 10 and 25 μM) significantly decreased intracellular ROS production in cells exposed to UV-B. It should be noted, however, that no significant differences were found between UV-B-exposed cells treated with 1, 5, 10 and 25 μM QCT and control cells (Figure 28A). When UV-B-exposed IOBA-NHC cells were treated with RES, only 50 μM RES significantly decreased intracellular ROS production ($p<0.05$; Figure 28B). In addition, there were no significant differences between exposed and unexposed cells treated with 25 and 50 μM RES (Figure 28B). Similar results were found when cells were treated with a combination of QCT and RES, although there were no significant differences between UV-B-exposed cells treated with vehicle and UV-B-exposed cells treated with 0.5 μM QCT, 5 μM RES or their combination. The latter (polyphenol combination) was not significantly different to its control (Figure 28C).

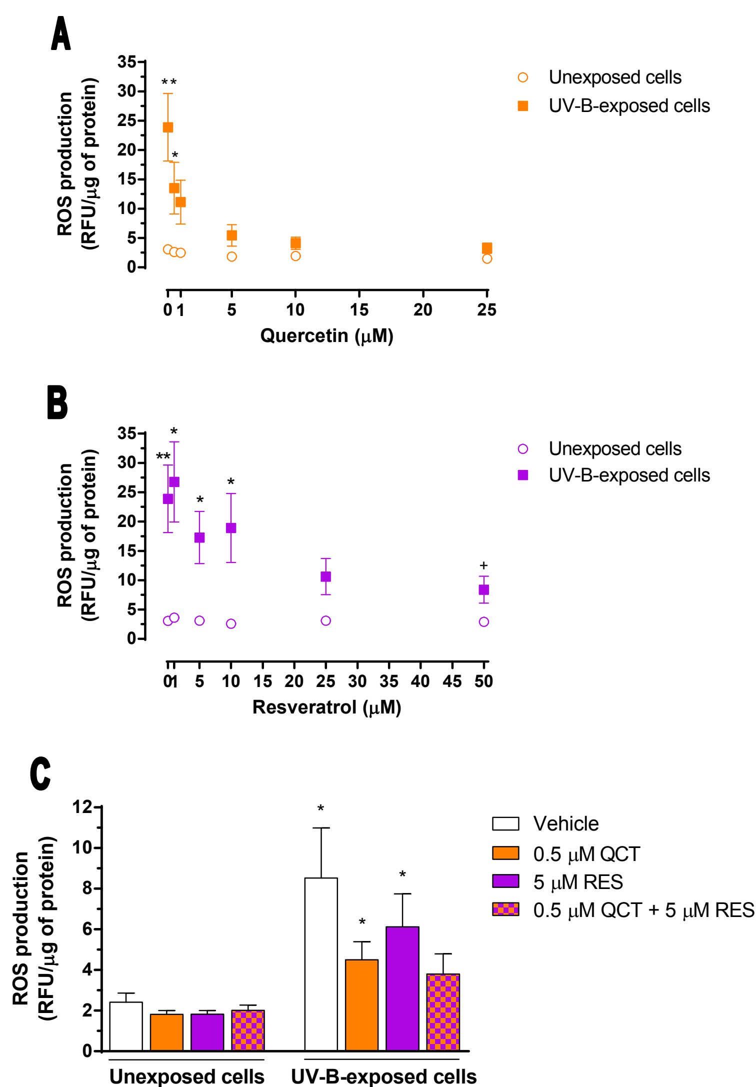

Figure 28. Effect of quercetin (QCT) (A), resveratrol (RES) (B) and their combination (C) on intracellular reactive oxygen species (ROS) induced by UV-B in IOBA-NHC cells. *p<0.05, **p<0.01, compared to unexposed cells; $^{+}$p<0.05, compared to vehicle-treated UV-B-exposed cells (0.5 % ethanol). RFU: relative fluorescence units. N=3.

VI.1.4.2. Intracellular ROS production by HCE cells

When HCE cells were irradiated with UV-B light, intracellular ROS increased dramatically (p<0.001; Figure 29). All doses of QCT tested (0.5, 1, 5, 10 and 25 μM) decreased intracellular ROS production significantly (p<0.01, p<0.05, p<0.001, p<0.001 and p<0.001, respectively; Figure 29A). Regarding RES treatments, 25 and 50 μM RES decreased significantly UV-induced intracellular ROS production (all p<0.001), whilst lower concentrations of RES did not have any effect (Figure 29B). Similar results to those found in IOBA-NHC cells were found in HCE cells treated with a combination QCT and RES, although there was no significant difference between UV-B-exposed

cells treated with 0.5 μM QCT, 5 μM RES or their combination and the vehicle-treated UV-B-exposed cells. The latter (polyphenol combination) was not significantly different to its control (Figure 29C).

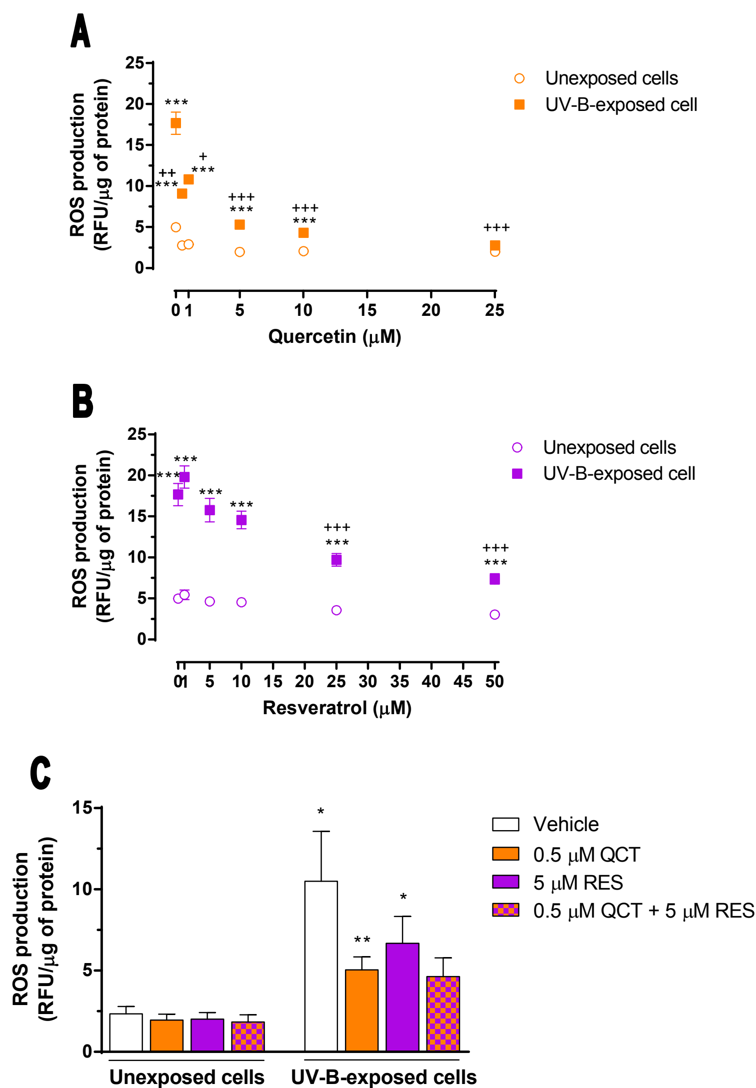

Figure 29. Effect of quercetin (QCT) (A), resveratrol (RES) (B) and their combination (C) on intracellular reactive oxygen species (ROS) induced by UV-B in HCE cells. $^{*}p<0.05$, $^{**}p<0.01$, compared to unexposed cells; $^{+}p<0.05$, $^{++}p<0.01$, $^{+++}p<0.001$, compared to vehicle-treated UV-B-exposed cells (0.5 % ethanol). RFU: relative fluorescence units. N=3.

VI.2. In-vitro proliferative response of human T cells

This section summarises all of the data obtained from testing the immunoregulatory effect of polyphenols on PBMCs from healthy donors.

VI.2.1. Cytotoxicity of polyphenols

The cytotoxicity of QCT, RES and QCT+RES in PBMCs stimulated with anti-CD3/anti-CD28 after 96 h exposure was tested using PI staining and flow cytometry.

Figure 30 summarises all of the data from this assay. Polyphenol concentrations used in this experiment were: 0.5, 1, 5, 10, 15, 20 and 25 μM QCT; 0.5, 1, 5, 10, 25, 50 and 100 μM RES; and the mix of all concentrations of QCT with all concentrations of RES.

PBMCs stimulated with anti-CD3/anti-CD28 antibodies increased significantly viability compared to unstimulated cells (p<0.001; Figure 30).

As it has been pointed out in "material and methods" section, polyphenol treatments have different concentrations of vehicle depending on whether QCT and RES were added separately (0.5 % EtOH) or mixed (1 % EtOH). However, there was not a significant difference in PBMC viability between either concentrations of EtOH in culture medium (Figure 30).

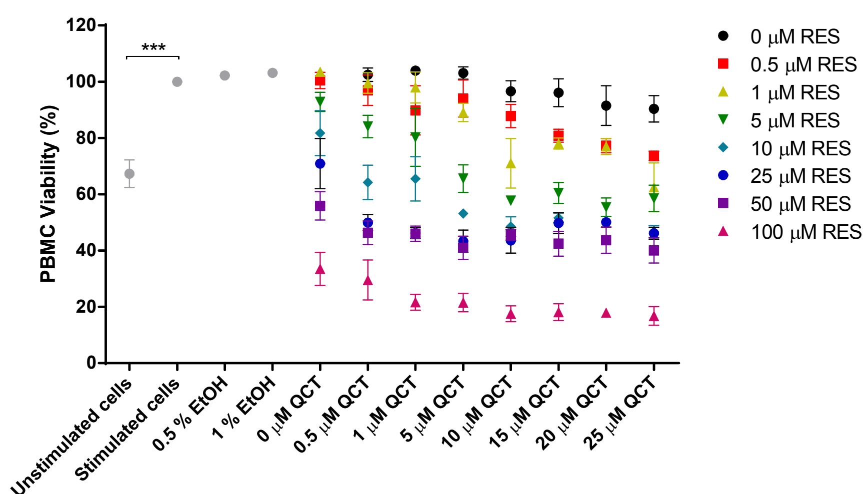

Figure 30. Cytotoxicity of quercetin (QCT), resveratrol (RES) and their combinations in peripheral blood mononuclear cells (PBMCs). ***p<0.001. EtOH: ethanol. N=3.

In order to clarify comparisons between polyphenol treatments, the cytotoxicity data are subdivided as follows: i) cytotoxicity of QCT, ii) cytotoxicity of RES and iii) cytotoxicity of combinations.

VI.2.1.1. Cytotoxicity of QCT

Figure 31 shows cytotoxicity of QCT in PBMCs. Seven concentrations of QCT were tested: 0.5, 1, 5, 10, 15, 20 and 25 μM. None of the QCT concentrations used were toxic for PBMCs, after 96 h of culture.

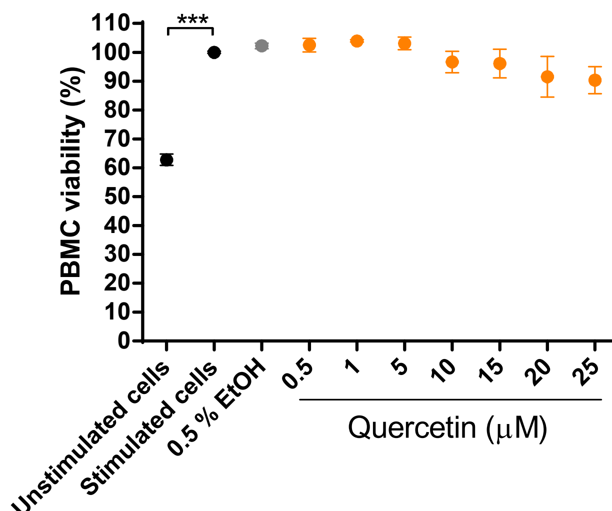

Figure 31. Cytotoxicity of quercetin (QCT) in peripheral blood mononuclear cells (PBMCs). ***p<0.001. EtOH: ethanol. N=3.

VI.2.1.2. Cytotoxicity of RES

Figure 32 shows the cytotoxicity of RES in PBMCs. Seven concentrations of RES were tested: 0.5, 1, 5, 10, 25, 50 and 100 μM. High concentrations of RES (50 and 100 μM) significantly decreased (p<0.05 and p<0.01, respectively) the percentage of viable cells.

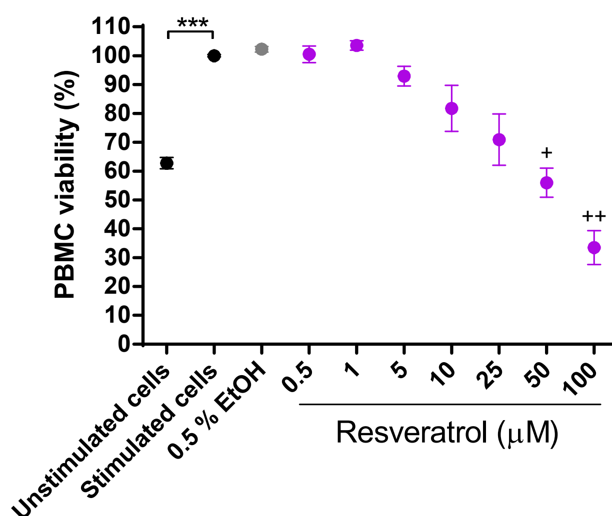

Figure 32. Cytotoxicity of resveratrol (RES) in peripheral blood mononuclear cells (PBMCs). ***p<0.001. $^{+}$p<0.05, $^{++}$p<0.01, compared to PBMCs treated with 0.5 % ethanol (EtOH). N=3.

VI.2.1.3. Cytotoxicity of combination of QCT and RES

Figure 33 shows the cytotoxicity of combinations of QCT and RES in PBMCs and Table 7 shows significant differences for each combination of polyphenols, compared to stimulated cells treated with EtOH. Seven concentrations of QCT (0.5, 1, 5, 10, 15, 20 and 25 µM) and seven concentrations of RES (0.5, 1, 5, 10, 25, 50 and 100 µM) were tested alone and in combination with each other. Cell viability decreased when QCT and RES concentrations increased separately and in combination.

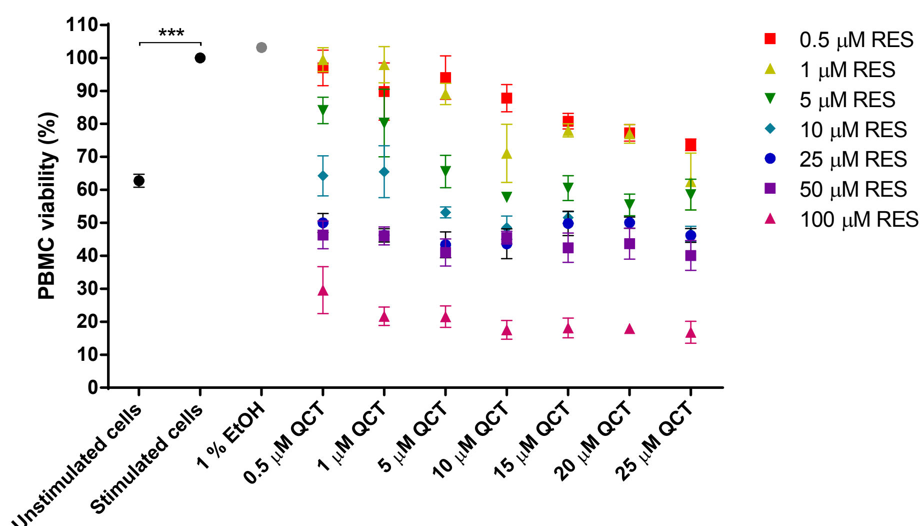

Figure 33. Cytotoxicity of combination of quercetin (QCT) and resveratrol (RES) in peripheral blood mononuclear cells (PBMCs). Data normalised to stimulated cells without treatments. EtOH: ethanol. N=3.

RES (µM)	QCT (µM)						
	0.5	1	5	10	15	20	25
0.5	ns	ns	ns	ns	0.001	0.001	0.001
1	ns	ns	ns	ns	0.001	0.001	0.05
5	ns	ns	0.05	0.001	0.001	0.001	0.05
10	0.05	0.05	0.001	0.001	0.001	0.001	0.001
25	0.001	0.001	0.001	0.001	0.001	0.001	0.001
50	0.001	0.001	0.001	0.001	0.001	0.001	0.001
100	0.001	0.001	0.001	0.001	0.001	0.001	0.001

Table 7. Statistical differences between quercetin (QCT) + resveratrol (RES) combinations and stimulated cells treated with 1 % ethanol. ns: no significant.

VI.2.2. Proliferation of PBMCs

After studying the cytotoxicity effect of polyphenols, it was investigated whether low doses of QCT and RES, and their combination, affect PBMC activation. Cells were activated with anti-CD3/anti-CD28 antibodies and treated with different concentrations of QCT (0.5, 1, 5 and 10 μM), RES (5 and 10 μM) and their combinations, as shown in Figure 34 (A-B). The CFSE proliferation assay (Figure 34A) demonstrated that 5 μM RES in combination with 5 and 10 μM QCT, and 10 μM RES in combination with 0.5, 1, 5 and 10 μM QCT inhibited PBMC activation in a dose-dependent manner (all $p<0.05$). In addition, although this did not reach significance, 10 μM RES alone tended to decrease PBMC proliferation ($p=0.0826$), compared to 0.5 % EtOH (Figure 34A). Moreover, there was not a decrease in the percentage of PI negative cells (Figure 34B), which confirmed that these inhibitory effects were not in any case due to a decrease in cell viability after 120 h of incuabation.

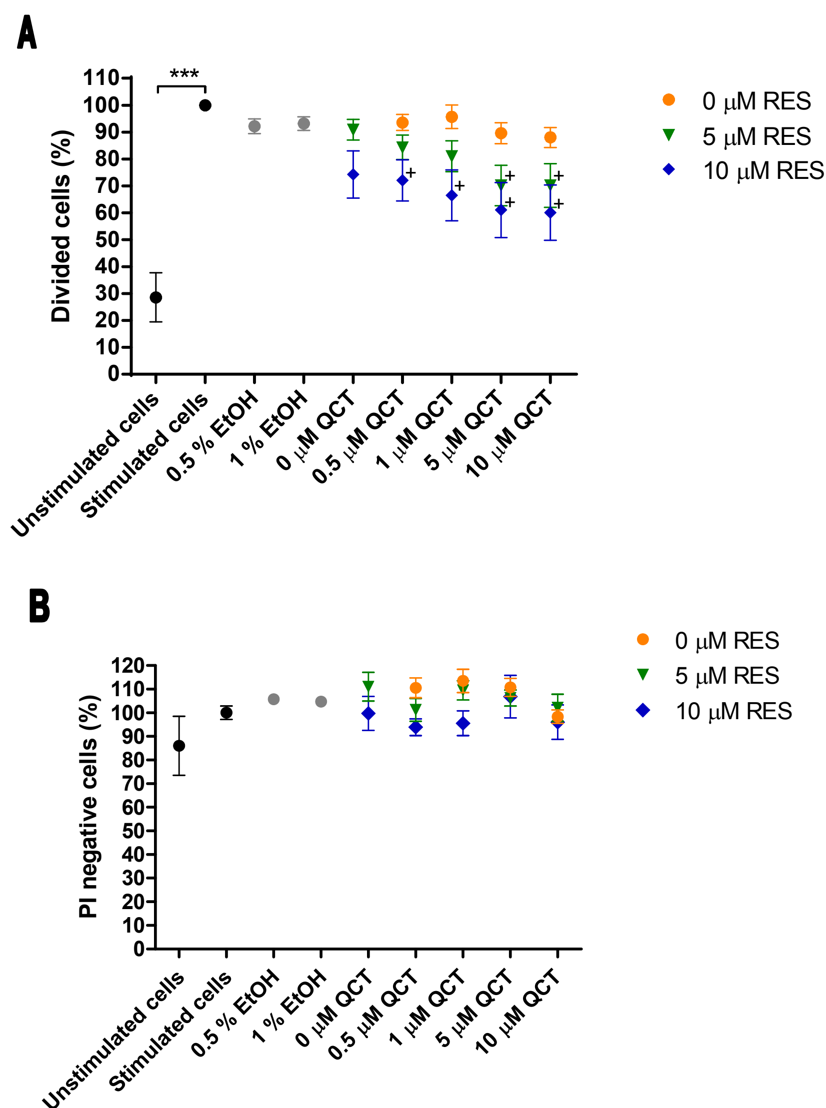

Figure 34. Peripheral blood mononuclear cells (PBMCs) treated with quercetin (QCT), resveratrol (RES) and their combination. A: The proliferation of PBMCs evaluated by CFSE labelling assay. B: Percentage of propidium iodide (PI) negative cells. Data are expressed as percentage of cells relative to stimulated cells without treatment. ***$p<0.001$. $^{+}p<0.05$, compared to its vehicle (ethanol: EtOH). N=3.

VI.3. Experimental murine model of dry eye and adoptive transfer model

This section summarises all of the data obtained from testing topical treatments of polyphenol on the in-vivo model of DED and adoptive transfer model.

VI.3.1. Desiccating stress-induced model of dry eye

In this study, mice exposed to DS for 10 days (donor mice) were treated topically with 0.01 % QCT, 0.1 % RES and their combination (0.01 % QCT + 0.1 % RES). CFS, TP, cytokine/chemokine concentration in tears, goblet cell density and $CD4^+$ T cell infiltration in conjunctiva were determined.

VI.3.1.1. Corneal fluorescein staining in donor mice

Visual evaluation of CFS was used as a measure of the effect of DS on corneal epithelial barrier function. Figure 36 shows the CFS score for donor mice. After 9 days, both DS mice and vehicle-treated DS mice showed a significant increase in CFS ($p<0.001$), compared to control group. There were no differences in staining between DS mice and vehicle-treated DS mice. Topical treatment with 0.01 % QCT ($p<0.001$) and 0.01 % QCT + 0.1 % RES ($p<0.05$) showed a significant decrease in CFS, compared to vehicle-treated DS mice. In addition, Figure 37 shows representative CFS images of corneas from each group. Minimal scattered punctate staining, or no staining, was observed on the corneas of control mice after instillation of fluorescein dye (A). Intense punctate fluorescein staining was observed in both DS mice (B) and vehicle-treated DS mice (C). This intense punctate staining decreased when mice were topically treated three times a day with 0.01 % QCT (D), 0.1 % RES (E) and 0.01 % QCT + 0.1 % RES (F).

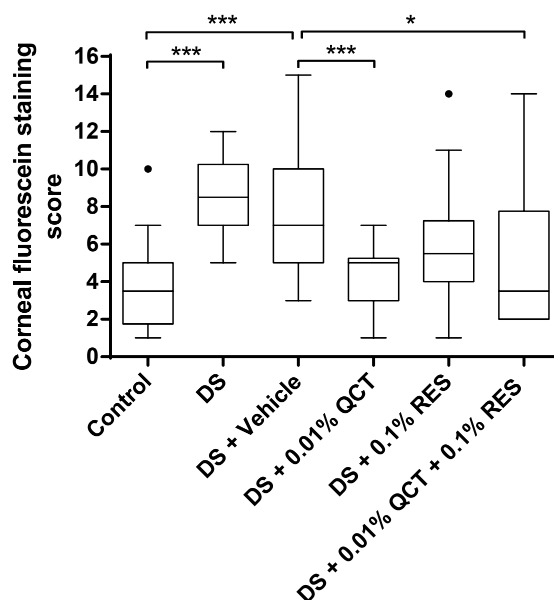

Figure 36. Corneal fluorescein staining in donor mice. Black dots represent outliers. DS: desiccating stress; QCT: quercetin; RES: resveratrol. *p<0.05; ***p<0.001. N=9.

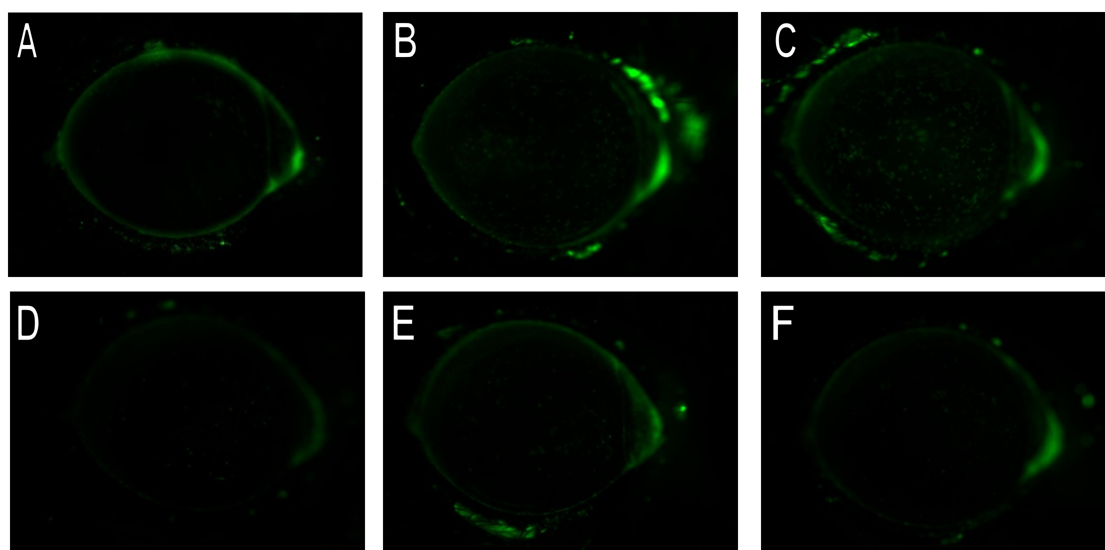

Figure 37. Representative corneal fluorescein staining images of corneas from each group. A: control group; B: desiccating stress (DS) mice; C: vehicle-treated DS mice; D: 0.01 % quercetin (QCT)-treated DS mice; E: 0.1 % resveratrol (RES)-treated DS mice; F: 0.01 % QCT + 0.1 % RES-treated DS mice. N=9.

VI.3.1.2. Aqueous tear production in donor mice

TP was measured using the phenol red thread test in donor mice, 1 day before DS challenge (baseline) and after 10 days of DS (post-DS). Figure 38 shows the data

for TP. DS caused a significant decreased in TP ($p<0.001$), compared to baseline. Neither QCT, nor RES, nor their combination had any effect on TP.

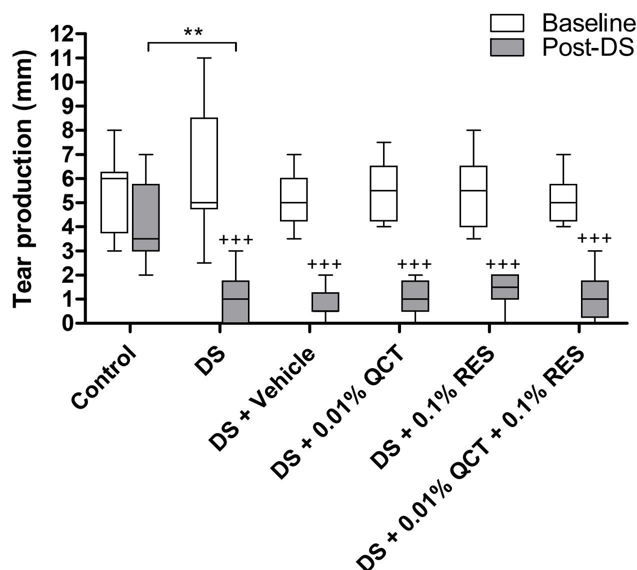

Figure 38. Tear production in donor mice. DS: desiccating stress; QCT: quercetin; RES: resveratrol. $^{**}p<0.01$. $^{+++}p<0.001$, compared to baseline data. N=9.

VI.3.1.3. Cytokine/chemokine concentration in tears of donor mice

The level of 15 cytokines/chemokines in tears was evaluated using a multiplex bead analysis with x-MAP® technology. Cytokines and chemokines IL1-α, IL-2, IL-4, IP-10/CXCL10, RANTES/CCL5 and TNF-α were detected in tears at day 6 (Figure 39; the percentage of detected cytokines was >70 % except for TNF-α: >50 %), whilst IL-2, IL-4, IP-10/CXCL10 and TNF-α were detected at day 10 (Figure 40; the percentage of detected cytokines was >60 % for all cytokines). Of the cytokines assessed, DS only significantly increased IL1-α in vehicle-treated DS mice and RANTES/CCL5 in DS mice (both $p<0.05$) at day 6, compared to control mice. Levels of IL1-α decreased when DS mice were treated with 0.01 % QCT ($p<0.05$), 0.1 % RES ($p<0.01$), and 0.01 % QCT + 0.1 % RES ($p<0.01$). At day 6, although this did not reach significance, there was a trend for decreased IL-4 levels with 0.01 % QCT ($p=0.0625$), compared to vehicle-treated DS mice. There was also a tendency for decreased levels of IP-10/CXCL10 by 0.01 % QCT ($p=0.0850$), at day 10 (Figure 40).

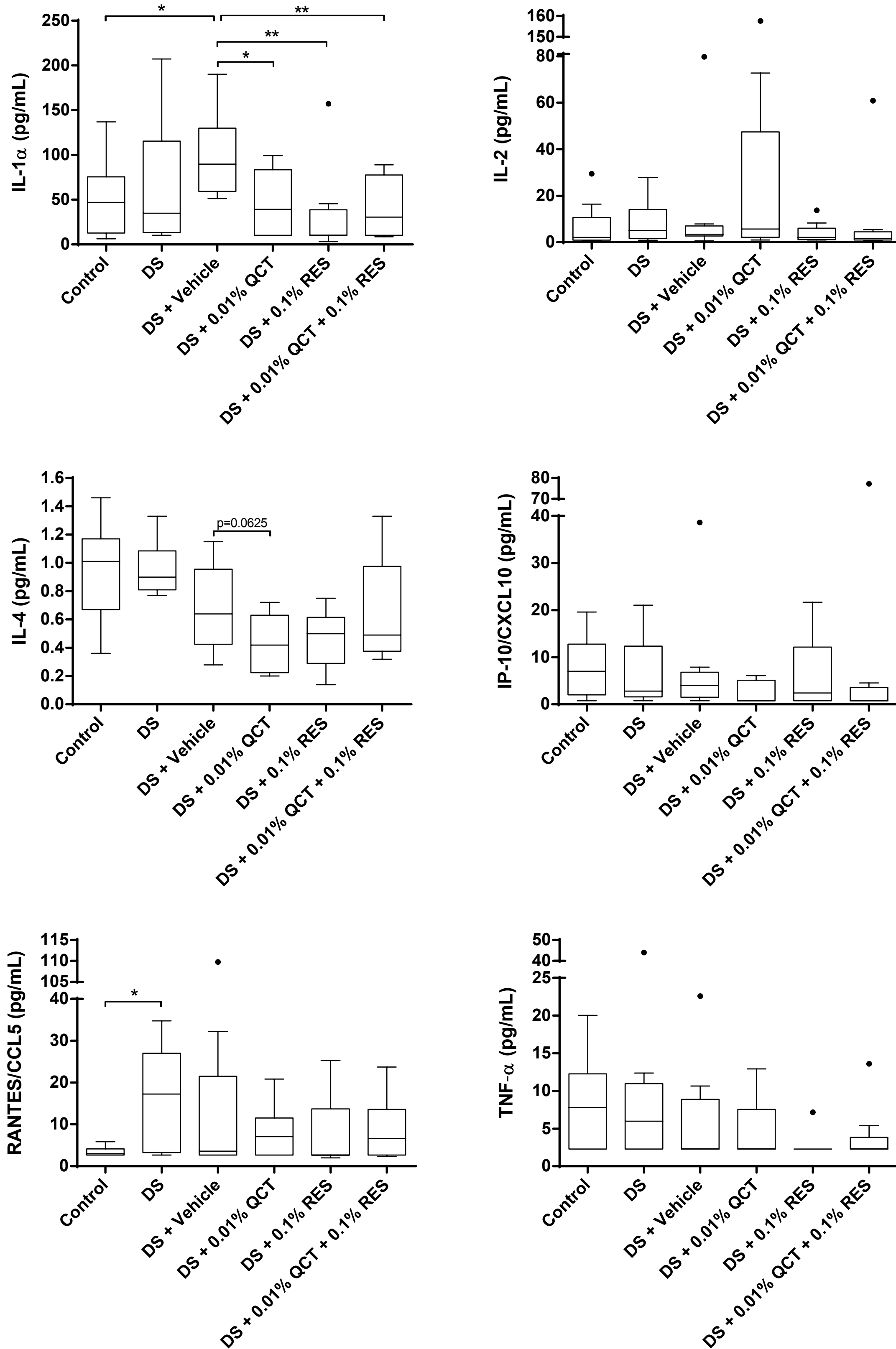

Figure 39. Cytokine/chemokine concentration in donor mouse tears at day 6. Black dots represent outliers. DS: desiccating stress; QCT: quercetin; RES: resveratrol. *p<0.05; **p<0.01. N=9.

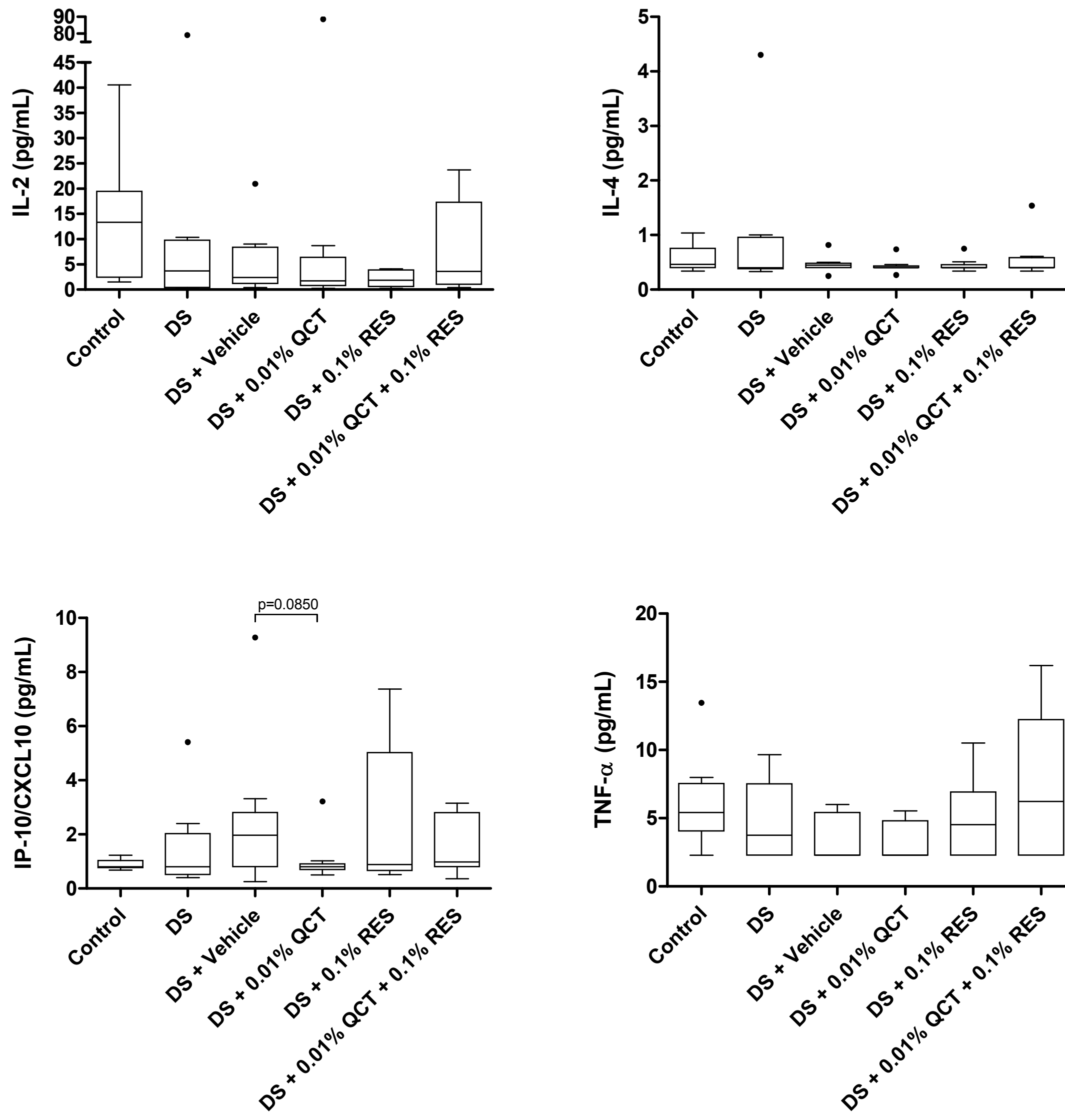

Figure 40. Cytokine/chemokine concentration in donor mouse tears at day 10. Black dots represent outliers. DS: desiccating stress; QCT: quercetin; RES: resveratrol. N=9.

VI.3.1.4. Goblet cell count in donor mice

Goblet cell numbers in donor conjunctiva were determined using PAS staining. Figure 41 shows the goblet cell count from superior and inferior conjunctivae. Neither DS nor polyphenols altered goblet cell number in donor mice.

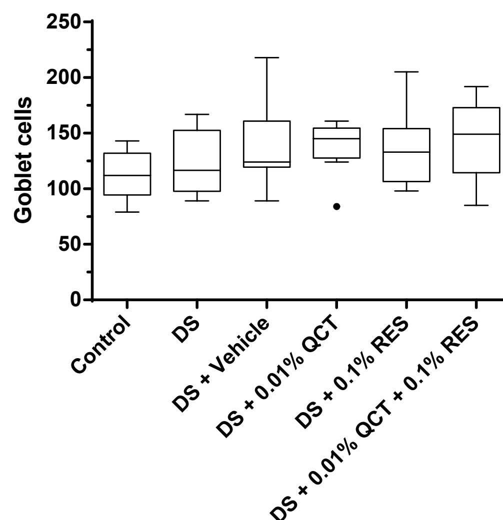

Figure 41. Goblet cell count in donor mouse conjunctiva. Black dots represent outliers. DS: desiccating stress; QCT: quercetin; RES: resveratrol. N=9.

VI.3.1.5. Immunohistochemistry in donor mice

CD4$^+$ T cell numbers in donor conjunctiva were determined by immunohistochemistry. Figure 42 shows CD4$^+$ T cell count from superior and inferior conjunctivae. Neither DS nor polyphenols altered CD4$^+$ T cell infiltration in donor mouse conjunctiva.

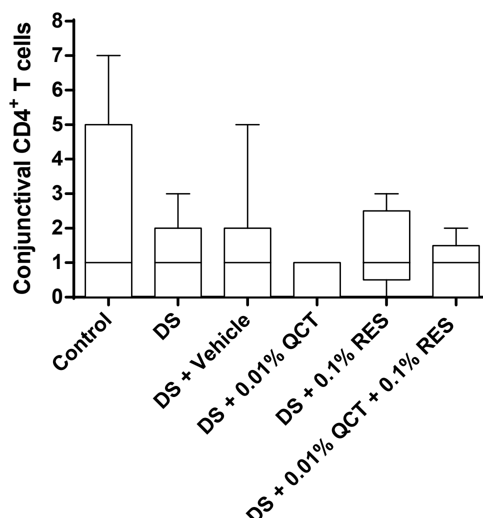

Figure 42. Conjunctival CD4$^+$ T cells in donor mice. DS: desiccating stress; QCT: quercetin; RES: resveratrol. N=9.

VI.3.2. Adoptive transfer model

After 10 days of DS, CD4$^+$ T cells were isolated from donor mice and adoptively transferred to athymic mice (recipient mice). The results from this model are shown in this section.

VI.3.2.1. Aqueous tear production of recipient mice

TP was measured using the phenol red thread test in donor mice 1 day before transferring CD4$^+$ T cells (baseline), and after 72 h (post-DS). Figure 43 shows the data for TP in recipient mice. DS tended to decrease TP in recipients of vehicle-treated DS mice (p=0.0643), compared to the control. Recipients of 0.01 % QCT + 0.1 % RES-treated DS mice significantly increased TP (p<0.01), compared to recipients of vehicle-treated DS mice. Neither 0.01 % QCT nor 0.1 % RES had any effect on TP in recipient mice.

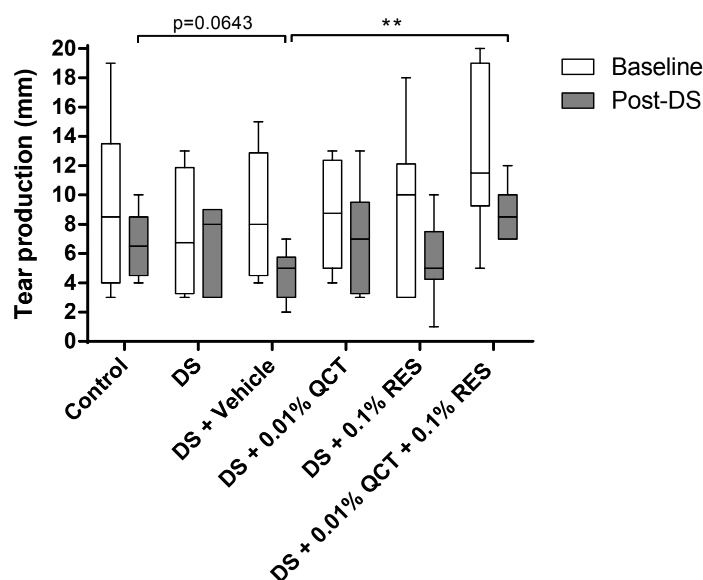

Figure 43. Tear production in recipient mice. DS: desiccating stress; QCT: quercetin; RES: resveratrol. **p<0.01. N=8.

VI.3.2.2. Cytokine/chemokine concentration in tears of recipient mice

The level of 15 cytokines/chemokines in tears was evaluated as shown in Figure 44. All analysed cytokines/chemokines were detected (percentage of detected cytokines was >80% for all cytokines). Cytokine IL-13 tended to decrease in recipient of DS mice (p=0.0766), compared to recipients of control mice, and IL-17 decreased significantly (p<0.05) in recipients of vehicle-treated DS mice, compared to recipients of control mice. Neither 0.01 % QCT, nor 0.1 % RES, nor 0.01 % QCT + 0.1 % RES had any effect on cytokine/chemokine levels in recipient mice.

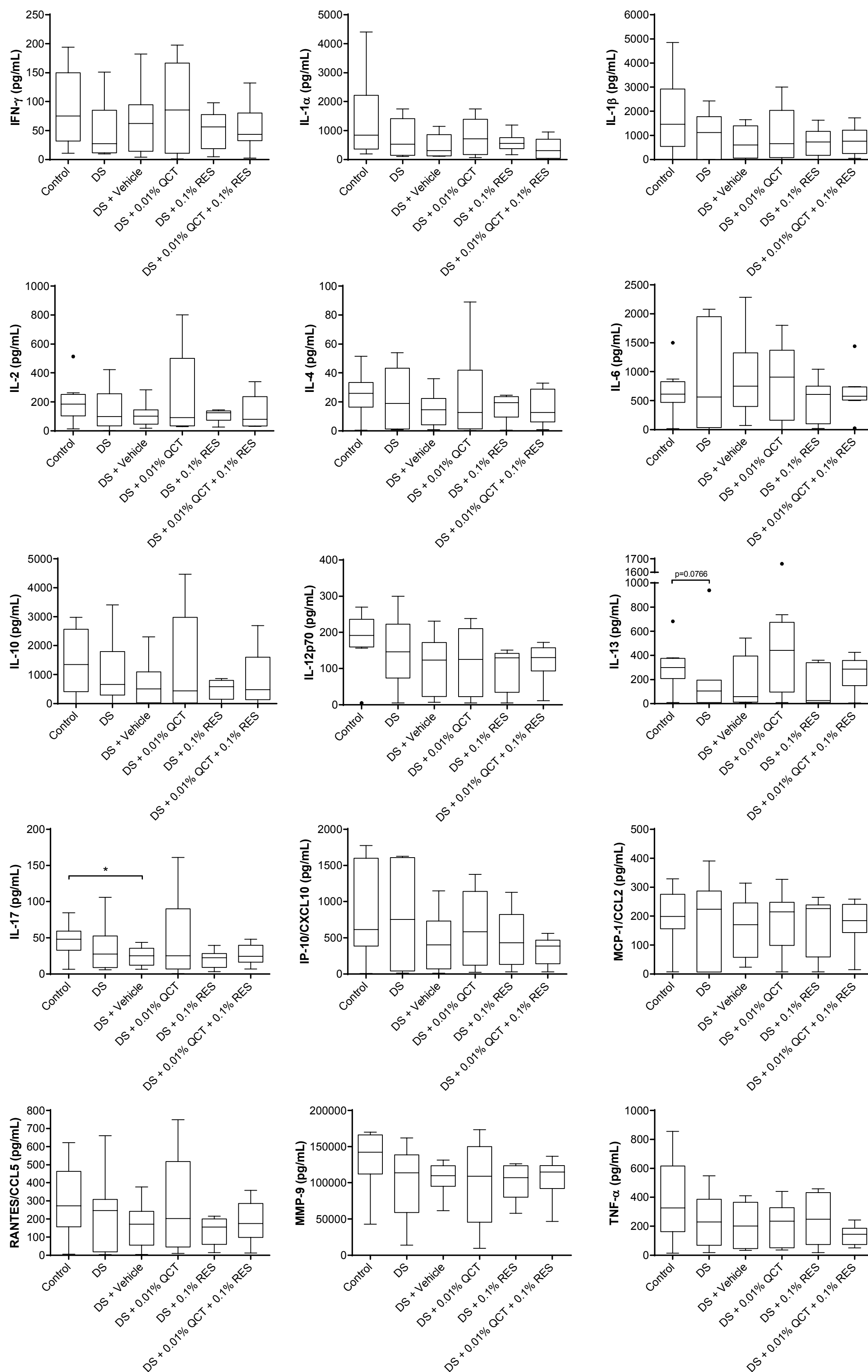

Figure 44. Cytokine/chemokine concentration in recipient mouse tears at day 3. Black dots represent outliers. DS: desiccating stress; QCT: quercetin; RES: resveratrol. *p<0.05. N=8.

VI.3.2.3. Goblet cell count in recipient mice

Goblet cell numbers in recipient conjunctiva were determined using PAS stain. Figure 45 shows goblet cell count from superior and inferior conjunctivae. Neither DS nor polyphenols altered goblet cell numbers in recipient mice.

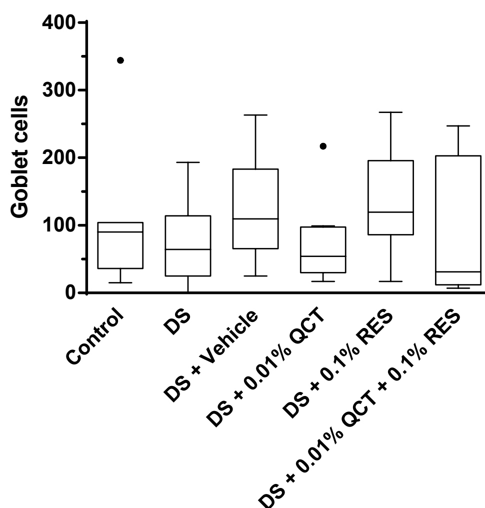

Figure 45. Goblet cell count in recipient mouse conjunctiva. Black dots represent outliers. DS: desiccating stress; QCT: quercetin; RES: resveratrol. N=8.

VI.3.2.4. Immunohistochemistry in recipient mice

$CD4^+$ T cell number was determined by immunohistochemistry. Figure 46A shows representative immunostaining images of $CD4^+$ T cells from each group and Figure 46B shows $CD4^+$ T cell infiltration in the superior and inferior recipient mouse conjunctiva. DS produced a tendency to increased $CD4^+$ T cells in conjunctiva of recipients of DS mice (p=0.0715). No significant differences were found in recipients of vehicle-treated DS mice and recipients of DS mice. Regarding topical treatments with polyphenols, 0.1 % RES decreased $CD4^+$ T cell infiltration in conjunctiva of recipients of RES-treated DS mice ($p<0.05$). QCT (0.01 %) showed a tendency for decreased $CD4^+$ T cell infiltration (p=0.0743), compared to recipients of vehicle-treated DS mice.

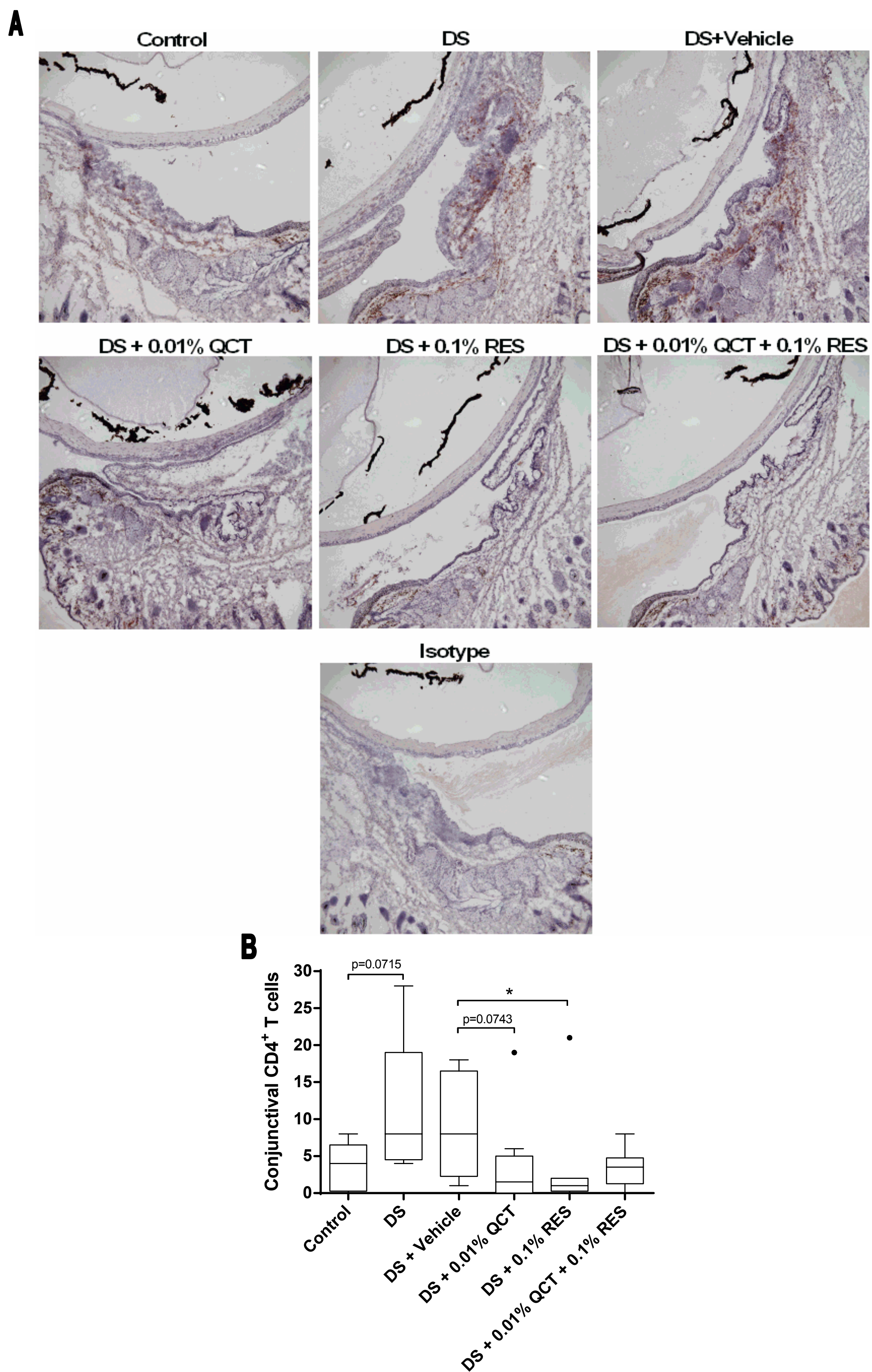

Figure 46. $CD4^+$ T cell infiltration in conjunctiva of recipient mouse. A: Representative immunostaining images from each group. Original magnification 10X. B: $CD4^+$ T cell infiltration in conjunctiva. Isotype were used as a negative control. Black dots represent outliers. DS: desiccating stress; QCT: quercetin; RES: resveratrol. *p<0.05. N=8.

VI.4. In-vitro model of conjunctival mast cell response

This section summarises all of the data obtained from testing the anti-allergic effect of polyphenols on CBMCs.

VI.4.1. Cytotoxicity of polyphenols

The cytotoxicity of QCT, RES and QCT+RES in CBMCs stimulated with IgE/anti-IgE after 24 h exposure was tested using PI staining and flow cytometry.

VI.4.1.1. Cytotoxicity of QCT

Figure 47 shows the cytotoxicity of QCT in CBMCs. Seven concentrations of QCT were tested: 0.5, 1, 5, 10, 15, 20 and 25 μM. None of the QCT concentrations used were toxic for CBMCs after 24 h of culture.

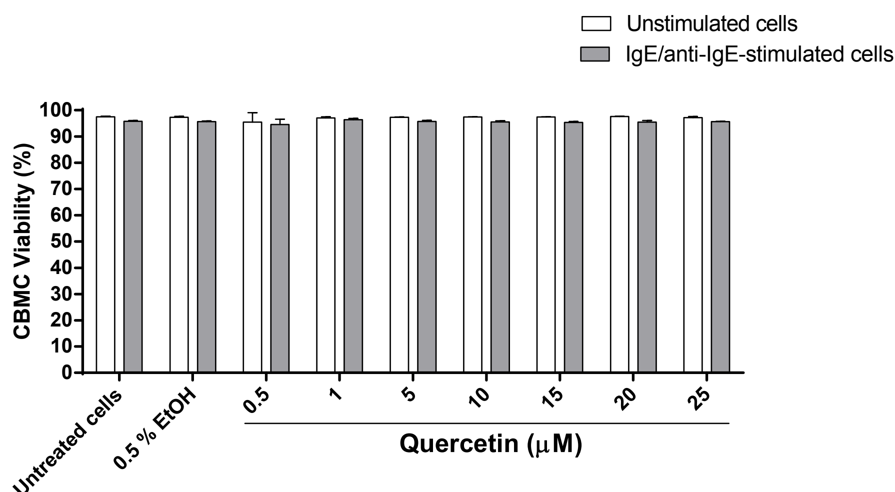

Figure 47. Cytotoxicity of quercetin (QCT) in cord blood mast cells (CBMCs). EtOH: ethanol. N=1.

VI.4.1.2. Cytotoxicity of RES

Figure 48 shows the cytotoxicity of RES in CBMCs. Seven concentrations of RES were tested: 0.5, 1, 5, 10, 25, 50 and 100 μM. None of the RES concentrations used were toxic for CBMCs.

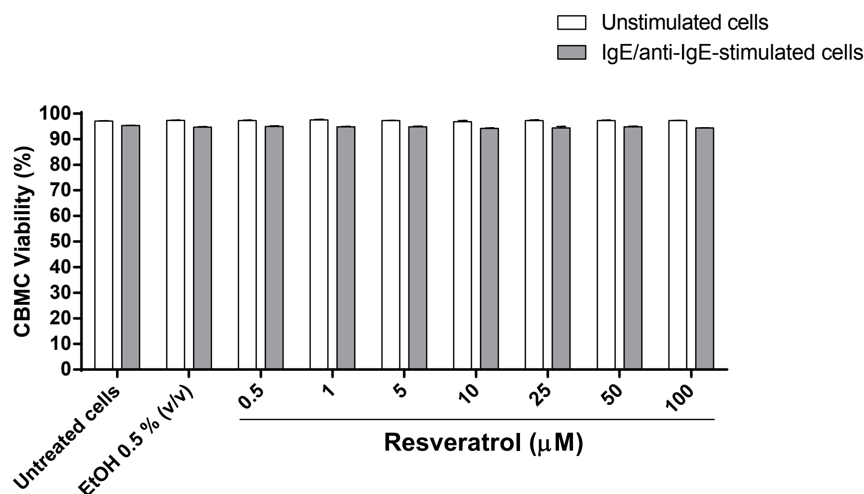

Figure 48. Cytotoxicity of resveratrol (RES) in cord blood mast cells (CBMCs). Ethanol: EtOH. N=1.

VI.4.1.3. Cytotoxicity of combinations of QCT and RES

Figure 49 shows the cytotoxicity of combinations of QCT and RES in CBMCs. Seven concentrations each of QCT (0.5, 1, 5, 10, 15, 20 and 25 μM) and RES (0.5, 1, 5, 10, 25, 50 and 100 μM) were tested alone and in combination with each other. None of the combinations of QCT+RES affected CBMC viability.

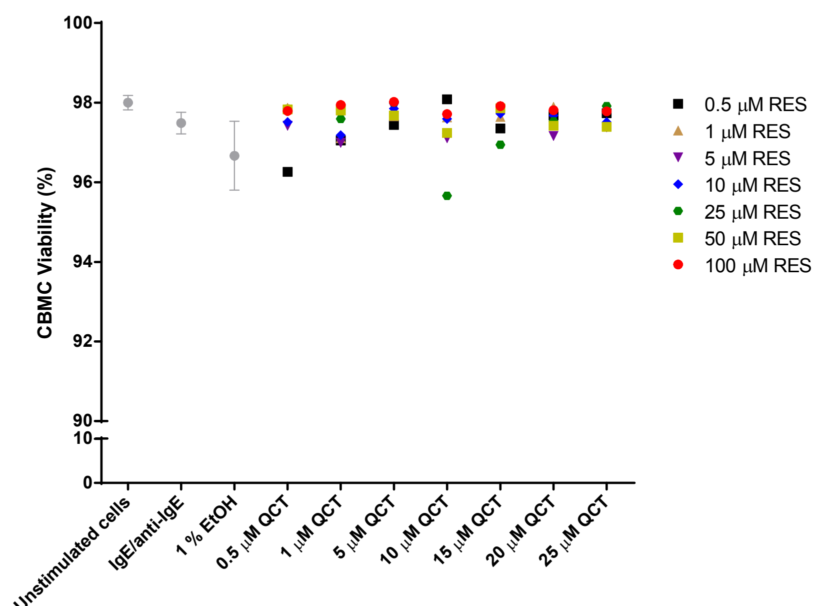

Figure 49. Cytotoxicity of combination of quercetin (QCT) and resveratrol (RES) in cord blood mast cells (CBMCs). EtOH: ethanol. N=1.

VI.4.2. Histamine release

Dose-response curves for studying the effect of QCT, RES and their combinations on histamine release were firstly obtained from two different experiments performed in single samples.

VI.4.2.1. Effect of QCT on histamine release

Figure 50 shows the effect of different concentrations of QCT on histamine release by CBMCs. One hour following IgE/anti-IgE stimulation, histamine secretion was increased ($p<0.001$). This increased secretion of histamine was significantly reduced by vehicle (0.5 % EtOH) ($p<0.001$). Histamine secretion by stimulated CBMCs treated with vehicle was only significantly decreased by 0.5 µM QCT ($p<0.01$). However, there was a tendency for decreased histamine secretion by QCT at concentrations of 1 µM ($p=0.0556$), 10 µM ($p=0.0504$), 15 µM ($p=0.0509$), 20 µM ($p=0.0556$) and 25 µM ($p=0.0513$).

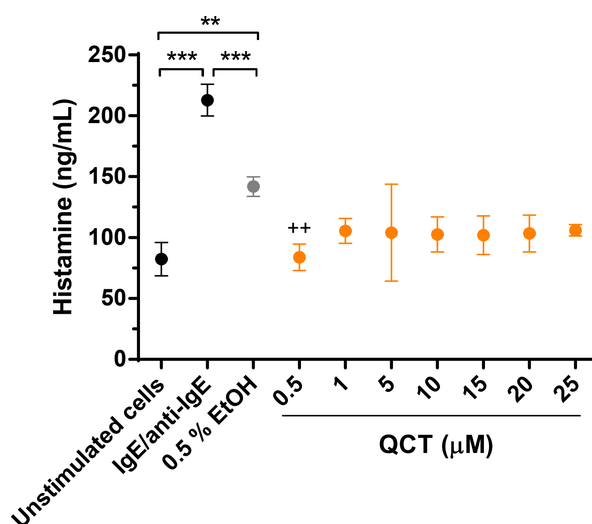

Figure 50. Effect of quercetin (QCT) on histamine secretion. **$p<0.01$, ***$p<0.001$; $^{++}p<0.01$, compared to stimulated cells treated with vehicle: ethanol (EtOH). N=2 (single samples).

VI.4.2.2. Effect of RES on histamine release

Figure 51 shows the effect of different RES concentrations on histamine release by CBMCs. RES did not have any significant effect on histamine release, except for 5 µM RES, which showed a trend for decreased histamine secretion ($p=0.0804$).

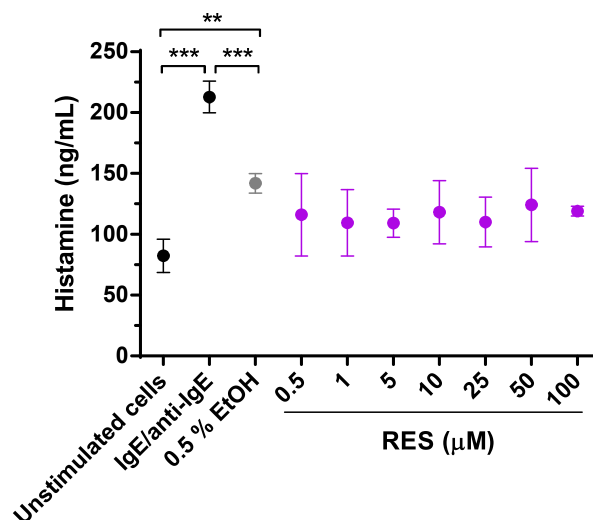

Figure 51. Effect of resveratrol (RES) on histamine secretion. EtOH: ethanol. **p<0.01, ***p<0.001. N=2 (single samples).

VI.4.2.3. Effect of QCT+RES combination on histamine release

Figure 52 (A & B) shows the effect of different QCT+RES combinations on histamine release by CBMCs. For better comprehension, Figures 52 A and B represent all data with and without plotting SEM, respectively. Table 8 shows the significant differences for each combination of QCT+RES, compared to stimulated cells treated with vehicle, 1 % EtOH. Histamine secretion by stimulated CBMCs treated with vehicle was significantly decreased by 5 µM QCT + 25 µM RES, 10 µM QCT + 25 µM RES, 0.5 µM QCT + 50 µM RES and 10 µM QCT + 100 µM RES (all p<0.05).

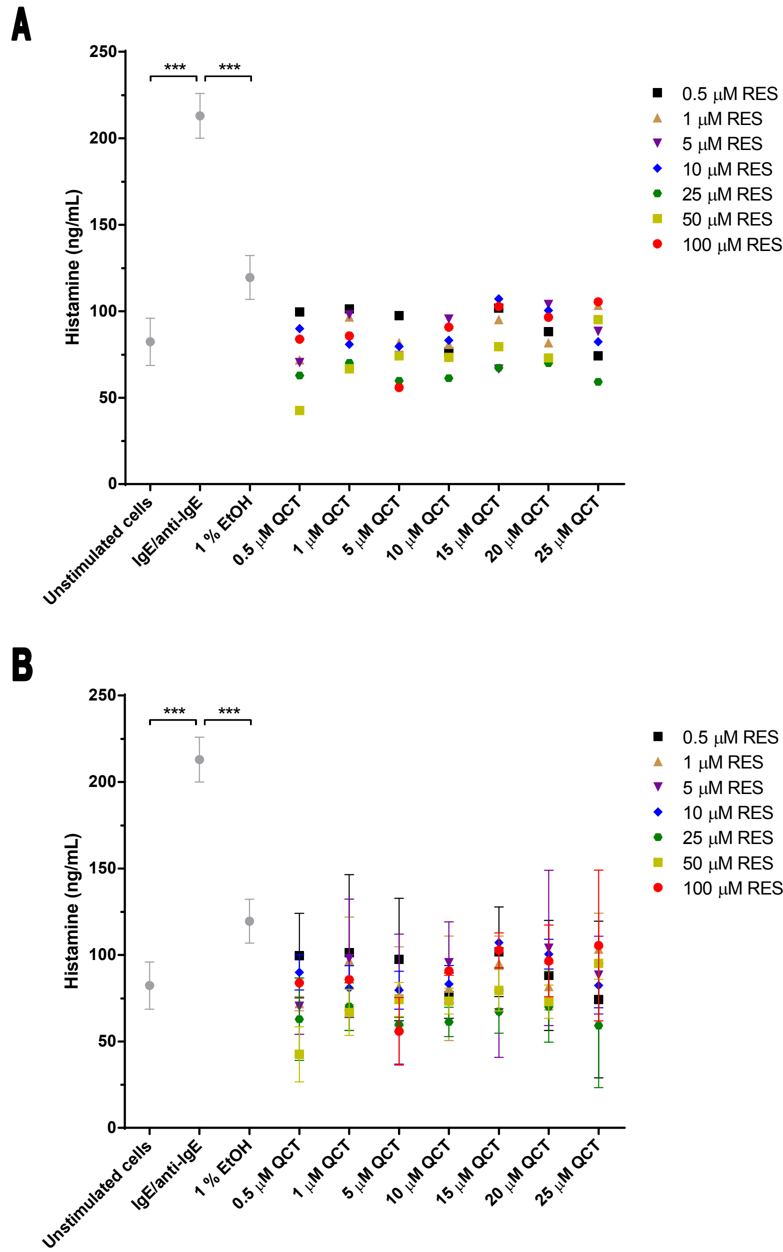

Figure 52. Effect of combination of quercetin (QCT) and resveratrol (RES) on histamine secretion. EtOH: ethanol. N=2 (single samples).

	QCT (µM)						
RES (µM)	**0.5**	**1**	**5**	**10**	**15**	**20**	**25**
0.5	ns	ns	ns	ns	ns	ns	ns
1	0.0861	ns	ns	ns	ns	ns	ns
5	ns	ns	ns	ns	0.0893	ns	ns
10	ns	ns	ns	ns	ns	ns	ns
25	ns	0.0859	0.05	0.05	0.0719	ns	0.0802
50	0.05	0.0706	ns	ns	ns	ns	ns
100	ns	ns	0.05	ns	ns	ns	ns

Table 8. Statistical differences between quercetin(QCT) + resveratrol(RES) combinations and stimulated cells treated with vehicle (1% ethanol). Ns: no significant.

After dose-response curves were obtained from two different experiments carried out in single samples, a combination of QCT+RES (10 µM QCT + 25 µM RES), which produced a significant decrease in histamine secretion, was chosen in order to study the effect of a polyphenol combination (in an experiment performed in triplicate). Figure 53 shows the effect of 10 µM QCT, 25 µM RES and 10 µM QCT + 25 µM RES on histamine release by CBMCs. Stimulated cells treated with 0.5 % EtOH showed a significant decrease of histamine release compared to control cells ($p<0.001$). Moreover, 10 µM QCT and 25 µM RES significantly decreased histamine release of stimulated CBMCs (both $p<0.01$). The mix of both compounds did not have any effect on histamine release in stimulated cells.

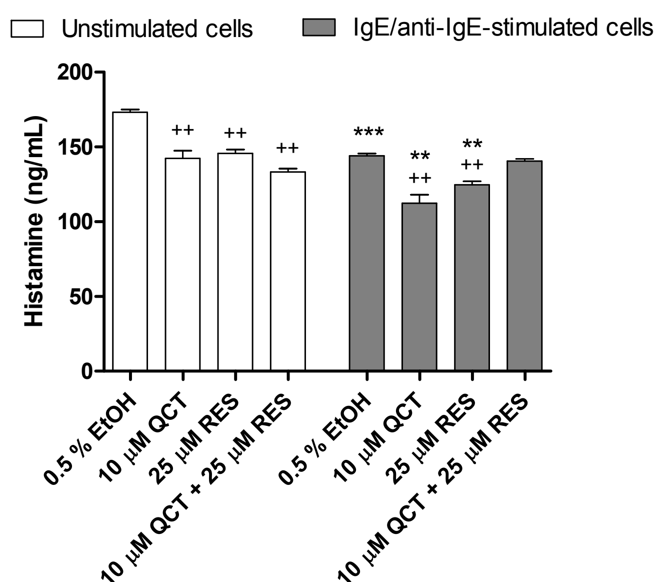

Figure 53. Effect of quercetin (QCT), resveratrol (RES) and their combination on histamine secretion. **$p<0.01$, ***$p<0.001$, compared to unstimulated cells; $^{++}p<0.01$, compared to cells treated with ethanol (EtOH). N=1 (triplicate).

VI.4.3. Cytokine/chemokine secretion

After determining the effect of different concentrations of QCT, RES and their combinations on histamine release, the effect of polyphenols on cytokine release after 24 h was studied. For this purpose, secreted cytokine dose-response curves were first obtained for QCT (0.5, 1, 5, 10, 15, 20 and 25 µM) and RES (0.5, 1, 5, 10, 25, 50 and 100 µM) in CBMCs stimulated by IgE/anti-IgE (from two different experiments performed in single samples). After that, the two concentrations of polyphenols tested on histamine release (10 µM QCT and 25 µM RES) and both compounds alone were

also tested in order to determine the effect of both compounds together (one experiment performed in triplicate).

For a better comprehension of all of the data presented herein, results are subdivided into nine sections, according to the studied cytokines/chemokines: IL-1β, IL-4, IL-5, IL-8/CXCL8, IL-9, IL-10, IL-13, TNF-α and IFN-γ.

VI.4.3.1. IL-1β secretion

IL-1β secretion was not detected in any sample.

VI.4.3.2. IL-4 secretion

Figure 54 (A-C) shows the effect of QCT, RES and their combination on IL-4 secretion. Stimulation with IgE/anti-IgE did not change IL-4 basal secretion by CBMCs. Neither QCT, nor RES, nor QCT+RES had any effect on IL-4 secretion.

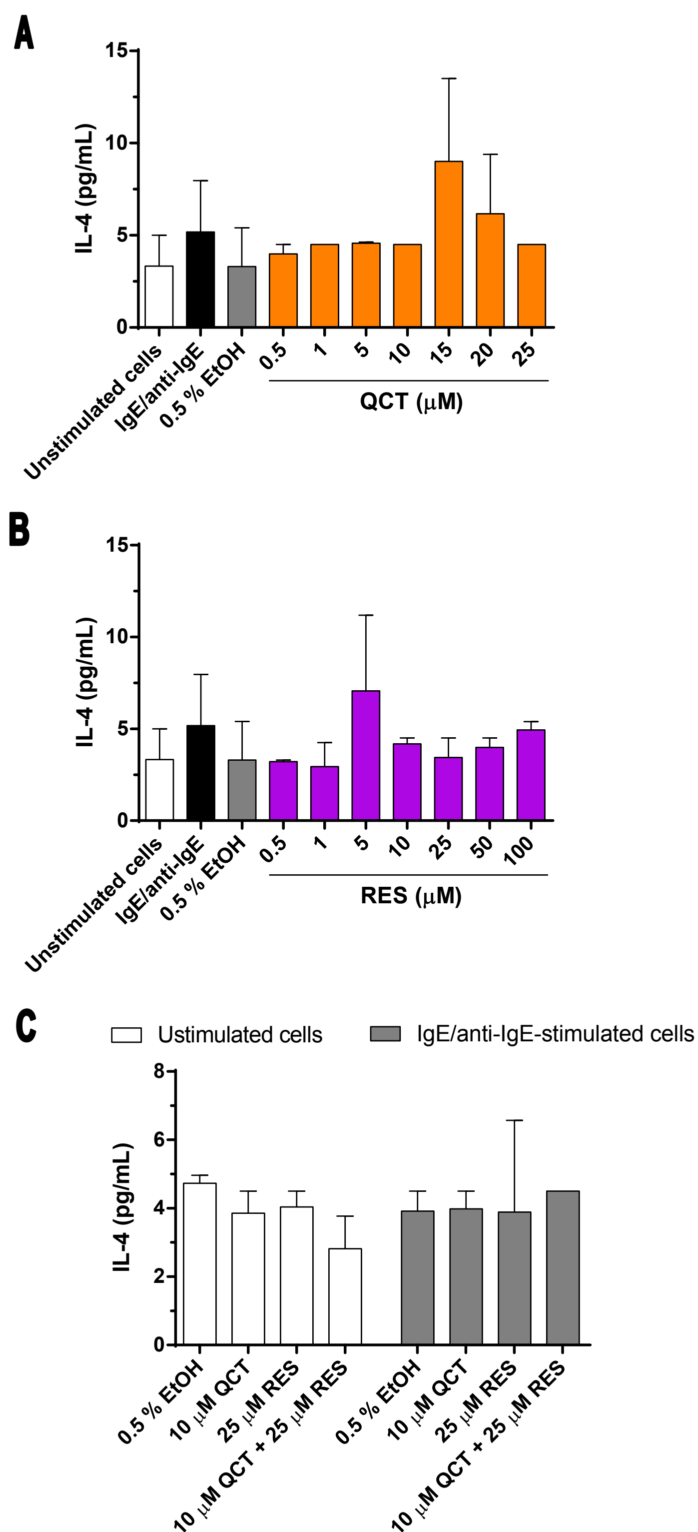

Figure 54. Effect of quercetin (QCT) (A), resveratrol (RES) (B) and QCT+RES (C) on IL-4 secretion. EtOH: ethanol. N=2 for A and B (single samples); and N=1 for C (triplicate).

VI.4.3.3. IL-5 secretion

Figure 55 (A-C) shows the effect of QCT, RES and their combination on IL-5 secretion. QCT increased IL-5 levels at 1 and 10 µM ($p<0.05$; Figure 55A). On the other hand RES decreased IL-5 secretion at 25 µM (Figure 55B). IL-5 secretion was only stimulated by IgE/anti-IgE in CBMCs when compounds were tested together (Figure 55C). QCT+RES had no effect on IL-5 secretion (Figure 55C).

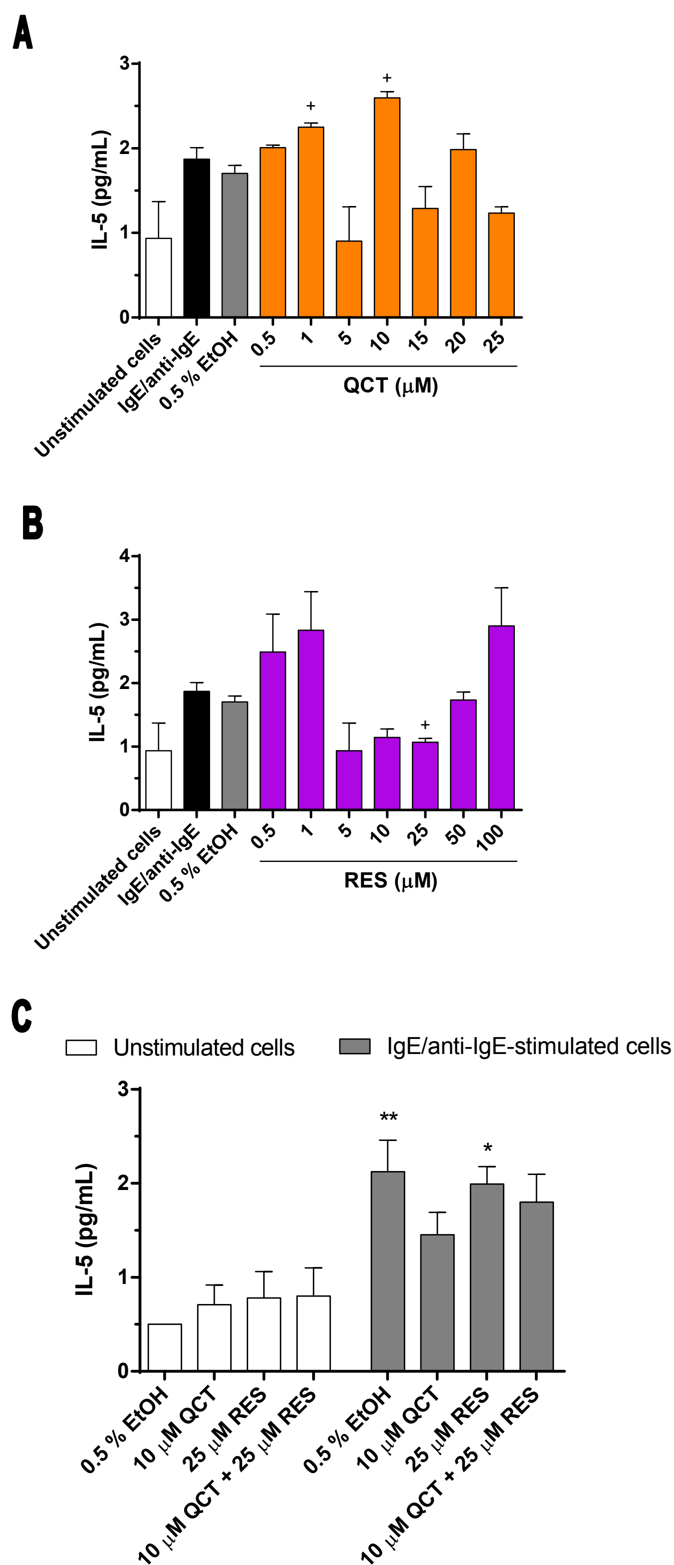

Figure 55. Effect of quercetin (QCT) (A), resveratrol (RES) (B) and QCT+RES (C) on IL-5 secretion. *p<0.05, **p<0.01, compared to unstimulated cells; +p<0.05, compared to stimulated cells treated with ethanol (EtOH). N=2 for A and B (single samples); and N=1 for C (triplicate).

VI.4.3.4. IL-8/CXCL8 secretion

Figure 56 (A-C) shows the effect of QCT, RES and their combination on IL-8 secretion. Stimulation of CBMCs with IgE/anti-IgE induced a significant increase of IL-8/CXCL8, compared to control cells ($p<0.05$; Figures 56 A and B). When cells were treated with polyphenols, this stimulated secretion of IL-8/CXCL8 was decreased at 10, 15 and 20 µM QCT (all $p<0.01$; Figure 56A), whilst RES decreased IL-8/CXCL8 secretion at 10, 25, 50 and 100 µM ($p<0.05$, $p<0.01$, $p<0.01$ and $p<0.01$, respectively; Figure 56B), compared to IgE/anti-IgE-stimulated cells treated with EtOH. The mix of both compounds did not have a significant effect on IL-8/CXCL8 production by CBMCs but its level was lower that stimulated cells treated with vehicle.

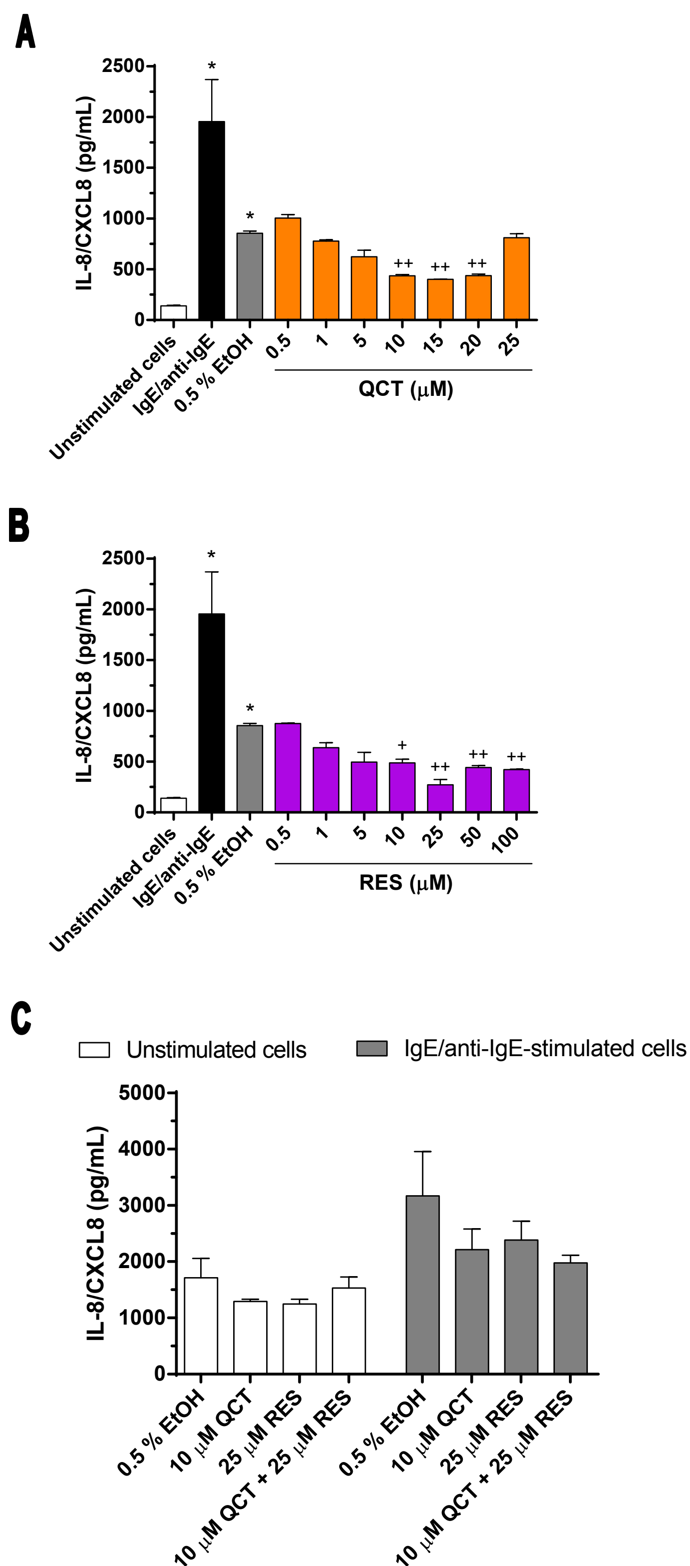

Figure 56. Effect of quercetin (QCT) (A), resveratrol (RES) (B) and QCT+RES (C) on IL-8/CXCL8 secretion. *$p<0.05$, compared to unstimulated cells; $^{+}p<0.05$, $^{++}p<0.01$, compared to stimulated cells treated with ethanol (EtOH). N=2 for A and B (single samples); and N=1 for C (triplicate).

VI.4.3.5. IL-9 secretion

IL-9 secretion was not detected in any sample.

VI.4.3.6. IL-10 secretion

Figure 57 (A-C) shows the effect of QCT, RES and their combination on IL-10 secretion. IgE/anti-IgE stimulation did not induce IL-10 secretion by CBMCs. Neither QCT, nor RES, nor their combination had any significant effect on IL-10 production.

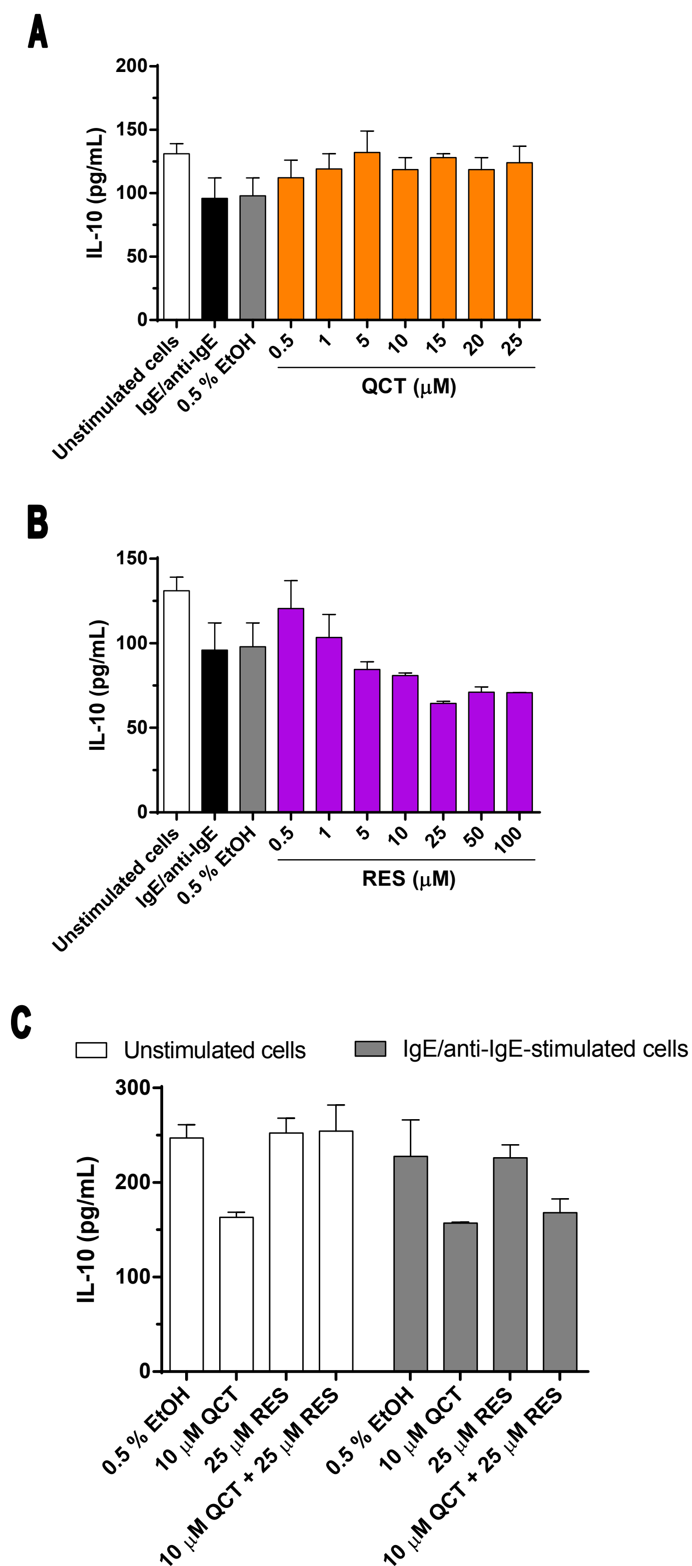

Figure 57. Effect of quercetin (QCT) (A), resveratrol (RES) (B) and QCT+RES (C) on IL-10 secretion. EtOH: ethanol. N=2 for A and B (single samples); and N=1 for C (triplicate).

VI.4.3.7. IL-13 secretion

Figure 58 (A-C) shows the effect of QCT, RES and their combination on IL-13 secretion. IgE/anti-IgE stimulation induced IL-13 secretion by CBMCs (Figures 58 A and B). QCT significantly decreased IL-13 secretion at concentrations of 10, 15 and 20 μM (all $p<0.05$; Figure 58A). IL-13 secretion was also significantly decreased when CBMCs were treated with 5, 10, 25 and 100 μM RES (all $p<0.05$; Figure 58B). The combination of QCT and RES did not have any effect on IL-13 secretion (Figure 58C).

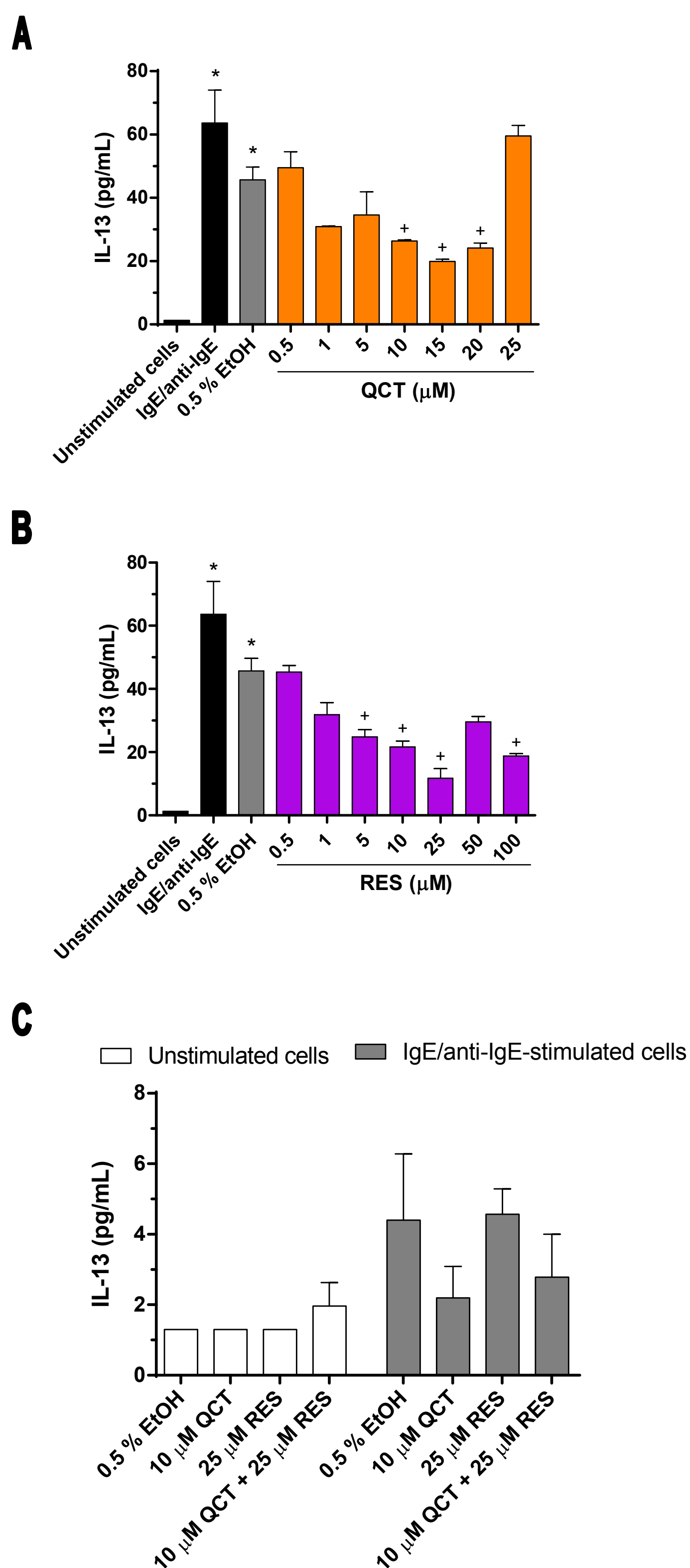

Figure 58. Effect of quercetin (QCT) (A), resveratrol (RES) (B) and QCT+RES (C) on IL-13 secretion. *$p<0.05$, compared to unstimulated cells; $^{+}p<0.05$, compared to stimulated cells treated with ethanol (EtOH). N=2 for A and B (single samples); and N=1 for C (triplicate).

VI.4.3.8. TNF-α secretion

Figure 59 (A-C) shows the effect of QCT, RES and their combination on TNF-α secretion. IgE/anti-IgE stimulation induced TNF-α secretion by CBMCs (Figures 59 A and B). This stimulated secretion of TNF-α was significantly decreased when QCT was added at 1, 10, 15, 20 and 25 μM QCT (all $p<0.05$; Figure 59A). Similar inhibitory effects on TNF-α secretion were found when IgE/anti-IgE-stimulated CBMCs were treated with 1, 5, 10, 25, 50 and 100 μM RES ($p<0.05$; Figure 59B). The mix of both compounds did not have any effect on TNF-α production (Figure 59C).

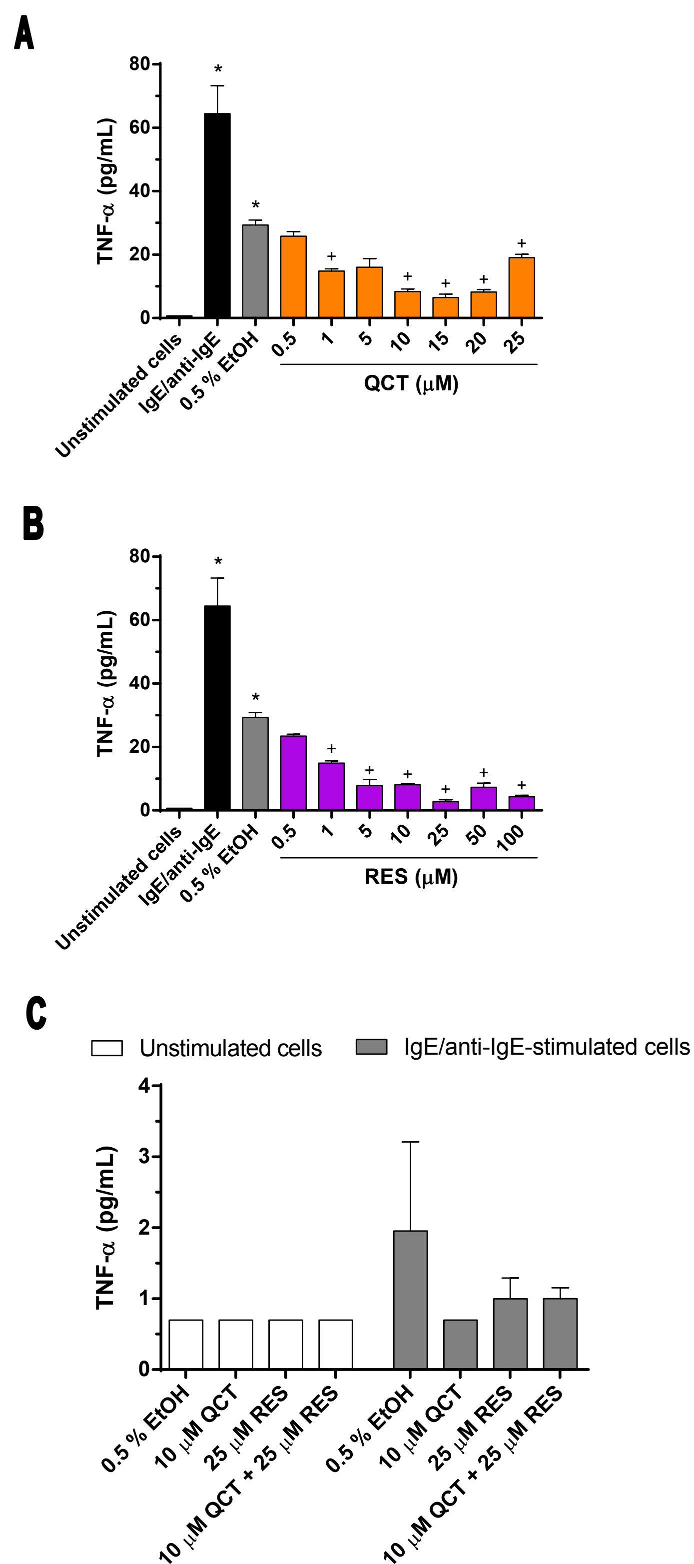

Figure 59. Effect of quercetin (QCT) (A), resveratrol (RES) (B) and QCT+RES (C) on TNF-α secretion. *p<0.05, compared to unstimulated cells; +p<0.05, compared to stimulated cells treated with ethanol (EtOH). N=2 for A and B (single samples); and N=1 for C (triplicate).

VI.4.3.9. IFN-γ secretion

Figure 60 (A-C) shows the effect of QCT, RES and their combination on IFN-γ secretion. IFN-γ secretion was not stimulated by IgE/anti-IgE in CBMCs. Neither QCT, nor RES, nor their combination had any effect on IFN-γ secretion.

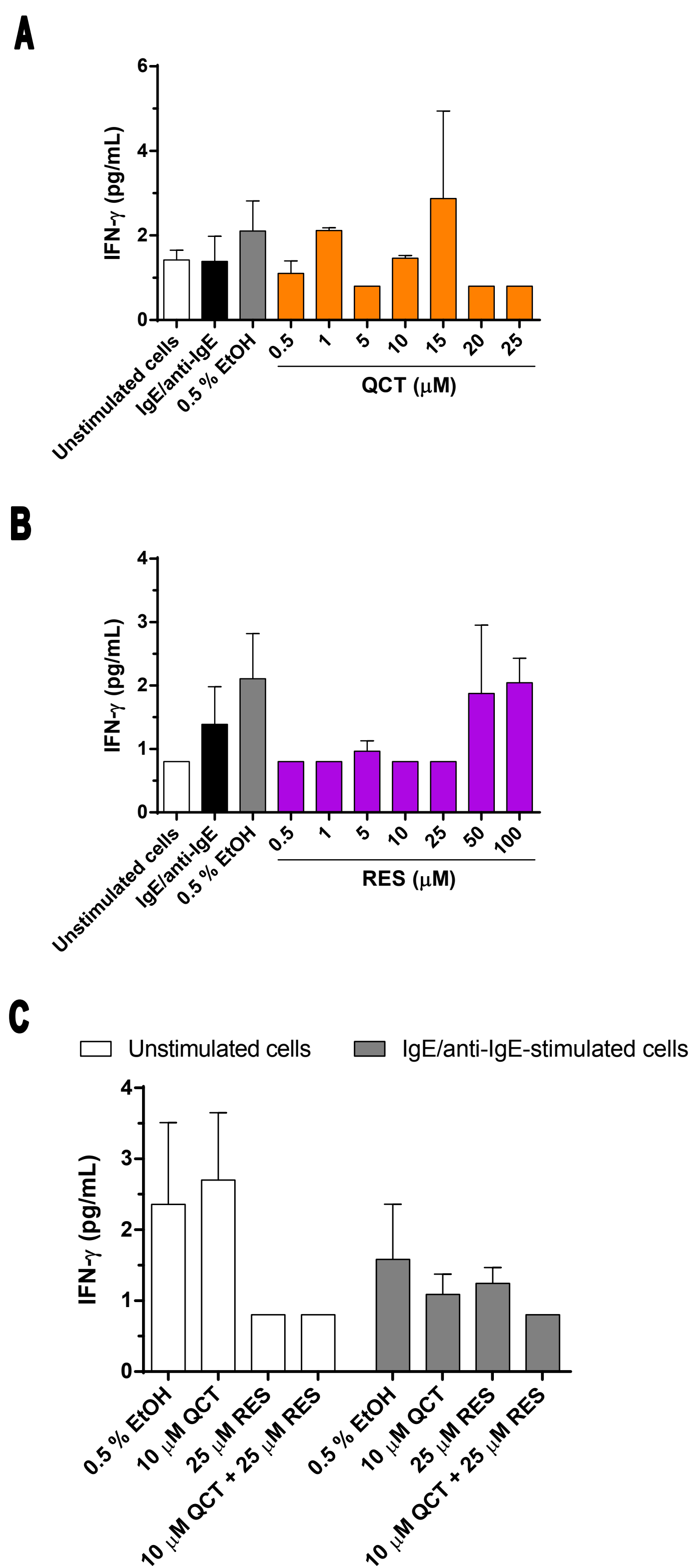

Figure 60. Effect of quercetin (QCT) (A), resveratrol (RES) (B) and QCT+RES (C) on IFN-γ secretion. EtOH: ethanol. N=2 for A and B (single samples); and N=1 for C (triplicate).

VII. Discussion

There are several ocular surface diseases, such as DED and allergy, in which inflammation is the underlying mechanism. It has been demonstrated that several types of cells play a key role in these inflammatory disorders. For example, it was demonstrated that pro-inflammatory cytokines in the conjunctival epithelium[71,79,81] and $CD4^+$ T cell infiltration into the conjunctiva and lacrimal glands are found in patients with DED.[4,10,79] Regarding ocular allergic diseases, acute allergy occurs as a result of IgE-mediated mast cell degranulation, whilst chronic allergies are mediated by T cells and continuous activation of mast cells.[119,120] This fact will help in the development of new treatments that can have a beneficial effect on the greatest number of cells involved in the inflammatory process.

The medicinal use of naturally-occurring compounds has had a great contribution to human health care, being the basis for some of today's common medicines, for example anticancer drugs. There are several sources of natural products, but interest focuses predominantly on plant sources, more specifically, on polyphenols. Two of the major polyphenols are QCT and RES because of their biological effects. These two compounds have been shown to have anticancer, antioxidant and antiviral activities, among others, but this work has focused mainly on their anti-inflammatory effects.

The aim of this thesis was to elucidate the role of both compounds and their combination as potential therapeutic agents for ocular inflammation, and more specifically, for DED and ocular allergy.

In order to confirm the hypotheses presented in this thesis, the anti-inflammatory and antioxidant effects of polyphenols were firstly determined in two human ocular surface epithelial cell lines (from cornea and conjunctiva) by measuring cytotoxicity, cytokine/chemokine secretion, COX-2 expression and intracellular ROS production. After that, the immunoregulatory properties of QCT, RES and their combination were studied in-vitro on activated PBMCs from healthy donors. Their effects of cytotoxicity and on proliferation of PBMCs were determined.

Once the anti-inflammatory effect of QCT, RES and their combination was studied in-vitro, their potential therapeutic effect on DED was corroborated using a murine model of DED, followed by an adoptive transfer model. Previous studies by Dr. Stern and colleagues pointed out that this murine model of DED allows imitation of the pathophysiological processes involved in the development of DED,[87,92-96] thus being a suitable in-vivo model for testing the polyphenols studied in this thesis. In addition, the major role of $CD4^+$ T lymphocytes in DS is corroborated by the adoptive transfer model, which could help to elucidate the mechanism underlying the anti-inflammatory properties of polyphenols.

Finally, the anti-allergy effects of both QCT and RES, and their combination was studied on sensitised/activated CBMCs. For this purpose, histamine secretion and cytokine/chemokine production by CBMCs were studied.

The use of compounds which have potent anti-inflammatory and antioxidant effects on inflammatory diseases are well documented. However, the potential effect of these compounds on ocular surface diseases has not been fully evaluated to date. This thesis has demonstrated that polyphenols, such as QCT and RES, have antioxidant and anti-inflammatory activities on cells involved in ocular surface diseases. Moreover, it has been demonstrated for the first time the immunoregulatory effect of both polyphenols in an in-vivo model of DED, inhibiting T cell activation in recipients of DS-exposed mice.

VII.1. In-vitro model of inflammation and oxidative stress in human conjunctival and corneal epithelial cells

The first objective of this thesis was to determine the anti-inflammatory effect of QCT, RES and their combination on two ocular surface epithelial cell lines, IOBA-NHC and HCE cells. For this purpose, the cytotoxicity of different concentrations of QCT, RES and their combinations were firstly determined. After that, non-toxic concentrations were evaluated for their anti-inflammatory activity on cytokine/chemokine release and COX-2 expression in an in-vitro model of ocular inflammation. Additionally, their antioxidant effects were tested on an in-vitro model of UV-induced oxidative stress.

It has been shown that cell culture medium plays a key role when polyphenols are tested in-vitro,[294] acting as pro-oxidant, degrading and decreasing polyphenol content. This degradation has been linked to the presence of $NaHCO_3$ in culture medium.[295,296] In addition, cell culture media usually contain many potential antioxidant compounds which can interfere with the antioxidant capacity of tested compounds, when studying oxidative stress in-vitro. Two of these compounds are pyruvate and phenol red.[297-300] Therefore, in order to avoid these artefacts in the results, all experiments from this part of the thesis were carried out using a culture medium devoid of $NaHCO_3$, sodium pyruvate and phenol red.

Some previous in-vitro studies have shown that polyphenols exhibit different cytotoxicity depending on the cell type used and/or specific type of polyphenol. The results from this thesis showed that QCT was not toxic for both IOBA-NHC and HCE cells, up to concentration of 25 µM. Concentrations of QCT above this value were not tested due to the very low solubility of QCT in EtOH (2 mg/mL). Thus, the amount of QCT tested in this work was restricted by the vehicle. However, as showed above in the Results section of this thesis, this solubility restriction was not a limitation to testing the biological properties of QCT. Regarding RES toxicity, this compound was not toxic up to 300 µM in both cell lines. Moreover, IOBA-NHC cells tolerated RES better than HCE cells, because the latter decreased more than conjunctival epithelial cells at 300 µM RES after 24 h exposure.

Both polyphenols were also mixed at different concentrations to test their toxicities when applied in combination. When 15, 20 and 25 µM QCT were mixed with 25, 50 and 100 µM RES, IOBA-NHC cell numbers decreased in some combinations of

QCT+RES, but viability remained above 80 % for all treatments. On the other hand, none of the combinations tested of QCT and RES were toxic for corneal epithelial cells.

Results from the cytotoxicity assays agreed with previous work. Stoddard et al. studied the antioxidant effect of some polyphenols in stratified human corneal limbal epithelial cells and found that QCT is not toxic at 56.5 µM in these cells.[301] Other authors have tested different polyphenols in ocular surface epithelial cells. For example, Chen et al. found that curcumin, a natural substance derived from the rhizome of the plant *Curcuma longa,* is not toxic up to 30 µM for immortalised human corneal epithelial cells.[302] While Cavet et al. found that the green tea polyphenol epigallocatechin gallate is not toxic at any of the doses tested (0.3-30 µM) for a human corneal epithelial cell line.[303] Taken together, these studies suggest that polyphenols in general, and QCT and RES in particular, are not toxic for ocular surface epithelial cell lines at concentrations up to 50 µM.

TNF-α is a cytokine that has pleiotropic effects including activation of apoptosis, inflammation and immune responses, in many cell types. Thus, TNF-α is a potent stimulator of cytokine/chemokine release such as IL-6, IL-8/CXCL8 and IP-10/CXCL10 by ocular surface epithelial cells.[154] However, not only does TNF-α stimulate the ocular surface epithelium, but it is also secreted by both corneal and conjunctival epithelial cells when they are stimulated by osmotic stress.[304,305]

In order to study the anti-inflammatory effect of polyphenols, TNF-α was selected as a pro-inflammatory stimulus to challenge both ocular surface epithelial cell lines. TNF-α was chosen as the stimulus for IOBA-NHC and HCE cells because this pro-inflammatory cytokine is involved in many ocular diseases, such as DED and ocular allergy. For example, TNF-α is elevated in patients with DED,[80,113] Sjögren syndrome,[71] ocular cicatricial pemphigoid,[306] and Steven-Johnson syndrome.[307] In addition, TNF-α is able to stimulate the recruitment of leukocytes to inflamed ocular surface and activate them, by promoting expression of adhesion molecules such as ICAM-1 on the ocular surface epithelium.[84] TNF-α is also released during acute allergic reactions by conjunctival mast cells, leading to upregulation of TNF receptor 1 (TNFR1) on human conjunctival epithelial cells,[308] however it has not been detected in patients with SAC.[309] Together with IL-4, TNF-α is a promoter of adhesion molecules expression and chemokine secretion from corneal keratocytes, conjunctival fibroblasts and epithelial cells, all contributing to inflammatory cell recruitment and induction of the allergic late phase reaction.[310] In addition, it is one of a number of cytokines found in patients with VKC[309,311] and AKC,[312] especially in their most severe forms.[139]

As expected, TNF-α stimulated IL-6, IL-8/CXCL8 and IP-10/CXCL10 secretion by both IOBA-NHC and HCE cells after 24 h exposure. However, TNF-α did not stimulate VEGF secretion by both cell lines. IL-6, IL-8/CXCL8, IP-10/CXCL10 and VEGF secretion were analysed because all of these cytokines/chemokines are involved in some inflammatory ocular surface diseases.

IL-6 is a pro-inflammatory cytokine that has a central role in B cell differentiation and polymorphonuclear cell infiltration,[313] being recognised as a primary mediator in the acute inflammatory response.[314] It can be synthesised by several cell types including monocytes, T lymphocytes and non-inflammatory cell types, such as epithelium and endothelium.[313,315] On the ocular surface, IL-6 has an established role in inflammatory diseases, such as DED and ocular allergy. IL-6 has been suggested as a possible biomarker for DED because it is increased in the conjunctival epithelium and tears of DED patients.[71,82,113,316,317] Moreover, IL-6 concentration in tears correlates with some clinical parameters in patients with DED, such as pain, tear film breakup time, Schirmer test, tear clearance, goblet cell density, tear lysozyme levels and conjunctival staining.[80,82,317,318]

IL-8/CXCL8 is a chemokine that belongs to the CXC chemokine subfamily that can be produced by any cell with a TLR (i.e. ocular surface epithelial cells and macrophages). It plays an important role in inflammation through its capacity to recruit T cells and non-specific inflammatory cells to sites of inflammation, and by its capacity to activate neutrophils.[319] In the ocular surface, IL-8/CXCL8 is involved in ocular inflammation and angiogenesis in the conjunctiva and cornea.[320] For example, patients with conjunctivochalasis have an increased level of IL-8/CXCL8 in tears, and this increase correlates with the severity of symptoms.[321] In addition, IL-8/CXCL8 has also been identified in tears and conjunctiva of patients with DED.[80,317] The expression of IL-8/CXCL8 is increased in the conjunctival epithelium of VKC patients and is significantly correlated with the presence of neutrophils and eosinophils.[322]

IP-10/CXCL10 is also a CXC chemokine identified as the product of a gene induced by IFN-γ.[323] IP-10/CXCL10 has been reported to attract human monocytes, T lymphocytes and NK cells,[324,325] because CXCR3, which is the specific receptor for IP-10/CXCL10, is selectively expressed on activated T cells and NK cells.[326] The restricted expression and the selectivity for a single receptor on T cells and NK cells suggest that IP-10/CXCL10 is involved in the regulation of lymphocyte recruitment and the formation of the lymphoid infiltrates observed in autoimmune inflammatory diseases on the ocular surface. For example, a high concentration of IP-10/CXCL10 is detected in tears of EDE patients,[80] and also in the conjunctiva and tears of Sjögren and non-

Sjögren syndrome DED patients.[81] In addition, an increased IP-10/CXCL10 expression in both corneal and conjunctival epithelia has been described in mice exposed to DS.[96]

VEGF (also known as VEGF-A) is a secreted growth factor peptide that belongs to a gene family that includes VEGF-B, VEGF-C, VEGF-D, VEGF-E and placental growth factor. VEGF is critical in regulating blood and lymphatic vessel development and maintenance in health and disease. Indeed, VEGF is identified in tissues of the eye that are normally vascularised such as conjunctiva, iris and the pigment epithelium of the choroid and retina.[327] Nevertheless, it plays a major role in ocular pathologies associated with tissue remodelling, inflammation and angiogenesis (growth of new blood vessels) such as pterygium,[328] age-related macular degeneration and diabetic retinopathy.[329]

It is well known that QCT and RES have an anti-inflammatory effect on many cell types, but this effect remains unknown in ocular surface epithelial cells. In order to elucidate this question, IOBA-NHC and HCE cells were treated with different doses of QCT or RES and stimulated with TNF-α. Regarding IOBA-NHC cells, both polyphenols QCT and RES inhibited the secretion of cytokines/chemokines tested. QCT decreased IL-6 (15, 20 and 25 μM), IL-8/CXCL8 (20 and 25 μM), and IP-10/CXCL10 (10, 15, 20 and 25 μM). For RES treatments, this compound also decreased IL-6 (25 and 50 μM), IL-8/CXCL8 (50 μM) and IP-10/CXCL10 (10, 25 and 50 μM). These data showed that low concentrations of both polyphenols (10 μM) were sufficient to decrease IP-10/CXCL10 secretion, whilst higher concentrations of QCT and RES were necessary to reduce IL-6 and IL-8/CXCL8 levels from conjunctival cells. In addition, although both compounds inhibited cytokine/chemokine secretion, QCT was more effective because the same inhibitory effect was reached at low doses, compared to doses of RES (i.e. QCT inhibited IL-8/CXCL8 at 25 μM, whilst RES had a similar effect at 50 μM).

Referring to HCE cells, QCT and RES also decreased the secretion of cytokines/chemokines tested. QCT decreased IL-6 (1, 5, 10, 15, 20 and 25 μM), IL-8/CXCL8 (5, 10, 15, 20 and 25 μM), and IP-10/CXCL10 (1, 5, 10, 15, 20 and 25 μM); and RES decreased IL-6 (10, 25 and 50 μM), IL-8/CXCL8 (50 μM), and IP-10/CXCL10 (0.5, 1, 5, 10, 25 and 50 μM). Both compounds significantly inhibited IL-6 and IP-10/CXCL10 dose-dependently from low doses. IL-8/CXCL8-stimulated secretion was only inhibited by QCT dose-dependently, whilst only a high dose of RES (50 μM) was able to inhibit IL-8/CXCL8. In contrast to conjunctival epithelial cells, QCT and RES had similar inhibitory effect on HCE cells because low doses of both compounds decreased cytokine/chemokine secretion (IL-6 and IP-10/CXCL10). This indicates that the anti-inflammatory effects of QCT and RES on IL-6 and IP-10/CXCL10 secretion is stronger

in HCE cells than IOBA-NHC cells, and QCT is more effective that RES in inhibiting IL-8/CXCL8 secretion by both cell lines.

Interestingly, 25 μM QCT decreased cytokine/chemokine concentrations (IL-6, IL-8/CXCL8 and IP-10/CXCL10) to control values in both cell lines, although significant differences were found in some data when compared to controls. This is due to the fact that there was a different amount of variability or spread, in the dataset. Data from cells exposed to TNF-α (treated or not with polyphenols) had higher variability than those found in control samples (cells not exposed to TNF-α). This may be solved by increasing the number of experiments performed.

Once the effects of QCT and RES were tested separately, both polyphenols were mixed and the anti-inflammatory properties of their combination were studied in both cell lines. For this purpose, one low dose from each compound was chosen based on dose-response curves: the concentrations selected were 0.5 μM QCT and 5 μM RES, because neither concentration had a significant effect on almost all cytokines/chemokines studied (except for RES on IP-10/CXCL10 secretion by HCE cells). Results from this thesis showed that the combination 0.5 μM QCT + 5 μM RES decreased stimulated secretion of IL-6 and IL-8/CXCL8 by IOBA-NHC cells. Although the inhibitory effect of 0.5 μM QCT + 5 μM RES on IL-8/CXCL8 was similar to that provoked by 0.5 μM QCT alone, in this case no significant difference was found when compared to the control group. In addition, 0.5 μM QCT did not show any effect in the dose-response curve, whilst it did in the experiment using the combination of polyphenols. The combination of QCT and RES inhibited IP-10/CXCL10 stimulated secretion by cells to a greater extent than both polyphenols did separately. Although this inhibition did not reach statistical significance compared to TNF-α-stimulated cells treated with vehicle.

Referring to HCE cells treated with the combination of polyphenols, the inhibition of cytokine/chemokine secretion was not significantly different from that provoked by polyphenols alone. These results suggest that QCT and RES are effective in inhibiting multiple cytokines and chemokines induced by a pro-inflammatory mediator (TNF-α), demonstrating that they might have a therapeutic potential in the treatment of ocular surface inflammatory diseases. A stronger inhibitory effect was observed in the conjunctival epithelial cells when treated with the combination of low doses of polyphenols, but this effect was not found in corneal epithelial cells. Thus, it is hypothesised that other combinations may have different effect compared to those found for 0.5 μM QCT + 5 μM RES.

Although there are very few studies regarding the effects of natural compounds on the ocular surface, similar results to those described in this thesis have been

described for other polyphenols in ocular surface epithelial cells. Chen et al. demonstrated that curcumin has an anti-inflammatory effect on HCE cells exposed to increasing osmolarities of cultured medium (with sodium chloride, NaCl).[302] They found that 5 μM curcumin can abolish the hyperosmoticity-induced production of cytokines such as IL-1β, IL-6 and TNF-α, as well as IL-1β mRNA and activation of p38. Therefore, they suggested that curcumin inhibits hyperosmoticity-induced increase of IL-1β production in HCE cells, through inhibition of p38, which leads to NF-kB p65 inhibition by curcumin. Cavet et al. also described that epigallocatechin gallate has an anti-inflammatory effect on HCE cells using two stimuli, IL-1β and hyperosmolarity with sucrose.[303] The authors found that IL-1β increases the release of IL-6, IL-8/CXCL8, MCP-1, G-CSF and GM-CSF, whereas hyperosmolarity only increases IL-6 and MCP-1. The polyphenol epigallocatechin gallate decreases IL-1β- and hyperosmolar-induced cytokine/chemokine secretion by HCE cells, in a dose-dependent manner from 3 to 30 μM. In addition, this polyphenol inhibits the IL-1β- and hyperosmolar-induced phosphorylation/activation of both p38 and JNK in HCE cells. Their results also indicated that inhibition of IL-1β-induced cytokine expression is, at least in part, mediated by inhibition of both AP-1 and NF-kB transcription activities by epigallocatechin gallate.

The present study has shown that TNF-α did not provoke VEGF secretion by any of both epithelial cell lines, although it has been demonstrated that normal and inflamed ocular surface epithelial cells can secrete VEGF.[330,331] For example, VEGF levels are increased in tears of mild-to-moderate DED patients[80] and in conjunctival biopsies and tears of patients suffering VKC or AKC,[332] playing a crucial role in the remodelling process of these severe allergic conjunctival disorders.[333] Yet, the effect of QCT and RES on VEGF secretion by ocular surface epithelial cells could not be determined in this in-vitro model. However, previous studies have reported that QCT and RES can affect VEGF production in other cell types. For example, stimulation of human retinal pigment epithelial cells (ARPE-19) with glucose in the presence of 0-10 μM RES dose-dependently inhibits VEGF, along with IL-6, IL-8/CXCL8 and COX-2.[334] Similar results were found when human retinal pigment epithelial cells from donors were stimulated with platelet-derived growth factor or cobalt chloride in the presence of 0-50 μM QCT, as QCT also inhibit VEGF in a dose-dependent manner.[335] In addition to that, it has been demonstrated that polyphenols can also inhibit VEGF in-vivo. Koh et al. evaluated the efficacy of topical application of the green tea polyphenol epigallocatechin gallate for the treatment of corneal neovascularisation in a rabbit model. They induced neovascularisation by placing a black silk suture in the corneal

stroma for a week. After that, they treated rabbits with topical epigallocatechin gallate for 2 weeks. Their results showed that epigallocatechin gallate effectively inhibits corneal neovascularisation in rabbits by decreasing VEGF messenger RNA levels, along with a decrease of COX-2 expression.[336] Our in-vitro model of inflammation demonstrated that TNF-α did not induce VEGF secretion by conjunctival and corneal epithelial cells. However, VEGF levels are increased in some ocular diseases as it has been pointed out above and it is hypothesised that both conjunctival and corneal epithelial cells may secrete VEGF using other stimuli such as hypoxia,[337] which could also permit the study of the effect of polyphenols on VEGF secretion by ocular surface epithelial cells.

Besides cytokines and chemokines, prostaglandins and thromboxanes (collectively termed prostanoids) play an important role in the generation of the inflammatory response.[338] They are formed when arachidonic acid is released from the plasma membrane by phospholipases and is metabolised by the sequential actions of enzymes. These are bifunctional enzymes (having both cyclooxygenase and peroxidase activities) and exist as two isoforms referred to as COX-1 and COX-2.[339] COX-1 is expressed constitutively in most cells, being the dominant source of prostanoids that subserve housekeeping functions, such as gastric epithelial cytoprotection and homeostasis.[340] COX-2 is the more important source of prostanoid formation in inflammation and in proliferative diseases, being induced by inflammatory stimuli, hormones and growth factors.[340] COX-2 is also involved in inflammatory disorders of the eye. For example, samples from patients suffering pterygium have COX-2 expression, while normal conjunctiva and limbus specimens do not express COX-2, indicating that COX-2 only exists in the pterygium and not in the normal conjunctiva and limbus.[341] Increased levels of prostaglandins in tears are found in patients with DED, which correlates with patients' symptom scores.[342] These clinical results are supported by increased COX-2 and prostaglandins expression levels in tear-producing tissues of DED mice.[342] In addition, it seems that prostaglandins, and therefore COX-2, are involved in allergic conjunctivitis as non-steroidal anti-inflammatory therapy, which inhibits COX enzymes, is commonly used to alleviate itching associated with allergic conjunctivitis.[343]

In order to complement the study of the anti-inflammatory properties of polyphenols, the effects of QCT and RES on basal and TNF-α-stimulated COX-2 expression in both epithelial cell lines, were analysed. These results showed that TNF-α induced COX-2 expression in IOBA-NHC cells. Regarding the effect of polyphenols, neither QCT, nor RES, nor their combination had any effect on COX-2 expression in

IOBA-NHC cells, compared to stimulated cells treated with 0.5 % EtOH. However, COX-2 levels expressed as a fold-change with respect to each control showed that QCT and QCT+RES decreased COX-2 expression but not significantly. Surprisingly, 25 µM QCT induced an increase of COX-2 expression in both unstimulated and stimulated IOBA-NHC cells, but this increase was less than control cells when the fold change was calculated. This unexpected result may suggest a pro-inflammatory role of QCT, however results from this thesis and scientific literature have shown that QCT is a COX inhibitor compound in several types of cells. For example, Qureshi et al. found that QCT inhibits LPS-induced expression of COX-2 in peritoneal macrophages from mice.[259] Similar results were reported by García-Mediavilla et al. who found that QCT produces a large concentration-dependent decrease of COX-2, as well as inhibition of mRNA levels, in Chang liver cells.[262]

Referring to HCE cells, TNF-α did not induce COX-2 expression in corneal cells. This is a notable limitation of this in-vitro assay because TNF-α did not stimulate a positive control in order to study the effect of polyphenols on COX-2 expression by HCE cells. Thus, comparison of how polyphenols change COX-2 expression relative to vehicle may not be relevant.

Nevertheless, results from this thesis demonstrated that QCT, RES and a mix of both compounds decreasing TNF-α-induced COX-2 production by conjunctival epithelial cells.

In this work it has also been demonstrated that polyphenols can protect conjunctival and corneal epithelial cells from UV-B radiation-induced oxidative stress. IOBA-NHC and HCE cells were irradiated with UV-B light for 15 s (107.25 mJ/cm^2). The average value of solar UV-B radiation reaching the human cornea is 105 mJ/cm^2 during a 1 h exposure.[344] Thus, the irradiance of the lamp used in this experiment was energetically relevant as the energy of 1 h exposure was condensed to 15 s. As expected, this UV-B radiation provoked an increase of intracellular ROS production in ocular surface epithelial cells.[280]

UV radiation is one of the largest causes of ROS induction in the eye and plays an important role in the pathogenesis of a large number of ocular diseases. For example, one of the most common acute effects of UV-B radiation on the cornea is photokeratitis.[345,346] Chronic UV radiacion can also provoke solar keratopathy,[347] pterygium,[348,349] and ocular melanoma in the conjunctiva.[347,350] Oxidative stress, along with inflammation, are involved in all of these pathologies. Cytokines, such as IL-6 and IL-9, are released by the epithelial cells in pterygium after UV-B exposure[349] and oxidative mediators have also been found in patients with primary pterygium.[351] In-vivo

models of photokeratitis have demonstrated that there is a decrease in antioxidant enzymes in the corneal epithelium, when eyes are excessively exposed to UV-B. This event provokes an oxidative stress, leading to transcriptional activation of inflammatory factors such as NF-κB and iNOS, and formation of cytotoxic nitric oxide and nitrogen-related oxidants.[352-354]

When IOBA-NHC and HCE cells were exposed to UV-B and treated with different doses of QCT or RES, ROS production decreased in a dose dependent-manner in both cell lines. This effect was stronger in HCE cells than that found in IOBA-NHC, suggesting that polyphenols are more effective at protecting corneal epithelial cells from UV-B light. Moreover, the antioxidant effect of QCT was stronger than the antioxidant effect of RES in HCE cells: a lower concentration of QCT (1 µM) was necessary for decreasing ROS production significantly, compared to RES (25 µM). In addition, the mix of both compounds abolished the significant increase of intracellular ROS production in both cell lines, compared to UV-B unexposed cells. These results indicate that QCT, RES and the mix of low doses of both compounds can decrease the oxidative stress involved in cells exposed to UV-B radiation. These results agree with previous studies. For example, the antioxidant effect of QCT was previously described by Stoddard et al. in stratified human corneal limbal epithelial cells.[301] Although they did not expose cells to UV-B radiation, they found that the concentration of QCT which gives 50 % of the maximum response (EC50) is 2.98 µM QCT, when cells are exposed to 2,2'-azobis(2-amidinopropane) dihydrochloride (to generate intracellular ROS).

Regarding the antioxidant effect of other polyphenols on ocular surface epithelial cells exposed to UV radiation, similar results were described by Larrosa et al. using two phenolic compounds: hydrocaffeic acid and p-coumaric acid.[355] They found that 10 µM hydrocaffeic acid alone and a mixture of both compounds (5 µM hydrocaffeic acid + 5 µM p-coumaric acid) reduce oxidation damage in human conjunctival cells exposed to UV-B radiation (4.4 mJ/cm^2). In addition, they also treated rabbit eyes with hydrocaffeic acid alone and a mixture of both compounds drops before UV-B exposure (7.9 mJ/cm^2), and showed that polyphenols reduce corneal and scleral DNA oxidation damage, xanthine oxidase activity and MDA levels in the corneal tissues of rabbits. Similar results have been reported recently by Chen et al.[356] They exposed rabbits to UV-B radiation (100 mJ/cm^2) and further treated them with topical epigallocatechin gallate (0.01 % and 0.1 %) for 8 weeks. Treatment with epigallocatechin gallate eye drops ameliorates corneal damage, as well as increasing SOD, catalase and GSH activity in the corneas, compared to the UV-B-treated group.

Corneal and conjunctival epithelial cells play a major role in ocular surface inflammation. Both types of cells can secrete inflammatory mediators when they are challenged by pro-inflammatory stimuli. This thesis has demonstrated for the first time that two naturally-occurring compounds, QCT and RES, and their combination modulate the inflammatory response of ocular surface epithelial cells in-vitro, decreasing cytokine/chemokine release when epithelial cells were exposed to TNF-α. Both polyphenols, QCT and RES, have shown to have similar effect on both ocular surface epithelial cells, but QCT is more effective than RES. In addition, the results described in this thesis have also demonstrated that QCT and RES can protect epithelial cells from UV-B-induced oxidative stress. The chronic inflammation is linked to the oxidative stress because this has been evidenced in ocular surface diseases, such as DED and ocular allergy. Results from this thesis suggest that QCT and RES may exert their protective effect via both pathways. This is an important and relevant result because a novel therapy using polyphenols may act as both an anti-inflammatory antioxidant therapy when applied topically to the ocular surface.

VII.2. In-vitro proliferative response of human T cells

One of the therapeutic objectives in tissue inflammation is to reduce the local inflammatory response by regulation of T cell activation and proliferation and cytokine production. $CD4^+$ T cells play a key role in several ocular surface diseases such as DED and allergy. Thus, after testing the anti-inflammatory effect of polyphenols in ocular surface epithelial cells, the second objective of this thesis was to test the immunoregulatory effect of QCT, RES and their combination on PBMCs from healthy donors. For this purpose, the cytotoxicity of polyphenols was firstly determined and thereafter the antiproliferative activity of QCT, RES and QCT+RES was tested on PBMCs.

Results from this thesis have demonstrated that cytotoxicity of QCT, RES and QCT+RES increased in a dose-dependent manner. It was also demonstrated that the polyphenol combination decreased proliferation of activated PBMCs, without affecting their viability. In this in-vitro model of inflammation, PBMCs from healthy donors were activated using anti-CD3/anti-CD28 antibodies.

A wide range of agents can be used to induce T cell activation and proliferation, such as calcium ionophore combined with phorbol ester and lectins (phytohemagglutinin (PHA) and concanavalin A). However, a direct way of inducing T cell activation involves stimulation with monoclonal antibodies that interact with the TCR/CD3 complex. This method, which was used in this thesis, has the advantage that it is a more physiologically relevant approach to stimulate T cells in a manner that partially imitates stimulation by APCs. Thus, the in-vitro model used in this thesis for activating PBMCs attempts to simulate immune-complex-mediated activation and proliferation of T cells following the two-signal model of lymphocyte activation, which has prevailed as a dominant paradigm to explain how T and B cells could be constrained into discriminating self from non-self.[357]

In order to test the anti-inflammatory effect of polyphenols, activated PBMCs were treated for 96 h with a wide range of QCT, RES or QCT+RES concentrations to determine which concentrations were toxic for PBMCs. The results showed that high doses of RES decreased activated PBMC numbers, whilst none of the QCT concentrations tested decreased cell viability. In contrast, QCT and RES combinations increased cytotocity when the concentration of both polyphenols increased. This indicates that RES is more toxic than QCT for PBMCs.

Once the cytotoxicity of polyphenols was tested, low concentrations of QCT and RES were studied individually and in combination, in order to discern whether polyphenols could affect cell proliferation after 120 h, using the CFSE assay counterstained with PI. Results showed that 5 μM RES in combination with 5 and 10 μM QCT, and 10 μM RES in combination with 0.5, 1, 5 and 10 μM QCT decreased PBMC proliferation, whilst only 10 μM RES showed a tendency to decreased cell proliferation. This is the most relevant result from this part of the thesis because it demonstrates the antiproliferative effect of QCT and RES on PBMCs. Moreover, this effect on proliferation was not due to a decrease in cell viability as PI staining demonstrated. More studies are necessary to elucidate whether other concentrations of QCT and RES, and other combinations of both compounds are more effective.

It is necessary to emphasise that the cytotoxicity experiments and the viability data from the proliferation assays were not comparable to each other, although they both showed PI negative cells. This is due to the fact that samples from the cytotoxicity assay were gated at the end of the experiment (96 h) and compensation was performed pre-acquisition by the instrument, prior to analysing all samples. In contrast, the CFSE assay was gated at day 0 and compensation was performed post-acquisition using specific software (WinList software) after 120 h of culture. All these circumstances make it very difficult to compare to both experiments. For example, some concerns may arise comparing the results from the cytotoxicity assay against the viability results from the proliferation assay: the combination of 5 and 10 μM RES with 5 and 10 μM QCT increased cytotoxicity significantly (Figure 33), whereas the same polyphenol combinations did not decrease cell viability in the proliferation assay as shown in Figure 34B. In addition, low doses of RES (0.5 and 1 μM) in combination with QCT were tested in the first experiment (n=1), along with 5 and 10 μM RES, but only these latter concentrations diminished proliferation (without affecting cell viability). Therefore, only 5 and 10 μM RES in combination with QCT were tested subsequently. Nevertheless, it was demonstrated that the combination of low doses of QCT and RES can inhibit PBMC proliferation in-vitro.

In addition, some data from the cytotoxicity and proliferation assays were above 100 %. This effect was due to the fact that all data were normalised to stimulated (no treated) cells (representing 100 %). Thus, values above 100 % only indicated that these values were higher that those found in stimulated (no treated) cells.

The antiproliferative effect in-vitro of QCT and RES individually on PBMCs has been reported in the scientific literature. For example, Lugli et al.[358] stimulated CFSE-labelled PBMCs with PHA for 72 h, adding QCT (10, 50 and 100 μM) at 24 or 48 h after

stimulation. They found that 10 μM QCT (added 48 h after PHA stimulation) slightly inhibited cell proliferation, while 50 and 100 μM concentrations are much more efficient at decreasing cell proliferation. This effect is more consistent when QCT is added 24 h after PHA stimulation. In contrast to these results, Sternberg et al.[359] stimulated PBMCs isolated from healthy donors with PHA in the presence of QCT (0, 0.2, 1, 5, 10, 25, 50, 100 and 200 μM) and found that QCT inhibits PBMC proliferation in a dose-dependent manner after 48 h of culture. The effect of QCT on cell proliferation is significant from the 5 μM concentration. In addition, QCT (at a concentration of 200 μM) reduces cell proliferation by 35 %, with no cytotoxicity (determined using the trypan blue dye exclusion test). Moreover, the antiproliferative activity of QCT was also reported in other normal cell types such as human umbilical vein cord blood cells[360] and normal $CD34^+$ bone marrow progenitors in-vitro.[361]

Regarding the immunomodulation role of RES, Falchetti et al. studied its effect on PBMC proliferation and viability.[362] They stimulated PBMCs with anti-CD3 for 3 days and further stimulated cells with anti-CD28 for 3 days. PBMCs were treated with RES (0-44 μM) at day 1 and 4. They found that RES (22 and 44 μM) completely blocks anti-CD3/anti-CD28-induced PBMC proliferation with no significant effect on cell viability, whereas RES has no effect on cell proliferation at lower concentrations. Other authors reported similar results. For example, Boscolo et al.[363] stimulated PBMCs with PHA in the presence of 0.1, 10 and 100 μM RES and analysed cell proliferation after 72 h, using 5-bromo-2'-deoxyuridine. This study showed that 100 μM RES strongly inhibits PHA-stimulated PBMC proliferation, but it does not exert this effect at 10 μM. Larbi et al. also reported similar results.[364] They stimulated CFSE-labelled PBMCs with anti-CD3/anti-CD28 coated beads and treated them with RES (5, 10, 25 and 50 μM) under hyperoxic conditions (culture in air) for 6 days. They found that RES has an inhibitory effect on T cell activation by anti-CD3/anti-CD28-coated beads at 25 and 50 μM concentrations, but not at 5 and 10 μM. However, this proliferation decrease is due to increased RES-induced apoptosis of PBMCs, explaining their decreased proliferation effect under hyperoxic conditions. In addition, it has been suggested that RES may exert its antiproliferative effect by down-regulating the expression of CD28 and CD80, as previously described in concanavalin A-stimulated splenocytes from mice.[214] Based on these published results, it might be hypothesised that QCT and RES could increase their antiproliferative effect on anti-CD3/anti-CD28-stimulated PBMCs at concentrations higher than those used in this thesis.

Results from this thesis have shown that the combination of QCT+RES at low doses was able to decrease proliferation of anti-CD3/anti-CD28-stimulated PBMCs more than QCT and RES separately. Thus, a mix of low doses of QCT and RES can

be more effective than the same concentration of polyphenols separately, as an immunomodulatory treatment in-vitro. Some studies also point out that mixing of polyphenols can affect cell proliferation at low concentrations. For example, Cosentino et al. studied the effect of *Achyrocline satureioides* (an aromatic herb from South America) infusion on PBMC proliferation.[365] They tested a range of total polyphenol concentrations expressed as QCT equivalents (0.0006-0.24 µg/mL QCT equivalents ~ 0.002-0.79 µM QCT equivalents), because QCT is one of the main flavonoids contained in *Achyrocline satureioides*.[366] This group found that treatment of PHA-stimulated PBMCs from healthy donors reduces concentration-dependent PHA-induced proliferation to complete inhibition in the 0.2-0.79 µM QCT equivalent concentration range (after 48 h of culture), without affecting cell viability. The authors concluded that the effect exerted by *Achyrocline satureioides* infusion at low concentrations seems unlikely to depend solely upon the activity of QCT, because *Achyrocline satureioides* has shown the presence of many flavonoids, terpenoids and other chemicals.[366]

In this thesis, it has been demonstrated that a combination of QCT and RES exert an inhibitory activity on normal immune functions, such as T cell activation and proliferation. This result showed that a combination of low doses of QCT+RES have a stronger antiproliferative effect on PBMCs than both compounds alone. More studies are necessary in order to elucidate whether this effect also occurs at higher doses.

The reduction in cell proliferation by QCT+RES is an important observation, since circulating autoreactive T cells in inflammatory ocular surface diseases may initiate a second cycle of inflammation, which subsequently may lead to the induction of a chronic process.

VII.3. Experimental murine model of dry eye and adoptive transfer mode

The results from the in-vitro experiments have shown the potential of QCT, RES and QCT+RES as an anti-inflammatory therapy on different types of cells, such as conjunctival and corneal epithelial cells and PBMCs. These cells, among others, play an important role in several immune-based ocular surface diseases such as DED. Thus, the third objective of this thesis was to test QCT, RES and QCT+RES combination in an in-vivo model of DED in order to confirm their anti-inflammatory activities.

The DED model used for this thesis has been extensively studied and characterised because the mice develop ocular surface lesions similar to those found in DED patients. Mice exposed to DS have an increased CFS, reduced tear production, decreased goblet cell density and $CD4^+$ T cell infiltration in conjunctiva, including an increase of cytokine/chemokine concentrations in tears.[87,93-96,99,367]

In this thesis, DS induced a significant decrease in tear production, as well as CFS in donor mice, as previously described.[87] In contrast to the literature, however, DS did not affect either goblet cell numbers or $CD4^+$ T cell infiltration of the conjunctiva. As suggested by Dr. Stern (personal communication), it may be hypothesised that it is due to the fact that the mouse exposed to DS has the complication of a fluctuating innate response that is superimposed over the adaptive immune result.

Prior to testing natural compounds in-vivo, the concentrations for both compounds were chosen based on published studies and a preliminary study carried out in Allergan in 2011. Cavet et al.[303] and Lee et al.[368] tested the effect of the green tea polyphenol epigallocatechin gallate in-vitro (using HCE cells) and in-vivo (in a DED model), respectively. Cavet et al. found that epigallocatechin gallate has a significant anti-inflammatory effect at 30 µM on HCE cells. Results from in-vitro studies of this thesis have shown that similar concentrations of QCT and RES, to that found by Cavet et al., also had an anti-inflammatory effect on HCE cells. In addition, Lee et al. found that epigallocatechin gallate has a significant anti-inflammatory effect on DED model at a concentration of 0.01 %. Thus, it was hypothesised that a similar concentration of QCT and RES might also show their anti-inflammatory properties in the DED model. As a result, 0.01 % QCT, 0.01 % RES and their combination were tested in a preliminary study using an in-vivo model of DED (the most important result from this experiment is shown in the patent derived from this thesis in Appendix I). Based on this preliminary

experiment, it was finally decided for experiments in this thesis to test 0.01 % QCT and increase RES concentration up to 0.1 %, and to test RES alone and in combination with 0.01 % QCT.

This part of the thesis has demonstrated that topical application of 0.01 % QCT, 0.1 % RES or 0.01% QCT + 0.1% RES can decrease the clinical signs (CFS) and inflammatory response (cytokine production) of the ocular surface in a murine model of DED.

CFS is a routine clinical test for determining the corneal epithelial integrity in patients. When corneal epithelial cells are damaged (leading to cell-cell junction disruption), fluorescein uptake occurs providing a visible staining.[369] This disruption renders the ocular surface susceptible to environmental insults. Corneal staining is usually found in patients suffering ocular surface diseases such as DED, and even in patients exposed to a controlled adverse environment,[66] which points out that the corneal epithelium is compromised. Current results from this study showed that topical treatment with 0.01 % QCT and 0.01 % QCT + 0.1 % RES decreased clinical signs associated with corneal staining, thus protecting the ocular surface by enhancing DS-exposed mouse corneal integrity. Surprisingly, these results did not correlate to TP because neither QCT nor QCT+RES had any effects on tear production. As it has been pointed out previously in this thesis based on scientific literature, DS and scopolamine induce clinical disease through different mechanisms.[88] As a result, the CFS score increases for both mechanisms but DS only causes a slight tear reduction at the early time points (days 3 and 7) that is not conserved at later time points (days 10 and 14), whilst scopolamine inhibits tear secretion at day 10.[88] Moreover, CFS score increases more in mice without lacrimal glands (extraorbital lacrimal gland excision) exposed to DS, than mice treated with scopolamine exposed to DS. According to Chen et al.,[88] these results correlate to a higher Th17 response in lacrimal gland excision+DS mice, compared to scopolamine+DS mice, indicating that Th17 is involved in regulating corneal integrity. Thus, it is hypothesised that the protective effect of QCT and RES on corneal integrity may be due to an inhibitory effect upon Th17 cells. More studies are necessary to elucidate the mechanisms underlying the improvement of corneal integrity by polyphenols.

This protective effect of studied polyphenols on corneal integrity agrees with a previous study by Lee et al.[368] They tested the effect of the green tea polyphenol epigallocatechin gallate in a similar in-vivo DED model. They applied 0.01 % and 0.1 % epigallocatechin gallate solutions topically to both mouse eyes twice daily starting 48 h after DS induction. They found that topical treatments at day 4, including vehicle (1 %

DMSO in PBS), have a significant decrease in corneal staining compared to the untreated group, but they also reported that there is no difference in corneal staining among these treatment groups. However, after 9 days of DS, 0.1 % epigallocatechin gallate-treated eyes show a significant decrease of corneal staining compared to the untreated group and vehicle-treated group, without differences between 0.01 % epigallocatechin gallate-treated and vehicle-treated groups.

DED was induced in mice by administering scopolamine (0.1 mg/day) via implanted osmotic pumps and then exposing them to DS in a controlled-environment chamber (23 °C, 20 % relative humidity, airflow from fans for 10 h a day). Cholinergic blockade with scopolamine and exposure to DS induce clinical disease through different primary pathogenic mechanisms. Scopolamine is a tropane alkaloid that antagonises muscarinic activity. Thus, it inhibits acetylcholine-mediated stimulation of the lacrimal gland by blocking muscarinic acetylcholine receptors. In the DED model, systemic administration of the anticholinergic scopolamine promotes pathogenic aqueous tear deficiency and inflammation of the lacrimal gland.[370] In contrast, environmental stress induced by low relative humidity and constant airflow does not lead to significant tear deficiency.[88] In this thesis, the results have shown that QCT and RES did not have any effect on tear production. Thus, topical administration of polyphenols did not affect lacrimal gland function. However, it has been recently shown that QCT is able to have an effect on the lacrimal gland, when it is administered orally in an in-vivo model of diabetes.[371] The authors found that diabetic mice fed *ad libitum* for 3 months with an experimental diet containing 0.5 % QCT have a significant recovery of tear volume, along with changes in the lacrimal gland morphology, which indicates improvements in the pathological features of diabetes. Based on these results, it is hypothesised that administration of polyphenols orally and topically might increase the anti-inflammatory effect on DED disease found in this thesis.

Cytokine/chemokine concentrations in tears were also analysed by immuno-bead assay: 6 and 4 out of 15 cytokines/chemokines included in the array were detected at days 6 and 10, respectively. This loss of cytokine/chemokine detection in the tear fluid at day 10 may be due in part to i) a concentration effect caused by decreased tear volume as the phenol red cotton thread test demonstrated, and/or ii) cytokine/chemokine levels increase at early stages of DS and decrease after 10 days of DS, as has been described previously for MMPs in tears of DS-exposed mice.[372] Nevertheless, the pro-inflammatory cytokine IL-1α and chemokine RANTES/CCL5 were increased in DS-exposed mouse tears. Topical treatment with 0.01 % QCT, 0.1 % RES or 0.01 % QCT + 0.1 % RES decreased IL-1α level. Although DS did not

significantly increase IL-4 at day 6 and IP-10/CXCL10 at day 10, there was a tendency to decreased cytokine/chemokine levels by 0.01 % QCT. All of these molecular mediators can induce immune-mediated inflammation. For example, RANTES/CCL5 is a chemokine involved in recruitment of T cells, IP-10/CXCL10 preferentially attracts Th1 cells, and IL-1α (a pro-inflammatory form of IL-1), is implicated in inducing inflammatory cytokine/chemokine expression (i.e. IL-6, IL-8/CXCL8, MCP-1/CCL2 and GM-CSF), as well as MMP synthesis by keratocytes, corneal fibroblasts and corneal epithelial cells.[373] All of these pro-inflammatory mediators are increased in patients suffering DED.[80,374] Thus, it is suggested that there is potential for QCT and RES as anti-inflammatory treatments of ocular surface diseases. In addition, Lee et al. demonstrated that polyphenols can also decrease inflammatory cytokine expression in mouse cornea.[368] They quantified the transcripts encoding IL-1β, TNF-α and MCP-1/CCL2 in corneas of DS-exposed mice and found that treatment with topical 0.01 % and 0.1 % epigallocatechin gallate decreases the relative expression of IL-1β and MCP-1/CCL2 transcripts, but not the TNF-α transcript. These results suggest that the anti-inflammatory effect of topical polyphenols on cytokine secretion may occur, in part, at early stages of the inflammatory response. Thus, more studies are necessary to elucidate the mechanisms of how polyphenols inhibit cytokine/chemokine concentrations in tears of the inflamed ocular surface.

After 10 days of DS, an adoptive transfer experiment was performed to determine the effect of polyphenols in $CD4^+$ T cells, in DS-induced ocular inflammation. As described in the “material and methods” section, draining CLN cells and spleen cells were collected from donor mice after 10 days of DS exposure and topical treatments with 0.01 % QCT, 0.1 % RES or the combination of both concentrations. $CD4^+$ T cells were then adoptively transferred to recipient athymic (nude) mice, which were maintained under non-stressed conditions for 72 h. The athymic mouse is an immunodeficient mouse that is not able to produce $CD4^+$ T cells. Thus, the recipient mouse is a *tabula rasa* (blank slate) in that it is not exposed to anything that will initiate an innate response, giving a clean evaluation of the cellular adaptive response in the recipient. It should be noted that inflammation in recipients of CLN and spleen cells from DS donors is restricted to the LFU (cornea, conjunctiva and lacrimal gland), and no inflammatory cell infiltrates are found in the salivary gland, oral mucosa, thyroid, heart, lung, colon, spleen, adrenal gland or vagina.[94] This model offers the advantage of more precisely dissecting T-cell-specific aspects (effector and regulatory) that contribute to the development of, and protection from, ocular surface autoimmunity.[375]

The results from this thesis have shown that recipients of DS-exposed donors had an increase of $CD4^+$ T cell infiltration in the conjunctiva, although recipient mice were never exposed to DS. This is an expected result, as previously described by Niederkorn et al., who also found an increase of pro-inflammatory cytokine levels in tears, an extensive cellular infiltration into the conjunctiva, cornea and lacrimal gland, a decrease of TP and loss of goblet cells.[94] These results described by Niederkorn and colleagues demonstrate that inflammation in a DS-induced mouse DED model is immune-mediated. Surprisingly, in our case, no decrease of TP and goblet cell numbers were observed, nor was there an increase of cytokine/chemokine concentration in tears of recipients after receiving CLN and spleen cells from DS-exposed donors. Bearing in mind that this adoptive model lasted 3 days, a more prolonged adoptive transfer model might be required in order to find significant changes in aqueous tear production, goblet cell number and cytokine/chemokine concentration in tears of recipients.

The results from this thesis have also clarified that the anti-inflammatory effect of polyphenols is, in part, due to the inhibition of $CD4^+$ T cells in recipient mice, which play an important role in the development of DED.[70] The results have shown that 0.01 % QCT and 0.1 % RES decreased $CD4^+$ T cell infiltration in the conjunctiva of recipients of polyphenol-treated DS mice. In addition, the 0.01 % QCT + 0.1 % RES combination did not significantly decrease $CD4^+$ T cell infiltration, but its value was clinically relevant because it was similar to that found in recipients of control mice. These results, which are likely to be the most important findings derived from this thesis, also corroborated the immunoregulatory effect of polyphenols on PBMCs in-vitro (described in Section VI.2).

Although more studies are needed to elucidate the mechanisms involved in the abolishment of the inflammatory response by polyphenols, it has been proposed that this inhibitory effect may also occur in cells involved with $CD4^+$ T cell activation. For example, Lee et al. also studied the effect of epigallocatechin gallate on $CD11b^+$ cells (monocytes/macrophages) in a DED model.[368] They found that an increased number of central and peripheral corneal $CD11b^+$ cells in mice exposed to DS, whilst tropical treatment with 0.1 % epigallocatechin gallate decreases $CD11b^+$ cells, compared to the untreated and vehicle-treated groups. However, as the authors pointed out, the mere presence or absence of $CD11b^+$ cells may not reflect their functional status. APCs, such as monocytes/macrophages ($CD11b^+$) and DCs ($CD11c^+$), play a pivotal role during the development and progression of DED by bridging the innate and adaptive immune response to activate autoreactive T cells and maintain primed and targeted T

cells at the ocular surface.[70,99] In fact, one of the most recent studies by Dr. Stern and co-workers demonstrated that the APCs responses are modulated by Treg cells (inducible and natural), which increase early in response to DS and have an increased ability to suppress DC maturation and T cell proliferation by paracrine-mediated inhibition.[376] Moreover, they also reported that stress-induced damage to the ocular surface resembling DED can occur independently of chronic T cell-mediated disease.[377] Thus, further studies are necessary to elucidate whether QCT, RES and their combination have any effect on other immune cells that play a critical role in the in-vivo model of DED, such as APCs and Treg cells.

The anti-inflammatory therapies have been accepted as treatment of DED since numerous evidences showing the role of inflammation in the pathogenesis of DED. Therefore, the use of anti-inflammatory drugs may prove beneficial for both the subjective and objective complaints of DED. In this section, it has been demonstrated that both polyphenols, QCT and RES, decrease the clinical signs and inflammatory process underlying DS-induced DED, suppressing the local activation of $CD4^+$ T cells in the conjunctiva of athymic mice. The anti-inflammatory effects of QCT and RES found in the two in-vitro models previously described, using ocular surface epithelial cells and T cells, have been corroborated in the in-vivo model of DED. Moreover, polyphenols are able to protect the ocular surface against DS and scopolamine as CFS data showed. All together indicates that polyphenols act at different levels of the inflammatory response. These scientific evidences may establish QCT and RES as novel therapeutic agents for treating DED.

VII.4. In-vitro model of conjunctival mast cell response

Mast cells are important effector cells in the conjunctiva because during allergic ocular diseases such as SAC, mast cells are activated following allergen cross-linking of surface IgE receptors, leading to degranulation and release of histamine, leukotrienes, prostaglandins and cytokines. In the present study, the effect of QCT, RES and their combinations were tested on CBMCs stimulated with IgE/anti-IgE.

One of the characteristics of an allergenic protein is that it induces specific IgE production and contains multiple IgE binding epitopes, so that the IgE on mast cells can be cross-linked. As a result of IgE cross-linking, mast cells release their well-known inflammatory mediators and cytokines such as histamine and TNF-α. The stimuli used to activate mast cells in-vitro is often not physiological and involves artificial chemicals such as compound 48/80, phorbol-12-myristate 13-acetate or calcium ionophore A23187. In the present study, IgE/Anti-IgE was used as a positive mast cell trigger for CBMCs.[292] This model attempts to simulate the physiological stimulation by cross-linking of the FcεRI (Figure 61). For this purpose, CBMCs are firstly sensitised with IgE. This sensitisation with IgE enhances FcεRI expression on the surface of mast cells. Then, IgE-sensitised CBMCs are activated using anti-IgE, which potentially cross-links FcεRI-bound IgE on mast cells. This event finally provokes mast cell degranulation.

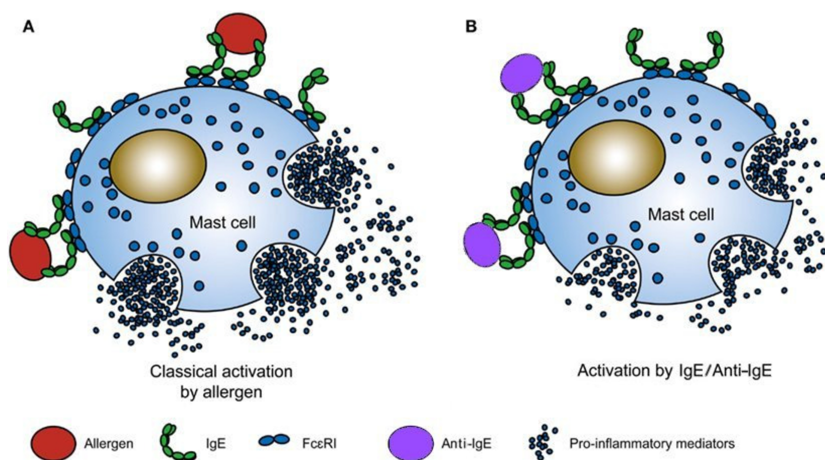

Figure 61. Mechanisms of real and in-vitro mast cell activation by IgE cross-linking. Picture modified from Bax et al.[378]

In order to test the polyphenols on mast cells, activated CBMCs were treated with a wide range of QCT, RES or QCT+RES concentrations for 24 h to determine the toxic concentrations for CBMCs. Results showed that neither of QCT, nor RES, nor

QCT+RES combinations decreased activated CBMC numbers. These results indicated that polyphenols are not toxic for CBMCs.

The anti-allergic effect of polyphenols was firstly tested on histamine release after 1 h of mast cell activation. IgE cross-linking with anti-IgE induced a significant increase of histamine. Surprisingly, the vehicle (EtOH) used to dissolve polyphenols decreased histamine secretion at both concentrations tested, 0.5 % and 1 %. In fact, histamine levels found in the study of polyphenol combination was less than that found in control cells (unstimulated cells treated with EtOH).

In order to test the effects of QCT, RES and their combination on histamine release from CBMCs, dose-response curves were obtained for different concentrations of QCT and RES, and their combinations in two different experiments performed in single samples. After that, one combination of QCT and RES (10 µM QCT + 25 µM RES) was chosen for studying its effect on the secretion of histamine by CBMCs, from one experiment performed in triplicate.

When CBMCs were treated with different concentrations of QCT, only 0.5 µM QCT significantly decreased histamine release. Other QCT concentrations tended to decrease histamine levels, and all calculated *p*-values were close to the significance level, but did not reach statistical significance. Regarding RES treatments, only 5 µM tended to decrease histamine secretion. Mean histamine values obtained from the remaining of RES concentrations were always lower, but not significant, than that obtained in stimulated cells treated with EtOH. Referring to the effect of QCT and RES combinations on histamine release, only 5 combinations out of 49 tested resulted in significantly decreased histamine levels. However, all of the mean values of histamine levels from each combination were also less than that found in stimulated cells treated with EtOH. This fact may be due to two reasons: i) the effect of vehicle on histamine release (a low histamine value was found in stimulated cells treated with EtOH, compared to unstimulated cells) and ii) the low number of samples (two experiments performed in single samples in dose-response curves). Therefore, increasing the number of samples may show significant differences, as found in the second experiment, in which 10 µM QCT and 25 µM RES alone decreased histamine levels significantly. This is a limitation commented on Section IX.

Previous studies have evidenced that QCT and RES have an inhibitory effect on histamine release. Kimata et al. tested the properties of QCT on human cultured mast cells stimulated with IgE/anti-IgE.[379] They found that QCT (1 to 100 µM) shows a concentration-dependent inhibition of IgE-mediated histamine release and GM-CSF, leukotrienes and prostaglandin D_2 secretion by human mast cells. The authors also

suggested that the target of QCT is not only FcεRI-mediated activation, but also other intracellular events because QCT also inhibits histamine secretion when mast cells are exposed to calcium ionophore A23187 (a non-immunological stimulus for histamine release), indicating that QCT also regulates Ca^{2+} signalling pathway in mast cells. Regarding RES, Baolin et al. studied its effect on bone marrow-derived mouse mast cells.[380] They reported that 100 μM RES inhibits the release of histamine, leukotrienes and prostaglandin D_2 mediated by both IgE and calcium ionophore A23187. These results suggested that RES also regulates Ca^{2+} signalling pathway in mast cells.

Degranulation of mast cells releases not only histamine, but also pro-inflammatory cytokines. Thus, the anti-allergic properties of polyphenols were also tested on the release of nine cytokines/chemokines: IL-1β, IL-4, IL-5, IL-8/CXCL8, IL-9, IL-10, IL-13, TNF-α and IFN-γ. Firstly, dose-response curves for different concentrations of QCT and RES were obtained from two experiments performed in single samples. After that, the same combination of QCT and RES used on histamine release (10 μM QCT + 25 μM RES) was tested on cytokine/chemokine secretion by CBMCs (one experiment performed in triplicate).

Cross-linking with anti-IgE induced a significant increase of IL-8/CXCL8, IL-13 and TNF-α. On the other hand, IL-4, IL-5, IL-10 and IFN-γ were detected but stimulation with IgE/anti-IgE did not induce a significant increase of these cytokines. As found in the histamine assay, the final concentration of vehicle (0.5 % EtOH) produced a decrease of IL-8/CXCL8, IL-13 and TNF-α levels, but these levels were significantly elevated compared to unstimulated cells.

IL-1β and IL-9 were not detected at 24 h, however it has been recently described by Mohd Zaki et al. that IL-9 is detected 24 h after anti-IgE stimulation of bone marrow-derived murine mouse mast cells, and reaches its maximum value at 48 h.[381] The authors also reported that IL-9 positive cells are increased in patients suffering SAC and increased mast cell numbers co-localised with IL-9, indicating that these cells secrete IL-9. Thus, it is hypothesised that IL-9 may be detected at longer times, such as 48 or 72 h after IgE/anti-IgE stimulation of CBMCs.

Regarding the effect of different concentrations on the polyphenols QCT and RES, both decreased cytokine/chemokine levels of IgE/anti-IgE-stimulated CBMCs. QCT decreased IL-8/CXCL8 (10, 15 and 20 μM), IL-13 (10, 15 and 20 μM) and TNF-α (1, 10, 15, 20 and 25 μM). For RES treatments, these compounds also decreased IL-8/CXCL8 (10, 25, 50 and 100 μM), IL-13 (5, 10, 25 and 100 μM) and TNF-α (1 to 100 μM). These data showed that low concentrations of QCT and RES inhibited TNF-α secretion, whilst higher concentrations of polyphenols were necessary for decreasing

IL-8/CXCL8 and IL-13 secretion. These data demonstrated that QCT and RES inhibit cytokine release from IgE/anti-IgE-stimulated CBMCs. Moreover, these results agree with previous studies, regarding the effect of both compounds on mast cells. For example, Min et al. investigated the effect of QCT on the expression of pro-inflammatory cytokines in a human mast cell line stimulated with phorbol-12-myristate 13-acetate and calcium ionophore A23187, and then treated with QCT.[382] They found that QCT decreases the gene expression and production of TNF-α, IL-1β and IL-8/CXCL8 in stimulated cells. QCT also attenuates activation of NF-κB and p38 mitogen-activated protein kinase. In addition, Park et al. found that QCT inhibits gene expressions and secretion of the pro-inflammatory cytokines TNF-α, IL-1β and IL-8/CXCL8 on a human mast cell line, following stimulation with IgE or phorbol-12-myristate 13-acetate and calcium ionophore A23187.[383] Kempuraj et al. treated human CBMCs with QCT (0.01, 0.1, 1, 10 or 100 μM) for 15 min before activation with anti-IgE. They found that the release of IL-8/CXCL8 and TNF-α is inhibited at 100 μM QCT.[384] Regarding RES properties, Kang et al. demonstrated the effects of RES on the expression of pro-inflammatory cytokines in a human mast cell line.[385] They stimulated cells with phorbol-12-myristate 13-acetate plus calcium ionophore A23187 in the presence of RES (10 and 50 μM) and found that RES significantly inhibits induction of the inflammatory cytokines IL-8/CXCL8 and TNF-α. While Catalli et al. studied the effect of RES in comparison to the anti-allergy drug tranilast in both human mast cell line and human primary $CD34^+$-derived mast cells. They found that both RES and tranilast inhibit mast cell degranulation induced by substance P, IgE/anti-IgE and compound 48/80. RES inhibition is immediate, preventing degranulation when added simultaneously to the physiological stimuli, and the effect is sustained for up to 24 h. RES also attenuates substance P-induced TNF-α and MCP-1/CCL2 production, and inhibits IgE-mediated release of leukotrienes, whilst tranilast is ineffective. Furthermore, RES reduces expression of the high-affinity IgE receptor, FcεRI, on a human mast cell line. The effects of RES on mast cell activation is more marked in human primary $CD34^+$-derived mast cells, with this compound being significantly more efficacious than tranilast in these cells.[386]

Referring to the combination of QCT and RES, 10 μM QCT + 25 μM RES did not have any effect on cytokine/chemokine levels. However in this experiment, the levels of IL-8/CXCL8, IL-13 and TNF-α were not increased, and the cytokine/chemokine levels were not significantly reduced by QCT and RES alone, as expected from the dose-response data. Only the IL-5 level was significantly increased. It is hypothesised that the vehicle (0.5 % EtOH) had an inhibitory effect on the secretion of these mediators. Therefore, it was not been possible to determine the

effect of both compounds together in this assay. In addition, above referenced authors dissolved RES and QCT with DMSO. In contrast to the results found in this thesis, they did not report any effect of this solvent on cytokine release and mast cell degranulation. Thus, DMSO may be better solvent for testing polyphenols on mast cells than EtOH. However, DMSO was discarded as a solvent for all in-vitro experiments due to its toxic effect on cells. Nevertheless, it has been suggested that combinations of polyphenols can have anti-allergic effects in-vitro and in-vivo. For example, Cruz et al. reported on the protective effect of *Kalanchoe pinnata* (a perennial plant found in tropical areas) extract in fatal anaphylactic shock, likewise a Th2-driven immunopathology.[387] They demonstrated that mice treated daily with oral *Kalanchoe pinnata* during hypersensitisation with ovalbumin are all protected against death when challenged with the allergen, as compared with the 100% mortality rate observed in the untreated group. Oral protection is accompanied by a reduced production of ovalbumin-specific IgE antibodies, reduced eosinophilia and impaired production of the IL-5, IL-10 and TNF-α cytokines. They also found that *Kalanchoe pinnata* prevented antigen-induced mast cell degranulation and histamine release in-vitro.[387] The authors did not find any inhibitory effect of the vehicle (PBS) on mast cells degranulation. Polyphenols used in these experiments from *Kalanchoe pinnata* were soluble in water-based solution because authors extracted polyphenols with distilled water.

Mast cells are one type of cells that take part in the acute allergic response owing to their release of synthesised and stored inflammatory mediators, such as histamine, cytokines and proteases. Thus, their activation plays an important role in the pathogenic process of ocular allergy. Activation of mast cells with IgE/anti-IgE results in the secretion of the preformed contents of their granules, such as histamine and cytokines. This thesis has shown that QCT and RES inhibit the release of histamine and cytokines mediated by IgE/anti-IgE activation of CBMCs. This indicated that both compounds are effective against IgE receptor-mediated mast cell degranulation. Thus, this study demonstrates that polyphenols differentially modulate two important effector functions, histamine release and cytokine expression of IgE/anti-IgE activated mast cells. However, the molecular targets of polyphenols on IgE-mediated allergy pathway need to be determined in further experiments.

VIII. Summary of thesis results

The aim of this thesis was to corroborate the following hypothesis:

"The topical application of QCT, RES and their combination has an anti-inflammatory effect on ocular surface diseases such as dry eye and ocular allergy, which is due to modulation of epithelial cells and immune cell responses"

The most important results from this thesis are shown in Figures 62 and 63, which summarise results from all of the in-vitro models and in-vivo model, respectively. QCT, RES and their combination have shown to target several inflammatory mediators/pathways that have been identified in ocular surface diseases, such as DED and ocular allergy.

In order to test the potential effect of QCT, RES and their combination, compounds were tested on three in-vitro models of inflammation and allergy, and one in-vivo model of DED. Firstly, polyphenols were tested on two ocular surface (conjunctival and corneal) epithelial cell lines stimulated with the cytokine TNF-α and UV-B light exposure by measuring cytokine secretion, COX-2 expression and intracellular ROS production. After that, the effect of polyphenols on T cell activation and proliferation was studied on anti-CD3/anti-CD28-stimulated PBMCs. Thirdly, the anti-inflammatory effects of topically-applied polyphenols were tested in a murine model of DED, followed by an adoptive transfer model. CFS, TP, goblet cell number, cytokine/chemokine levels in tears and $CD4^+$ T cell infiltration in conjunctiva were measured. Finally, the anti-allergic effects of polyphenols were studied on CBMCs stimulated by IgE cross-linking with anti-IgE. Histamine secretion and cytokine/chemokine release were determined.

Regarding the in-vitro models, experiments performed on ocular surface (conjunctival and corneal) epithelial cells demonstrated that QCT, RES and their combination caused inhibition of TNF-α-stimulated cytokine/chemokine secretion (IL-6, IL-8/CXCL8 and IP-10/CXCL10) and COX-2 expression, as well as decreased intracellular ROS production in UV-B-exposed cells. The immunoregulatory effect of QCT+RES was demonstrated on anti-CD3/anti-CD28-stimulated PBMCs by inhibiting cell proliferation. Moreover, the anti-allergic properties of QCT, RES and QCT+RES were finally demonstrated on IgE/anti-IgE-stimulated CBMCs by decreasing histamine levels and cytokine/chemokine secretion (IL-8/CXCL8, IL-13 and TNF-α), after mast cell degranulation (Figure 62).

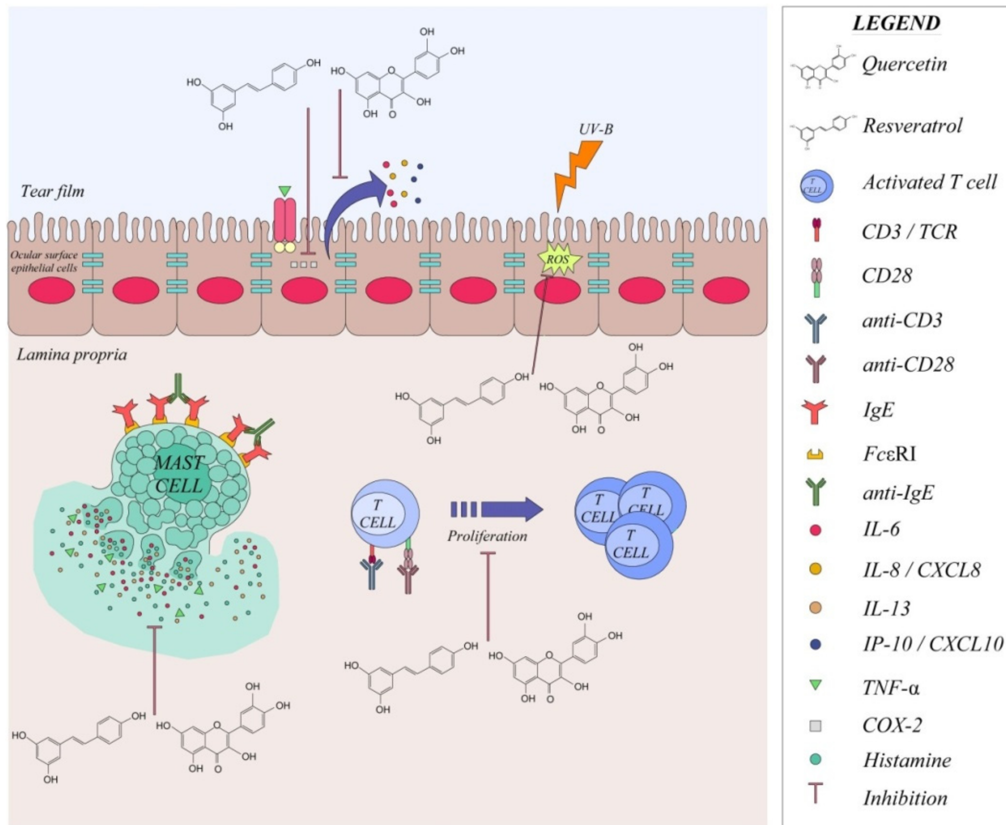

Figure 62. Summary of the most important results from in-vitro experiments.

Regarding the in-vivo model of DED, results from this thesis have shown that QCT, RES and QCT+RES protect the ocular surface from DS exposure. In addition, QCT, RES and QCT+RES reduce the infiltration of $CD4^+$ T cells in the conjunctiva of athymic mice (Figure 63).

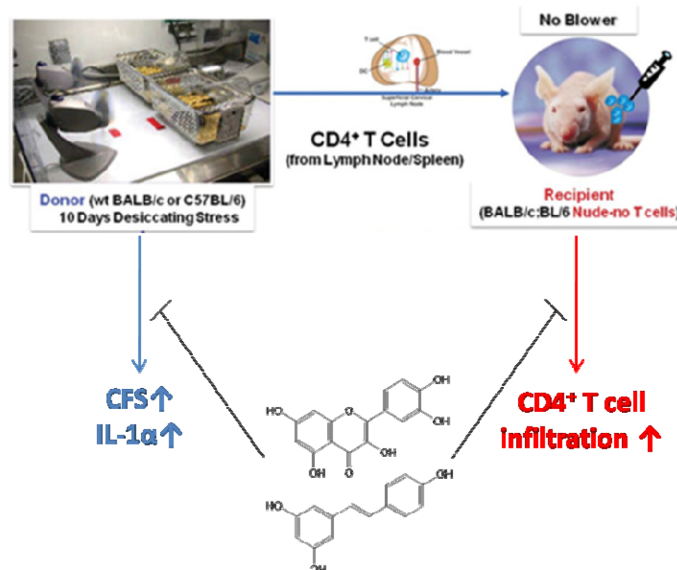

Figure 63. Summary of the most important results from in-vivo experiments. CFS: corneal fluorescein staining. Adapted from Stern et al. 2013.[70]

QCT and RES have demonstrated their anti-inflammatory properties in different types of cells that are involved in several ocular surface diseases, and were corroborated in an animal model of DED. Consequently, the topical application of QCT, RES or their combination might be promising anti-inflammatory therapy for ocular surface diseases such as DED and ocular allergy. Moreover, they may have the potential to help improve UV-B exposure-induced ocular surface disorders, because current clinical therapy for ocular surface inflammation does not offer any protection against UV-B radiation.[388]

Summary of results of this thesis:

1. Quercetin, resveratrol and their combination have an anti-inflammatory effect on ocular surface epithelial cells by inhibiting cytokine/chemokine secretion and COX-2 expression in an in-vitro model of ocular inflammation.

2. Quercetin, resveratrol and their combination have an antioxidant effect on ocular surface epithelial cells by decreasing intracellular reactive oxygen species production in-vitro in cells exposed to ultraviolet light.

3. Quercetin and resveratrol combination have an immunoregulatory effect on T cells by inhibiting the activation and proliferation of human peripheral blood mononuclear cells in-vitro.

4. Quercetin, resveratrol and their combination have anti-allergic effects on mast cell degranulation by inhibiting histamine release and cytokine/chemokine secretion in-vitro.

5. Topical application of quercetin, resveratrol and their combination decreases ocular signs and cytokine levels in tears of mice in an in-vivo model of DED.

6. Topical application of quercetin and resveratrol avoid $CD4^+$ T cell infiltration in conjunctiva of mice in an adoptive transfer model.

IX. Limitations and future studies

This work has some limitations that are addressed in this section.

The anti-inflammatory and antioxidant effects of polyphenols were firstly tested in two ocular surface cell lines (conjunctival –IOBA-NHC–and corneal –HCE– epithelial cells) stimulated by TNF-α. Both cell lines are commonly used to study physiological and pathophysiological mechanisms involved in inflammation, and also to test new potential compounds for treating ocular surface diseases. The cellular responses in cell lines do not reflect the complexity of the human ocular surface epithelia. However, in-vitro models offer several advantages, such as the study of the underlying biological mechanisms of action of drugs and they allow the testing of several drugs without a large financial investment. Another significant limitation is the fact that these tests were performed using a monolayer rather than a stratified structure, as is found in the ocular surface. Finally, the anti-inflammatory effect of polyphenols has only been tested using the pro-inflammatory cytokine TNF-α (which did not stimulate COX-2 secretion by HCE cells), and inflammatory pathways involved in ocular surface inflammation (such as MAPK) have not been studied in these in-vitro models. Thus, it is proposed for further studies in ocular surface epithelial cells:

i) to test the anti-inflammatory effect of polyphenols in stratified cultures of human epithelial cells;

ii) to use other pro-inflammatory cytokines involved in moderate-severe ocular surface inflammation for stimulating cells, i.e. IL-4, IL-13, IFN-γ and/or TGF-β; and

iii) to study cellular pathways involved in ocular surface inflammation.

In addition to that, the antioxidant effect of polyphenols was studied using UV-B radiation. For this purpose, only intracellular ROS was measured. However, it is well known that ROS are involved in apoptosis. Moreover, UV-B radiation is one of the most common inductors of ocular surface epithelial apoptosis. Thus, future studies might address the effect of both polyphenols in ocular surface epithelial cell apoptosis. Regarding this point, a preliminary study was performed during the stay in Allergan, looking at the effect of polyphenols in the mitochondrial permeability transition pore, using IOBA-NHC cells. These preliminary results have been included in Appendix II.

After testing the anti-inflammatory effects of polyphenols in conjunctival and corneal epithelial cells, the immunomodulatory properties of QCT, RES and QCT+RES were studied in PBMCs isolated from healthy donors, analysing viability and proliferation of PBMCs treated with polyphenols. This part of the thesis also has some limitations. For example, PBMCs used in this study were only obtained from three subjects. In addition, experiments from this thesis have demonstrated the antiproliferative effect of QCT+RES, but not for both compounds alone at the

concentrations tested. Moreover, there was a lack of studies on the molecular mechanisms underlying their antiproliferative effects. In addition, PBMCs are a mix of different cell subsets that may be affected by polyphenols in different ways. Therefore, further studies may include:

i) experiments with a larger population;

ii) to test more concentrations of QCT and RES individually;

iii) assays for studying molecular mechanisms involved in inflammation; and

iv) characterisation of the modulatory effect of polyphenols on the different subsets of T cells.

The anti-inflammatory effects of QCT, RES and QCT+RES were studied in a murine model of DED, followed by an adoptive transfer model. For this purpose, 0.01 % QCT and 0.1 % RES and their combination were tested. There were some limitations regarding these experiments. Firstly, $CD4^+$ T cell infiltration in conjunctiva was studied by immunohistochemistry, but this cell marker is expressed by several cell types and it is well known that the different subtypes of $CD4^+$ T cells (i.e. Th1, Th2, Th17 and Treg cells) are involved in the development of experimental DED model. Therefore, future studies might evaluate the effect of polyphenols on $CD4^+$ T cell subtypes. Secondly, APCs play an important role in the development of DED being necessary for the initiation and development of the disease. Moreover, a previous study supports the idea that polyphenols may act on $CD11b^+$ DCs. Therefore, it would be very interesting to look into the effect of QCT, RES and their combination on APCs. In addition, it is also well known that oxidative stress is involved in DED because increase markers of DNA oxidative damage (i.e. 8-OHdG), and lipid peroxidation markers (such as 4-hydroxy-2-nonenal and MDA) are found in a mouse model of DED.[389] Thus, the antioxidant effect of both polyphenols on experimental DED may be tested as their antioxidant effects have been demonstrated in ocular surface epithelial cells in this thesis. All of these future experiments on cells of the immune system would allow characterisation of the mechanisms underlying polyphenols as anti-inflammatory compounds in DED. Finally, only two concentrations of QCT and RES, and their combination were tested. In-vitro results demonstrated that the mixed low concentrations of both polyphenols proved the anti-inflammatory activity of QCT and RES, compared to those results found in cells treated with polyphenols separately. Thus, future studies could evaluate the effect of low concentrations of QCT, RES and their combinations in this in-vivo model of DED.

The anti-allergic properties of polyphenols QCT and RES on CBMCs have also been demonstrated in this thesis, but with some limitations. Firstly, polyphenols have been demonstrated to have inhibitory effects on histamine and cytokine release in response to IgE cross-linking. Yet other mediators, such as leukotrienes and prostaglandin D_2, have not been studied, as well as the intracellular mechanism underlying the effects of both polyphenols. Secondly, this is an in-vitro model using mast cells, which play a key role in ocular allergic diseases, but there are other cells that are involved in these disorders such as neutrophils, basophils and eosinophils. Another limitation of this study was the fact that the vehicle (EtOH) used for dissolving polyphenols had an effect on CBMCs degranulation at low doses. Lastly, the in-vitro model does not reflect the complexity of the allergic response to the human ocular surface. Therefore, further studies should include:

i) another vehicle to avoid artefacts (i.e. DMSO);

ii) experiments for studying the effect of polyphenols and their combinations on leukotrienes and prostaglandin D_2 and their molecular mechanisms;

iii) assays for testing the polyphenols effects on other cell types involved in allergy; and

iv) an in-vivo model of ocular allergy as an approximation to the response in the ocular allergic process.

This thesis has demonstrated the anti-inflammatory effect of QCT, RES and their combination on ocular surface epithelial cells, T cells, mast cells and in a murine model of DED. The main target of any research is to bring discoveries from the bench to the bedside. This purpose inspires the target of IOBA: "*investigar para curar mejor*" (to research to heal better). Therefore, the most important future study would be to test the effect of both QCT and RES topically in patients suffering DED. For this aim, experiments could be performed in a controlled environmental chamber (CER-lab) located in the IOBA building, in which pressure, temperature and relative humidity can be controlled. This future study could provide clinical evidence that QCT and RES may be useful in the treatment of DED.

X. Conclusions

1. Quercetin and resveratrol are naturally-occurring compounds with anti-inflammatory and antioxidant properties on human epithelial cells from the cornea and conjunctiva, and immunoregulatory effects preventing activation of cells of the immune system, such as lymphocytes and mast cells.
2. Both compounds have shown these properties separately and in combination, indicating the necessity for future experiments to determine what concentrations of each individually, or both in combination, are most effective to use.
3. Their properties have shown that these compounds could be used as topical treatment of inflammatory diseases of the ocular surface, such as dry eye or ocular allergy, as results from the murine model of dry eye have shown.

XI. Resumen en español

1. Objetivos

Los objetivos generales y específicos planteados para comprobar la hipótesis establecida en esta tesis son:

Objetivo general:

Estudiar el efecto de los compuestos quercitina, resveratrol y su combinación en los procesos inflamatorios de la superficie ocular con base inmune como el ojo seco y la alergia ocular.

Objetivos específicos:

- Determinar el efecto anti-inflamatorio de quercitina, resveratrol y su combinación en dos líneas celulares procedentes de los epitelios conjuntival y corneal humano estimuladas con TNF-α.
- Determinar el efecto antioxidante de quercitina, resveratrol y su combinación en dos líneas celulares procedentes de los epitelios conjuntival y corneal humano irradiadas con luz UV-B.
- Determinar la actividad inmunorreguladora de quercitina, resveratrol y su combinación en células mononucleares procedentes de sangre periférica humana.
- Determinar el efecto terapéutico de la aplicación tópica de de quercitina, resveratrol y su combinación en un modelo murino de ojo seco.
- Determinar el efecto de de quercitina, resveratrol y su combinación en la degranulación de mastocitos.

2. Material y métodos

2.1. Modelo in-vitro de inflamación y estrés oxidativo en células epiteliales conjuntivales y corneales humanas

El efecto anti-inflamatorio y antioxidante de QCT, RES y su combinación fue estudiado in-vitro en dos líneas celulares, la primera derivada del epitelio conjunctival humano (IOBA-NHC) y la segunda derivada del epitelio corneal humano (HCE), utilizando dos estímulos diferentes: 1) inflamación inducida por TNF-α y 2) estrés oxidativo provocado por luz UV.

La citotoxicidad de los polifenoles fue estudiada en primer lugar para determinar las concentraciones no tóxicas para ambas líneas celulares. Después, el efecto de QCT, RES y su combinación fue estudiado en la secreción de citoquinas y quimioquinas, la expresión de COX-2 y la producción de ROS.

2.1.1. Reactivos

Todos los reactivos y anticuerpos (tabla 1) usados en esta parte de la tesis han sido agrupados según el fabricante:

- *Invitrogen* (Inchinnan, Reino Unido): Dulbecco's Modified Eagle Medium/Nutrient Mixture F12 (DMEM/F12), test alamarBlue® para la viabilidad celular, ácido 4-(2-hidroxietil)-1-piperazineetanosulfónico (HEPES).
- *Nunc* (Roskilde, Dinamarca): Frascos y placas de cultivo celular.
- *Panreac* (Barcelona, España): EtOH y D-glucosa.
- *PeproTech EC* (Londres, Reino Unido): TNF-α.
- *Sigma-Aldrich* (St Louis, MO, EE.UU.): RES, QCT, DMEM (medio de cultivo sin $NaHCO_3$, piruvato sódico y rojo fenol), diacetato de 2',7'-diclorodihidrofluoresceína (H_2DCF-DA), L-glutamina, suero fetal bobino (FBS), toxina colérica, factor de crecimiento epitelial humano (EGF), insulina bobina, penicilina, estreptomicina, fungizona, hidrocortisona, dimetil sulfóxido (DMSO), tampón fosfato salino (PBS), tris-ácido clorhídirico (Tris-HCl), cloruro sódico (NaCl), ácido desoxicólico, Triton X-100, dodecil sulfato de sodio (SDS), ácido etilendiaminotetraacético (EDTA), fluoruro de fenilmetilsulfonilo (PMSF), aprotinina, ortovanadato sódico (Na_3VO_4), glicerol, 2-mercaptoetanol (2-ME), azul de bromofenol, albumina de suero bovino (BSA), Tween 20, tris-tampón salino (TBS).

- Thermo Fisher Scientific (Rockford, IL, EE.UU.): ácido bicinconínico (BCA).

Anticuerpo	Dilución	Fabricante	Referencia
Conejo anti-COX-2	1/100	Novus Biologicals (Littleton, CO, USA)	NB100-689
*Ratón anti-g*liceraldehído-3-fosfato deshidrogenasa *(GAPDH)*	1/500	Santa Cruz Biotechnology (Heidelberg, Germany)	SC-166545
HRP-conjugado humano anti-IgG	1/2000		SC-2004
HRP-conjugado ratón anti-IgG	1/5000	Jackson Laboratory (Bar Harbor, ME, USA)	715-035-150

Tabla 1. Anticuerpos utilizados en esta parte de la tesis.

2.1.2. Preparación de las disoluciones de los polifenoles

QCT y RES fueron disueltos en EtOH. Cada disolución de QCT y RES se preparó antes de cada experimento por dilución seriada a partir de disoluciones madre recién preparadas. Las concentraciones finales en cada experimento fueron 0,5-25 μM de QCT y 0,5-300 μM de RES.

La disoluciones en las cuales se combinaron QCT y RES fueron preparadas mezclando disoluciones individuales de QCT y RES para conseguir las concentraciones finales deseadas para cada experimento.

Todas las disoluciones fueron preparadas para conseguir una concentración final no tóxica del vehículo (EtOH) del 0,5 % en todas las muestras tratadas con los polifenoles. Las disoluciones se mantuvieron en todo momento en oscuridad para evitar su degradación por la luz solar.

2.1.3. Líneas celulares y condiciones de cultivo

Se utilizaron dos líneas celulares procedentes del epitelio de la superficie ocular, IOBA-NHC y HCE, para los experimentos de esta parte de la tesis.

A) La línea celular IOBA-NHC es una línea inmortalizada espontáneamente, no transfectada, procedente de la conjuntiva humana normal. Las células IOBA-NHC se cultivaron en medio DMEM/F-12 L-glutamina suplementado con 10 % de FBS, 0,1 μg/mL de toxina colérica, 2 ng/mL EGF, 1 μg/mL de insulina bovina, 5000 U/mL de

penicilina, 5 mg/mL de estreptomicina, 2,5 µg/mL de fungizona y 0,5 µg/mL de hidrocortisona.

B) La línea celular HCE es una línea inmortalizada procedente del epitelio corneal humano donada por el Profesor Arto Urti (Universidad de Helsinki, Finlandia). Las células HCE se cultivaron en medio DMEM/F-12 L-glutamina suplementado con 15 % de FBS, 0,5 % DMSO, 0,1 µg/mL toxina colérica, 10 ng/mL de EGF, 5 µg/mL de insulina, 100 U/mL de penicilina y 0,1 mg/mL de estreptomicina.

Todos los experimentos se llevaron a cabo utilizando un medio de cultivo DMEM sin $NaHCO_3$, piruvato sódico y rojo fenol y suplementado con 3.15 g/L de D-glucosa, 2 mM de L-glutamina y 25 mM de HEPES. Se hará referencia a este medio como medio completo (CM) a lo largo de todo el texto.

2.1.4. Ensayo de citotoxicidad

La citotoxicidad de QCT, RES y QCT+RES en las células epiteliales se determinó por la reducción del reactivo resazurina (alamarBlue®). Para realizar los ensayos, las células IOBA-NHC y HCE se plantaron en placas de 96 pocillos y se dejaron crecer durante 3 días. Después, el medio de cultivo se sustituyó por medio puro, sin suero y sin suplementos, en el cual se mantuvieron las células durante 24 h. Entonces, el medio puro se reemplazó por CM y las células se trataron con diferentes concentraciones de QCT, RES o QCT+RES y se incubaron durante 24 h a 37 ºC. Las células control fueron tratadas con el vehículo (0,5 % EtOH). Después de las 24 h de incubación, se eliminaron los sobrenadantes y se añadió a cada muestra una disolución de alamarBlue® al 10 %, preparada en el medio de cultivo suplementado DMEM/F12. Las células se incubaron durante 4 h a 37 ºC. Finalmente, el medio de cada muestra fue recogido y se midió la fluorescencia de cada uno de ellos en un espectrofotómetro UV/Vis (SpectraMax® M5, Molecular Devices, Sunnyvale, CA, EE.UU.).

2.1.5. Estimulación celular con TNF-α y tratamiento con los polifenoles

Para este experimento, las células IOBA-NHC and HCE se plantaron en placas de 24 pocillos y se dejaron crecer durante 3 días. Para el ensayo de la expresión de COX-2, las células se plantaron en frascos de cultivo de 25 cm^2 hasta confluencia. Pasado el tiempo de incubación, el medio de cultivo se reemplazó por medio de cultivo

puro, sin suero ni suplementos, y las células se mantuvieron en él durante 24 h a 37 ºC. Después de las 24 h de incubación, el medio puro se reemplazó por medio CM y las células fueron pretratadas con QCT, RES, QCT+RES o vehículo durante 2 h a 37 ºC. Después, se quitaron los pretratamientos, las células se estimularon con 25 ng/mL de TNF-α en presencia de QCT, RES, QCT+RES o vehículo en medio CM y se dejaron en incubación durante 24 h. Se utilizaron como controles células no estimuladas con TNF-α pero tratadas con los polifenoles. Después de las 24 h, el medio de cada muestra se recogió y se centrifugó a 59 x g durante 5 min. Las placas y los frascos con las células adheridas y los sobrenadantes se guardaron a -80 ºC antes de ser analizados.

2.1.6. Lisis celular y determinación de la proteína total

El contenido total de proteína fue determinado tanto en los frascos como en las placas con las células adheridas. En primer lugar, las células fueron lisadas con buffer de lisis (RIPA) [10 mM tris-HCl (pH 7,4), 150 mM de NaCl, 1 % de ácido desoxicórico, 1 % de Triton X-100, 0,1 % de SDS y 1 mM de EDTA] suplementado con 0,1 mg/mL de PMSF, 60 µg/mL de aprotinina y 0,1 µM de Na_3VO_4. Las muestras se incubaron en hielo durante 30 min y finalmente se centrifugaron y se almacenaron a -80 ºC antes de ser usadas.

La cantidad de proteína total fue determinada con el test BCA. Brevemente, 25 µL de las muestras estándar y de los lisados celulares fueron tratados con el reactivo de trabajo BCA (BCA en 0,1 mM hidróxido sódico y 4 % de una solución de sulfato de cobre (II)) e incubados durante 30 min a 37 ºC. Entonces, las muestras se enfriaron a temperatura ambiente y se midió la absorbancia de cada muestra a 562 nm utilizando el espectrofotómetro SpectraMax® M5 UV/Vis (Molecular Devices). Los datos fueron analizados con el software SoftMax® Pro (Molecular Devices).

2.1.7. Medida de la secreción de citoquinas y quimioquinas

La secreción de citoquinas y quimioquinas fue medida de manera simultánea con un ensayo multianalito utilizando tecnología x-MAP®. Los niveles de IL-6, IL-8/CXCL8, IP-10/CXCL10 y VEGF fueron determinados en los sobrenadantes celulares. Brevemente, 25 µL de cada muestra se cultivaron con las respectivas microesferas o "beads" a 4 ºC durante toda la noche. Después, se incubaron con el anticuerpo biotinilado durante 1 h a temperatura ambiente seguida de otra incubación

con estreptavidina-ficoeritrina durante 30 min. Finalmente, las microesferas se analizaron con un Luminex™ 100-IS (Luminex Corporation, Austin, Tx, EE.UU.) y con el software BeadView™ Software (Upstate, Reino Unido).

2.1.8. Expresión de ciclo-oxigenasa-2

La expresión de COX-2 en las células epiteliales se determinó mediante gel de poliacrilamida SDS-PAGE y Western blotting. El homogeneizado celular se mezcló con 2X Laemmli [4 % de SDS, 20 % de glicerol, 10 % de 2-ME, 0,004 % de azul de bromofenol y 0,125 mM de Tris-HCl, pH~6,8].

Las proteínas en las muestras se desnaturalizaron por calor a 110 ºC durante 5 min. Entonces, 20 µg de proteína total se separaron con un 10 % de gel SDS-PAGE. Después, se transfirieron a una membrana porosa donde se bloquearon con 5 % de leche desnatada, 3 % de BSA y 0,05 % de Tween 20 en TBS durante 1 h. La membrana se incubó con anticuerpo para COX-2 toda la noche a 4 ºC. Seguidamente, se incubaron con anticuerpo para IgG durante 1 h a temperatura ambiente. Finamente, se detectaron las bandas inmunorreactivas de COX-2 con solución quimioluminiscente (Santa Cruz) durante 1 min. Las imágenes se capturaron con la cámara CCD incorporada en un ChemiDoc XRS (Bio-Rad, Inc., Hercules, CA, EE.UU.). La banda de GAPDH se utilizó como control para normalizar los niveles de COX-2.

2.1.9. Medida de especies reactivas de oxígeno inducidas por luz UV-B

La generación intracelular de ROS por radiación UV-B se determinó con el compuesto H_2DCF-DA. Las células IOBA-NHC and HCE se plantaron y cultivaron en placas de 24 pocillos transparentes para la luz UV-B. Una vez eliminados los medios de cultivo y mantenidas las células en medio puro durante 24 h, se pretrataron las células con QCT, RES, QCT+RES o vehículo durante 1 h a 37 ºC. Después, se eliminaron los tratamientos y se cargaron las células con una disolución 10 µM de H_2DCF-DA en medio CM durante 30 min. Entonces, se eliminó la disolución de H_2DCF-DA, se volvieron a tratar las células con los polifenoles y se irradiaron con una lámpara UV-B de 8W con el pico máximo a 302 nm durante 15 s. La energía final que recibieron las células fue de 107,25 mJ/cm^2. Las células control no se irradiaron. Seguidamente, las células se dejaron en incubación durante 1 h y finalmente se determinó la fluorescencia con un espectrofotómetro SpectraMax® M5 UV/Vis (Molecular Devices).

2.2. Modelo in-vitro de proliferación de linfocitos T

El efecto inmunosupresor de los compuestos QCT, RES y su combinación se estudió en células procedentes de sangre periférica humana (PBMCs). Se determinó la citotoxicidad, y la proliferación y viabilidad.

2.2.1. Reactivos

Todos los reactivos se adquirieron en Sigma-Aldrich si no se especifica otra cosa. Todos los ensayos se realizaron con el medio de cultivo modificado Roswell Park Memorial Institute (RPMI)-1640 HEPES suplementado con 2 mM de L-glutamina, 10 % de FBS, 50 U/mL de penicilina, 50 de µg/mL de estreptomicina, aminoácidos no esenciales, 1 mM de piruvato sódico y 2,5 µM de 2-ME. El ioduro de propidio (PI) también se adquirió en Sigma.

Los anticuerpos anti-CD3 (clon HIT3a) y anti-CD28 (clon 28.2) se adquirieron en Becton Dickinson Biosciences (BD, Oxford, Reino Unido). El succinimidil éster de diacetato de carboxifluoresceína (CFSE) y Ficoll-Hypaque se adquirieron en Invitrogen. El suero fetal bovino (FCS) se adquirió en Labtech International (Ringmer, Reino Unido).

2.2.2. Preparación de las disoluciones de polifenoles

Las disoluciones de QCT y RES se prepararon a partir de disoluciones madre en EtOH por dilución. Las concentraciones finales fueron de 0,5-25 µM de QCT y 0,5-100 µM de RES. La concentración del vehículo para los tratamientos individuales de los polifenoles fue siempre de 0,5 % de EtOH.

Las concentraciones finales de QCT+RES se consiguieron añadiendo cada disolución de QCT y RES a cada muestra, de esta manera la concentración final de vehículo fue siempre el 1 % de EtOH.

2.2.3. Donantes

La sangre periférica se obtuvo de tres pacientes anónimos que leyeron y firmaron el consentimiento informado. Los protocolos utilizados fueron revisados y aprobados por los comités éticos de la UCL y de la Universidad de Valladolid.

2.2.4. Aislamiento de las células mononucleares

Las células PBMC se aislaron mediante gradiente de densidad con Ficoll-Hypaque. La sangre se diluyó en medio de cultivo y se añadió lentamente sobre el Ficoll para luego centrifugar la muestra a 624 x g durante 40 min. Las células mononucleares se recogieron, se lavaron, se volvieron a centrifugar y, finalmente, se resuspendieron en medio de cultivo para su uso.

2.2.5. Ensayo de citotoxicidad

La citotoxicidad de los polifenoles en las células PBMC se determinó con PI por citometría de flujo.

Las células PBMC se plantaron en placas de 96 pocillos y se trataron con diferentes concentraciones de QCT, RES y QCT+RES. Después, las células se estimularon con 50 ng/mL de anti-CD3 y 10 µg/mL de anti-CD28 y se incubaron durante 96 h a 37 ºC. Las células control se estimularon pero no se trataron con los polifenoles. Después del tiempo de incubación, se añadieron 5 µg/mL de PI y se analizaron las células con un citómetro FACSCalibur™ (BD).

2.2.6. Ensayo de proliferación y viabilidad

El efecto de los polifenoles sobre la viabilidad y proliferación de las células PBMC se determinó con el compuesto CFSE.

Las células se incubaron con 5 µM de CFSE en medio de cultivo sin suero durante 10 min a 37 °C en un baño de agua. Después, se añadió 1 mL de medio de cultivo con 10 % de FCS y se incubaron durante 30 min a temperatura ambiente. Después se recogieron, se plantaron en placas de 96 pocillos, se trataron con los polifenoles y se estimularon con anti-CD3 y anti-CD28 durante 120 h. Finalmente se añadió PI y se analizaron las muestras por citometría de flujo.

2.3. Modelo murino de ojo seco

El efecto anti-inflamatorio de QCT, RES y su combinación (QCT+RES) fue estudiado en un modelo murino de ojo seco seguido de un modelo de transferencia adoptiva. Se evaluaron la tinción corneal con fluoresceína (CFS), la producción lagrimal (TP), el recuento de células caliciformes, los niveles de citoquinas en lágrima y la infiltración de linfocitos $CD4^+$ T en conjuntiva.

2.3.1. Animales

Se utilizaron dos tipos de ratón: C57BL/6 y C57BL/6 atímicos (sin linfocitos T) de entre 8 y 10 semanas de edad. El estudio con animales fue aprobado por el comité ético de Allergan y se siguieron las normas de “Association for Research in Vision and Ophthalmology (ARVO)”.

2.3.2. Inducción del estrés por desecación y aplicación tópica de los tratamientos.

El estrés por desecación (DS) se provocó en los ratones C57BL/6 (denominados donantes). Los ratones se colocaron en cajas con rejillas en los laterales y se expusieron a un flujo de aire continuo durante 10 h al día en una cámara de ambiente controlado, con una humedad relativa del 20 % y una temperatura mantenida de 23 °C durante 10 días. Además, se administraron 0.1 mg/día de escopolamina sódica mediante una bomba osmótica implantada subcutáneamente. Los ratones control se mantuvieron en una sala aislada con una humedad relativa del 80 % y una temperatura de 21-23 °C, sin flujo de aire.

Los ratones se dividieron en 6 grupos (9 ratones por grupo) de acuerdo al tratamiento seguido:

1) Grupo control (sin DS y sin tratamiento tópico).
2) DS (sin tratamiento tópico).
3) DS + vehículo.
4) DS + 0.01 % QCT.
5) DS + 0.1 % RES.
6) DS + 0.01 % QCT + 0.1 % RES.

Los tratamientos comenzaron el día antes de inducir DS y se administraron bilateralmente (5 µL/ojo) 3 veces al día. Al finalizar el experimento, los ojos izquierdos con los párpados fueron embebidos en OCT y congelados; y los ojos derechos con los párpados sumergidos en paraformaldehído y conservados a 4 ºC.

2.3.3. Tinción corneal

CFS se evaluó en los ratones donantes después de 9 días de DS. Se instilaron 5 µL de 0,125 % de fluoresceína sódica (Sigma) en el saco inferior conjunctival y después de 1 min se examinaron ambas corneas con un microscopio bajo una luz azul cobalto. El punteado superficial se evaluó en las 5 áreas corneales (central, superior, inferior y laterales) según la escala del National Eye Institute.

2.3.4. Modelo de transferencia adoptiva

Se aislaron las células $CD4^+$ de los nódulos linfáticos cervicales y del bazo de los ratones donantes mediante un kit de aislamiento de células $CD4^+$ (MACS System, Miltenyi Biotec, Auburn, CA, EE.UU.). Después se transfirieron a los ratones atímicos (denominados receptores) intraperitonealmente (8 ratones receptores por cada grupo de ratones donantes) y se mantuvieron durante 3 días. Al finalizar el experimento, los ojos izquierdos con los párpados fueron embebidos en OCT y congelados; y los ojos derechos con los párpados sumergidos en paraformaldehído y conservados a 4 ºC.

2.3.5. Producción lagrimal

TP se midió en los ratones donantes y receptores el día antes de comenzar los experimentos y un día antes de terminarlos. TP se midió con tiras impregnadas de rojo fenol (Zone-Quick; Lacrimedics, Eastsound, WA, EE.UU.). La tira se mantuvo con unas pinzas sobre el canto lateral externo del ojo derecho durante 30 s y se midió la zona humectada.

2.3.6. Recogida de lágrima

La recogida de lágrima se realizó en los ratones donantes los días 6 y 10 y en los receptores el día 3. Se instilaron 1,5 µL de tampón para el ensayo de citoquinas (Millipore) en cada ojo e inmediatamente se recogieron 2 µL (1 µL/ojo) de lágrima con

tampón del menisco lagrimal inferior con un capilar. Las muestras se diluyeron en 8 µL de tampón y se almacenaron a -80 °C.

2.3.7. Medida de los niveles de citoquinas y quimioquinas en lágrima.

Se midieron los niveles de 15 citoquinas y quimioquinas (IFN-γ, IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12(p70), MMP-9, IL-13, IL-17, IP-10/CXCL10, MCP-1/CCL2, RANTES/CCL5 y TNF-α) utilizando un ensayo multianalito con tecnología x-MAP® siguiendo el mismo protocolo que el apartado 2.1.7 de esta sección. Las muestras se analizaron con un Luminex 200 (Luminex Corporation).

2.3.8. Recuento de células caliciformes

Los ojos derechos con sus respectivos párpados mantenidos en paraformaldehído se fijaron en parafina y se realizaron secciones que posteriormente se tiñeron con ácido de Schiff (PAS). Las secciones se analizaron con un microscopio utilizando un objetivo 20x. El recuento de células caliciformes se realizó en toda la conjuntiva, superior e inferior.

2.3.9. Inmunohistoquímica

Los ojos izquierdos con sus respectivos párpados embebidos en OCT se seccionaron con un microtomo en un criostato (CM3050 S; Leica Microsystems, Buffalo Grove, IL, USA). Posteriormente, las secciones se secaron a 37 ºC y se fijaron con acetona a -20 ºC. Después, se bloquearon las peroxidasas endógenas con H_2O_2 y se secaron al aire durante 1 h. Se bloquearon las uniones no específicas con suero de conejo y se añadió anticuerpo para CD4. Después de lavar las muestras con PBS se añadió el anticuerpo secundario anti-IgG y, posteriormente, se añadió el reactivo Vectastain Elite ABC (Vector laboratories, Burlingame, CA, EE.UU.). Finalmente, las muestras se incubaron con el sustrato NovaRED (Vector Laboratories) y se realizó una contratinción con hematoxilina (Invitrogen). El recuento de células $CD4^+$ se realizó en toda la conjuntiva, superior e inferior.

2.4. Modelo in-vitro de respuesta de mastocitos conjuntivales

El efecto antialérgico de los compuestos QCT, RES y su combinación fue estudiado en mastocitos derivados de sangre de cordón umbilical humano (CBMC). Se evaluaron la viabilidad celular y la secreción de histamina y citoquinas.

2.4.1. Reactivos

El medio Stemspan™ se adquirió de StemCell Technologies (Grenoble, France). El factor de crecimiento celular (SCF) y las citoquinas IL-3 e IL-6 se adquirieron en Peprotech. FCS se adquirió en Labtech. IgE humano fue comprador en Abcam (Cambridge, MA, USA). PI y anti-IgE humano fueron adquiridos en Sigma.

2.4.2. Preparación de las disoluciones de los polifenoles

Las disoluciones de QCT, RES and QCT+RES se prepararon de la misma manera que se describe en los apartados 2.1.2 y 2.2.2 de esta sección.

2.4.3. Mastocitos derivados de sangre de cordón umbilical

Las células madre $CD34^+$ disponibles comercialmente (Lonza, Wokingham, UK) se cultivaron y diferenciaron como previamente se describió en la bibliografía.[293]

2.4.4. Sensibilización y activación de los mastocitos

Las células CBMC se activaron por la unión cruzada de IgE de superficie con anti-IgE siguiendo el protocolo descrito en la bibliografía.[292,293]

2.4.5. Tratamiento con los polifenoles

Después de sensibilizar las células CBMC con IgE durante 18 h se añadieron los tratamientos con los polifenoles. Las células se incubaron durante 30 min antes de añadir anti-IgE. Se recogieron sobrenadantes 1 h y 24 h después de añadir anti-IgE.

2.4.6. Ensayo de citotoxicidad

La citotoxicidad de los polifenoles se determinó 24 h después de activar las células con anti-IgE. Para ello, se añadieron 5 μg/mL de PI a cada muestra y, seguidamente, las células se analizaron por citometría de flujo (FACSCalibur).

2.4.7. Secreción de histamina

La concentración de histamina en los sobrenadantes 1 h después de la estimulación con anti-IgE se realizó con un kit comercial de ELISA (IBL, Hamburg, Germany) siguiendo las instrucciones del fabricante. En primer lugar, se realizó la acilación de los sobrenadantes y las muestras estándares. Después, se añadió suero de anti-histamina y se reveló con una solución de tetrametilbencidina para, finalmente, medir la absorbancia con un espectrofotómetro.

2.4.8. Secreción de citoquinas

Se determinó el nivel de 9 citoquinas (IL-1β, IL-4, IL-5, IL-8/CXCL8, IL-9, IL-10, IL-13, TNF-α y IFN-γ) en los sobrenadantes a las 24 h siguiendo el protocolo descrito en el apartado 2.1.7 de esta sección.

3. Resultados más relevantes

1. Quercitina, resveratrol y su combinación tienen un efecto anti-inflamatorio sobre las células del epitelio de la superficie ocular mediante la inhibición de la secreción de citoquinas y quimioquinas, y la expresión de COX-2 en un modelo in-vitro de inflamación ocular.

2. Quercitina, resveratrol y su combinación tienen un efecto antioxidante sobre las células del epitelio de la superficie ocular disminuyendo la formación de especies reactivas de oxígeno cuando las células se expusieron a luz UV-B.

3. La combinación de quercitina y resveratrol tienen un efecto inmunorregulador sobre los linfocitos T inhibiendo la activación y proliferación in-vitro de células mononucleares procedentes de sangre periférica humana.

4. Quercitina, resveratrol y su combinación tienen un efecto anti-alérgico sobre los mastocitos inhibiendo la secreción de histamina y citoquinas in-vitro.

5. La aplicación tópica de quercitina, resveratrol y su combinación disminuye los signos y los niveles de citoquinas en la lágrima de ratones expuestos a estrés por desecación.

6. La aplicación tópica de quercitina y resveratrol evita la infiltración de linfocitos $CD4^+$ en la conjuntiva de ratones receptores en un modelo de transferencia adoptiva.

4. Conclusiones

1. Quercetina y resveratrol son compuestos naturales con propiedades anti-inflamatorias y antioxidantes en células epiteliales procedentes de la córnea y la conjuntiva humana, y con efectos inmunorreguladores sobre la activación de células del sistema inmune como los linfocitos y los mastocitos.

2. Ambos compuestos han mostrado estas propiedades tanto de forma aislada como en combinación, siendo necesario en el futuro estudiar qué concentraciones de ambos compuestos de forma aislada, o conjuntamente, son más efectivas para su uso.

3. Estas propiedades han demostrado que ambos compuestos pueden ser usados en el tratamiento tópico de enfermedades inflamatorias de la superficie ocular, tales como el ojo seco y la alergia ocular, como han confirmado los resultados obtenidos en el modelo animal de ojo seco.

XII. Appendix I

Patente: *Composición para su uso en el tratamiento y/o prevención de la inflamación, el estrés oxidativo y la neovascularización ocular*. Número de publicación: P201230664. Asignado a: Universidad de Valladolid y Allergan, Inc. Inventores: María Jesús González García, Amalia Enríquez de Salamanca y Aladro, Margarita Calonge Cano, Michael E. Stern y Antonio Abengózar Vela.

COMPOSICIÓN PARA SU USO EN EL TRATAMIENTO Y/O PREVENCIÓN DE LA INFLAMACIÓN, EL ESTRÉS OXIDATIVO Y LA NEOVASCULARIZACIÓN OCULAR

DESCRIPCIÓN

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La presente invención se encuadra en el campo de la oftalmología, específicamente dentro de las composiciones oftálmicas útiles para el tratamiento y/o prevención de lesiones o enfermedades que cursan con inflamación, estrés oxidativo y/o neovascularización ocular, preferiblemente del sistema lagrimal (secretor y excretor), anejos oculares (párpados, incluyendo las glándulas de Meibomio) y del 10 segmento anterior del ojo, el cual comprende conjuntiva (incluyendo las células productoras de mucinas y las glándulas del sistema lagrimal secundario), córnea, limbo esclero-corneal, iris, pupila, cristalino, zónula de Zinn, cuerpo ciliar, cámara anterior, humor acuoso, cámara posterior; y más preferiblemente de la superficie ocular, que comprende los epitelios de la córnea, limbo esclero-corneal y conjuntiva, la película lagrimal suprayacente y el estroma subyacente a estas estructuras.

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ESTADO DE LA TÉCNICA

La inflamación ocular es uno de los problemas de mayor incidencia en la patología oftalmológica. El tratamiento de esta afección se puede realizar de forma específica, atacando la etiología, de forma 20 inespecífica, reduciendo los síntomas inflamatorios, o uniendo ambas cosas. En este sentido, los corticosteroides se han utilizado como fármacos de elección en el tratamiento inespecífico de la inflamación ocular, pero los efectos adversos que presentan han planteado la necesidad de incrementar el uso de otros fármacos como los antiinflamatorios no esteroideos (AINEs) (Thadani SM., Foster CS., 2004, *Pediatr Drugs*, 6: 289-301). La actividad antiinflamatoria de estos últimos agentes radica en la inhibición 25 reversible de la actividad de la enzima ciclooxigenasa (COX) en sus isoformas COX-1 y COX-2, impidiendo la formación de prostaglandinas, sustancias mediadoras de la inflamación.

La inflamación ocular puede deberse a diversas causas y tener unas características u otras dependiendo de la zona del ojo en la que se desencadene. Puede estar provocada, por ejemplo, por una intervención 30 quirúrgica, sin embargo, ésta es solo una de las causas de la patología, la cual debe tratarse con antiinflamatorios en cualquiera de sus manifestaciones para paliar los síntomas y evitar las graves complicaciones que puede acarrear y que pueden empeorar la calidad de vida de los pacientes. Dichas complicaciones dependen de la localización de la inflamación y pueden ir desde la pérdida de las pestañas, si se da en los párpados, empeoramiento del pronóstico de los traumatismos oculares e incluso 35 ceguera en el caso de uveítis o patología inflamatoria intraocular.

La inflamación puede darse en todas las estructuras de la anatomía ocular. Así, serían susceptibles de verse afectados tanto los anejos oculares como el globo ocular o el nervio óptico, pudiéndose encontrar inflamación tanto de origen infeccioso como no infeccioso. Y así, se hablará de inflamaciones orbitarias, 40 palpebrales, intraoculares (uveítis, también denominadas, según las partes a las que afecte predominantemente, retinitis, coroiditis, papilitis o vasculitis) o inflamaciones de la superficie ocular (blefaritis, conjuntivitis, queratitis, afecciones del limbo esclero-corneal y patología lagrimal, el síndrome de ojo seco (SOS) incluido).

45 Se estima que alrededor del 7% de las personas jóvenes padece algún fenómeno de inflamación ocular en España, mientras que en la edad adulta la aparición de otras enfermedades que provocan este fenómeno, como el SOS, hace que su prevalencia aumente hasta el 30% a partir de los 60 años.

El SOS es una enfermedad inflamatoria inmune, crónica, de origen multifactorial que podría originarse en 50 cualquiera de los componentes de la unidad funcional lagrimal, formada por la superficie ocular, las glándulas lagrimales principal y accesorias y la inervación de interconexión entre ellas -ramas aferentes y eferentes-, y que, en algún momento, provoca daño en la superficie ocular (epitelios de córnea, limbo esclero-corneal y conjuntiva, película lagrimal suprayacente y estroma subyacente a estas estructuras) que se detectan con las pruebas convenientes.

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En cuanto a la neovascularización ocular, ésta se encuentra ligada directamente al estímulo de la hipoxia, a la necrosis tisular y a la activación de complejas cascadas de interacción entre citoquinas/quimioquinas (Ej: IL-8) y factores de crecimiento estimuladores de la formación de neovasos (VEGF, bFGF, TGF-α, PDGF, IGF). Todos estos eventos son desencadenados, más frecuentemente, por inflamación intensa y/o 60 prolongada y/o recurrente, independientemente de la causa que la origine. Por ejemplo, un evento agudo y muy intenso como una causticación (quemadura química) o un evento menos intenso pero recurrente (queratitis herpética) o prolongado (SOS severo) podrán ser el origen de una neovascularización corneal. En los últimos años, estudios de investigación en neovascularización han demostrado una interrelación

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directa entre citoquinas, factores de crecimiento, moléculas de adhesión celular (integrinas) y metaloproteinasas (MMP). Dichas MMP cumplen un rol esencial en la invasión tisular por neovasos en diferentes enfermedades corneales (inflamatorias, autoinmunes, infecciosas), por lo que la inhibición de las MMP por los inhibidores naturales (o TIMPs) y por drogas inhibidoras de MMP, resultaría en la
5 inhibición de la neovascularización.

Uno de los casos en que puede aparecer neovascularización es, por ejemplo, cuando se produce la destrucción extensa de la superficie corneal, y en especial de las células madre pluripotenciales localizadas a nivel del limbo esclero-corneal, lo que llevaría a la pérdida de la re-epitelización corneal
10 normal y finalmente a una conjuntivalización o pannus corneal con una invasión por neovasos. Este proceso, en la actualidad, se define como Síndrome de Insuficiencia Límbica (SIL).

Los hallazgos experimentales (*in vitro* e *in vivo*), que permiten una mejor comprensión de la biología molecular de la neovascularización y cicatrización, han ayudado a realizar nuevos tratamientos médicos
15 con drogas que inhiben factores de crecimiento angiogénicos o MMP, así como con el uso de nuevos factores de crecimiento que inhiben la neovascularización ("Pigment Epithelium Derived Factor" o PEDF), y mediante tratamientos quirúrgicos tales como el trasplante de células limbares y membrana amniótica.

En lo que se refiere al estrés oxidativo, las especies de oxígeno reactivas (ROS), que producen efectos
20 adversos a nivel celular, se generan por múltiples mecanismos endógenos, como por ejemplo, las reacciones inflamatorias o la isquemia; y exógenos, como por ejemplo, la exposición a la luz ultravioleta. A nivel ocular, cabe destacar por ejemplo el proceso inflamatorio de la unidad funcional lagrimal que se produce en el SOS, el cual tiene asociado un proceso oxidativo que tiene lugar en la superficie ocular.

25 En referencia a la película lagrimal, la capa lipídica está formada por ésteres de colesterol y fosfolípidos. Si tenemos en cuenta que la capa lipídica está en contacto directo con el aire y los procesos de oxidación que sufren los lípidos en presencia de moléculas radicalarias, la generación de ROS en el proceso oxidativo inflamatorio puede degradar los ácidos grasos, aumentado la evaporación de la capa acuosa de la película lagrimal. Esto se corrobora con el aumento de la concentración de peroxidasa lipídica en la
30 superficie ocular.

Además de las ROS, existe también la formación de especies oxidantes relativas al nitrógeno. El óxido nítrico y las especies oxidantes relativas al nitrógeno pueden tener un papel muy importante en el SOS. La expresión de la óxido nítrico sintasa 2 y 3 (NOS2, NOS3) en el epitelio conjuntival está aumentada en
35 el SOS respecto al epitelio conjuntival de ojos sanos, lo que agrava la sintomatología.

Este proceso oxidativo produce una elevada concentración, y por ello una elevada actividad, de mieloperoxidasa (MPO) y xantina oxidoreductasa/xantina oxidasa en el epitelio conjuntival, entre otras. Dichas enzimas generan especies radicalarias que podrían estar implicadas en los procesos oxidativos
40 que ocurren en el SOS. Además, la concentración de las enzimas antioxidantes, encargadas de neutralizar las moléculas radicalarias, se encuentra disminuida en el ojo seco, lo que podría contribuir aun más al daño oxidativo producido en el proceso inflamatorio.

Por otro lado, los compuestos polifenólicos, dentro de los cuales se encuentran los polifenoles flavonoides
45 y no flavonoides, son compuestos cuya característica principal es la presencia de grupos bencilo e hidroxilo en su estructura y cuyas propiedades antioxidantes han sido ampliamente estudiadas. Dentro de los compuestos polifenólicos flavonoides destaca la molécula quercetina (QCT), 3,3',4',5,7-pentahidroxi-2-fenilcromen-4-ona. Es el compuesto flavonoide más abundante, con muchos efectos potencialmente beneficiosos para la salud humana (Kelly GS, 2011, *Alternative Medicine Review*, 16(2):172-194). Del
50 mismo modo, dentro de los compuestos polifenólicos no flavonoides, dentro de los cuales se encuentran los estilbenoides que son compuestos cuya característica principal es la presencia de grupos bencilo e hidroxilo en su estructura, cabe destacar una molécula cuya importancia ha aumentado en los últimos años debido también a sus múltiples cualidades, usos y beneficios para la salud (Frémon L., 2000, *Life Sciences*, 66(8): 663-673), la molécula resveratrol (RES), 5-[(E)-2-(4-hidroxifenil)etenil]benzen-1,3-diol.
55 Ambos compuestos se pueden obtener sintéticamente pero se encuentran de forma natural en más de 70 especies de plantas, frutas y verduras.

En cuanto a su aplicación clínica, tanto los polifenoles en general como los derivados del estilbeno o estilbenoides en particular son moléculas utilizadas para diferentes tratamientos. En este sentido, algunos
60 estudios indican que RES presenta varios beneficios para la salud, tales como la prevención de problemas cardiovasculares, oncológicos e inflamatorios (Das S., Das D. K., 2007, *Inflamm Allergy Drug Targets*, 6(3):168-173). Por estos motivos, se han descrito algunas composiciones oftálmicas que

comprenden compuestos polifenólicos para el tratamiento de la inflamación ocular, como por ejemplo, la que se describe en US20060127505.

Sin embargo, debido a que un gran número de patologías y lesiones oculares cursan frecuentemente con procesos inflamatorios, estrés oxidativo y/o neovascularización ocular, continúa existiendo como objetivo en el campo de la oftalmología la identificación de compuestos y composiciones oftálmicas que permitan tratar y/o prevenir eficientemente dichos procesos patológicos.

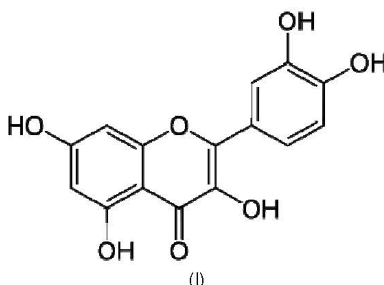
DESCRIPCIÓN DE LA INVENCIÓN

La presente invención propone el uso de la quercetina (QCT), o de cualquiera de sus sales o derivados, para el tratamiento y/o prevención de la inflamación, del estrés oxidativo y/o de la neovascularización ocular, preferiblemente del segmento anterior, más preferiblemente de la superficie ocular, y aun más preferiblemente del epitelio corneal. Además, la presente invención proporciona una composición, de ahora en adelante “composición de la invención”, que comprende QCT, o cualquiera de sus sales o derivados, y al menos otro compuesto polifenólico, o cualquiera de sus sales o derivados, preferiblemente resveratrol (RES), la cual también es de utilidad en dicha aplicación clínica.

La QCT, o cualquiera de sus sales o derivados, así como la composición de la invención, son por tanto útiles para el tratamiento y/o prevención de la inflamación, del estrés oxidativo y de la neovascularización ocular, preferiblemente del segmento anterior, más preferiblemente de la superficie ocular, y aun más preferiblemente del epitelio corneal. Como demuestran los ejemplos de la presente invención, tanto la QCT sola como dicha composición son capaces de reducir los niveles de, por ejemplo, aunque sin limitarnos, citoquinas, quimioquinas, ciclooxigenasa 2 (COX-2) y especies oxidativas en, por ejemplo, aunque sin limitarnos, células de epitelio corneal humano (HCE) estimuladas con TNF-α o irradiadas con luz ultravioleta. También son capaces de disminuir significativamente la cantidad de linfocitos CD4+ en conjuntiva en modelos animales de síndrome de ojo seco (SOS).

Por ello, un primer aspecto de la invención se refiere al uso de la QCT, o de cualquiera de sus sales o derivados, para la elaboración de un medicamento para el tratamiento y/o prevención de la inflamación ocular, preferiblemente de la superficie ocular, más preferiblemente del epitelio corneal. Alternativamente, este aspecto de la invención se refiere a la QCT, o a cualquiera de sus sales o derivados, para su uso como medicamento en el tratamiento y/o prevención de la inflamación ocular, preferiblemente de la superficie ocular, más preferiblemente del epitelio corneal.

La “quercetina” o “QCT” o “3,3',4',5,7-pentahidroxi-2-fenilcromen-4-ona” es un compuesto polifenólico flavonoide y natural, que se encuentra en altas concentraciones en una gran variedad de especies vegetales y, por tanto, en alimentos tales como, por ejemplo aunque sin limitarnos, fruta, verduras, cereales, té o vino tinto; aunque también puede ser sintetizada químicamente. Su fórmula molecular es $C_{15}H_{10}O_7$ y su estructura química (I) es la que se indica a continuación:



(I)

Así, el medicamento que comprende QCT, o cualquiera de sus sales o derivados, es de utilidad para el tratamiento y/o prevención de enfermedades o lesiones que cursan con inflamación ocular.

Otro aspecto de la invención se refiere al uso de la QCT, o de cualquiera de sus sales o derivados, para la elaboración de un medicamento para el tratamiento y/o prevención del estrés oxidativo ocular, preferiblemente de la superficie ocular, más preferiblemente del epitelio corneal. Alternativamente, este

aspecto de la invención se refiere a la QCT, o a cualquiera de sus sales o derivados, para su uso como medicamento en el tratamiento y/o prevención del estrés oxidativo ocular, preferiblemente de la superficie ocular, más preferiblemente del epitelio corneal.

5 Así, el medicamento que comprende QCT, o cualquiera de sus sales o derivados, es de utilidad para el tratamiento y/o prevención de enfermedades o lesiones que cursan con estrés oxidativo ocular.

Otro aspecto de la invención se refiere al uso de la QCT, o de cualquiera de sus sales o derivados, para la elaboración de un medicamento para el tratamiento y/o prevención de la neovascularización ocular, 10 preferiblemente del segmento anterior, más preferiblemente de la superficie ocular, aun más preferiblemente de la córnea. Alternativamente, este aspecto de la invención se refiere a la QCT, o a cualquiera de sus sales o derivados, para su uso como medicamento en el tratamiento y/o prevención de la neovascularización ocular, preferiblemente del segmento anterior, más preferiblemente de la superficie ocular, aun más preferiblemente de la córnea.

15

Así, el medicamento que comprende QCT, o cualquiera de sus sales o derivados, es de utilidad para el tratamiento y/o prevención de enfermedades o lesiones que cursan con neovascularización ocular.

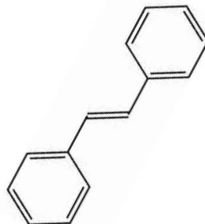
En otra realización preferida, el medicamento que comprende QCT, o cualquiera de sus sales o 20 derivados, es para el tratamiento y/o prevención de síndrome de ojo seco (SOS), blefaritis, disfunción de las glándulas de Meibomio, meibomitis, procesos alérgicos oculares, distrofias corneales, conjuntivitis, alteración de la superficie ocular por el uso de lentes de contacto, enfermedades autoinmunes que afecten al segmento anterior del ojo, postcirugía del segmento anterior, quemaduras (tanto químicas o “causticaciones” como térmicas) o lesiones o patologías oculares producidas por radiación ultravioleta. 25 Más preferiblemente, el medicamento que comprende QCT, o cualquiera de sus sales o derivados, es para el tratamiento y/o prevención de SOS.

Otro aspecto de la invención se refiere al uso de la QCT, o de cualquiera de sus sales o derivados, como agente antimicrobiano, antioxidante y antiinflamatorio en soluciones de limpieza y mantenimiento de 30 lentes de contacto. Otro aspecto de la invención se refiere a una solución para la limpieza y mantenimiento de lentes de contacto que comprende QCT o cualquiera de sus sales o derivados.

Un segundo aspecto de la invención se refiere a una composición, “composición de la invención”, que comprende QCT, o cualquiera de sus sales o derivados, y al menos otro compuesto polifenólico, o 35 cualquiera de sus sales o derivados.

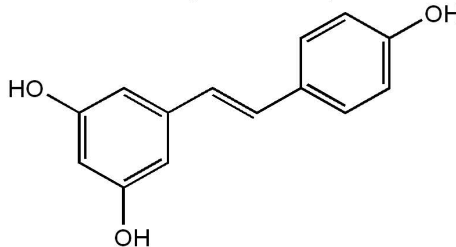
El término “otro compuesto polifenólico” empleado en la presente invención, hace referencia a un metabolito secundario biosintetizado por, aunque sin limitarnos, organismos del reino vegetal que se puede encontrar en, por ejemplo, alimentos derivados de fuentes vegetales, pero que también se puede 40 obtener sintéticamente, y que posee más de un grupo fenol. Los polifenoles son un grupo de moléculas cuya característica principal es la presencia de grupos bencilo e hidroxilo en su estructura. Estos polifenoles se pueden clasificar de forma general como: ácidos fenólicos, flavonoides, estilbenos y lignanos. A su vez, los compuestos flavonoides se pueden subclasificar como chalconas, flavonoles, flavonas, flavanonas, antocianidinas e isoflavonoides. El otro compuesto polifenólico de la composición de 45 la invención puede ser, aunque sin limitarnos, cualquier compuesto polifenólico dentro de las cuatro familias. Por ello, en una realización preferida, el otro compuesto polifenólico comprendido en la composición de la invención es un estilbenoide, más preferiblemente el RES, o cualquiera de sus sales o derivados.

50 Un “compuesto estilbenoide” es cualquier compuesto cuya estructura principal sea la molécula de estilbeno (II), tales como por ejemplo, aunque sin limitarnos, resveratrol, piceid piceatanol, oxiresveratrol, rhapontigenin o pterostilbeno.



(II)

5 El “resveratrol”, “RES” ó “5-[(E)-2-(4-hidroxifenil)etenil]benzen-1,3-diol” se encuentra en una gran variedad de plantas, aunque también puede ser sintetizado químicamente. Su número CAS es 501-36-0, su fórmula molecular es $C_{14}H_{12}O_3$ y su estructura química (III) es la que se indica a continuación:



(III)

10

Tal como aquí se utiliza, el término “derivado” incluye a compuestos farmacéuticamente aceptables, es decir, derivados de la QCT o del otro compuesto polifenólico comprendido en la composición de la invención, preferiblemente RES, que pueden ser utilizados en la elaboración de la composición de la invención, y a derivados farmacéuticamente no aceptables, ya que éstos pueden ser útiles en la
15 preparación de derivados farmacéuticamente aceptables.

En una realización más preferida, la composición de la invención además comprende un vehículo farmacéuticamente aceptable. Además, dicha composición puede comprender uno o más excipientes.

20 El término “excipiente” hace referencia a una sustancia que ayuda a la absorción de los elementos de la composición de la invención, estabiliza dichos elementos, activa o ayuda a la preparación de la composición en el sentido de darle consistencia o aportar sabores que la hagan más agradable. Así pues, los excipientes podrían tener la función de mantener los ingredientes unidos, como por ejemplo es el caso de almidones, azúcares o celulosas, la función de endulzar, la función de colorante, la función de
25 protección de la composición, como por ejemplo, para aislarla del aire y/o la humedad, la función de relleno de una pastilla, cápsula o cualquier otra forma de presentación, la función desintegradora para facilitar la disolución de los componentes y su absorción en el intestino, sin excluir otro tipo de excipientes no mencionados en este párrafo.

30 El “vehículo farmacéuticamente aceptable”, al igual que el excipiente, es una sustancia que se emplea en la composición para diluir cualquiera de los componentes comprendidos en ella hasta un volumen o peso determinado. El vehículo farmacéuticamente aceptable es una sustancia inerte o de acción análoga a cualquiera de los elementos comprendidos en la composición de la presente invención. La función del vehículo es facilitar la incorporación de otros elementos, permitir una mejor dosificación y administración o
35 dar consistencia y forma a la composición.

6

Preferiblemente, la composición de la invención comprende QCT, o cualquiera de sus sales o derivados, y al menos otro compuesto polifenólico, o cualquiera de sus sales o derivados, preferiblemente RES, en una cantidad terapéuticamente efectiva, entendiéndose por "cantidad terapéuticamente efectiva" el nivel, cantidad o concentración de QCT, o de cualquiera de sus sales o derivados, y de al menos otro compuesto polifenólico, o de cualquiera de sus sales o derivados, preferiblemente de RES, que produzca el efecto deseado tratando y/o previniendo la inflamación, el estrés oxidativo y/o la neovascularización ocular, sin causar efectos adversos. La dosificación para obtener una cantidad terapéuticamente efectiva depende de una variedad de factores, como por ejemplo, la edad, peso, sexo o tolerancia del individuo al que le va a ser administrada la composición de la invención.

La composición de la presente invención puede formularse para su administración en una variedad de formas conocidas en el estado de la técnica. Como ejemplos de preparaciones se incluye cualquier composición sólida (comprimidos, píldoras, cápsulas, gránulos, etc.) o líquida (soluciones, suspensiones o emulsiones) para administración oral, tópica o parenteral. La composición de la presente invención también puede estar en forma de formulaciones de liberación sostenida de drogas o de cualquier otro sistema convencional de liberación, así puede estar contenida, aunque sin limitarnos, en nanopartículas, liposomas o nanosferas, en un material polimérico, en un implante biodegradable o no biodegradable o en micropartículas biodegradables, como por ejemplo, microesferas biodegradables.

Tal composición y/o sus formulaciones pueden administrarse a un animal, incluyendo un mamífero y, por tanto, al hombre, en una variedad de formas, incluyendo, pero sin limitarse, intraperitoneal, intravenosa, intradérmica, intraespinal, intraestromal, intraarticular, intrasinovial, intratecal, intralesional, intraarterial, intramuscular, intranasal, intracraneal, subcutánea, intraorbital, intracapsular, tópica, mediante parches transdérmicos, percutánea, espray nasal, implante quirúrgico, pintura quirúrgica interna o bomba de infusión.

En una realización aun más preferida, la composición de la invención se encuentra formulada para su administración oftálmica. La expresión “formulada para su administración oftálmica” se refiere a una formulación que permita que la composición de la invención pueda ser administrada ocularmente, por ejemplo aunque sin limitarnos, de manera tópica o de manera intraocular, sin que dicha administración afecte negativamente a las propiedades, por ejemplo ópticas y/o fisiológicas, del ojo. Ejemplos de la composición de la invención formulada para su administración oftálmica son, aunque sin limitarnos, dicha composición asociada a agua, a sales, a un vehículo líquido polimérico o semi-sólido, a un tampón fosfato o a cualquier otro vehículo líquido oftálmicamente aceptable de los conocidos en el estado de la técnica.

Como se ha explicado anteriormente, la composición de la invención es capaz de reducir los niveles de, por ejemplo, aunque sin limitarnos, citoquinas, quimioquinas y COX-2, factores implicados en el proceso inflamatorio, en células oculares que presentan un fenotipo inflamado, así como de especies oxidativas en células oculares que han sido inducidas a sufrir estrés oxidativo. Por ello, la composición de la invención es útil para el tratamiento y/o prevención de la inflamación, del estrés oxidativo y de la neovascularización ocular, preferiblemente del segmento anterior, más preferiblemente de la superficie ocular, y aun más preferiblemente del epitelio corneal.

Así, otro aspecto de la invención se refiere al uso de la composición de la invención para la elaboración de un medicamento o, alternativamente, a la composición de la invención para su uso como medicamento, de ahora en adelante, “medicamento de la invención”.

Los “medicamentos” a los que se refiere la presente invención pueden ser de uso humano o veterinario. El “medicamento de uso humano” es toda sustancia o combinación de sustancias que se presente como poseedora de propiedades para el tratamiento o prevención de enfermedades en seres humanos o que pueda usarse en seres humanos o administrarse a seres humanos con el fin de restaurar, corregir o modificar las funciones fisiológicas ejerciendo una acción farmacológica, inmunológica o metabólica, o de establecer un diagnóstico médico. El “medicamento de uso veterinario” es toda sustancia o combinación de sustancias que se presente como poseedora de propiedades curativas o preventivas con respecto a las enfermedades animales o que pueda administrarse al animal con el fin de restablecer, corregir o modificar sus funciones fisiológicas ejerciendo una acción farmacológica, inmunológica o metabólica, o de establecer un diagnóstico veterinario.

En una realización preferida de este aspecto de la invención, el medicamento es para el tratamiento y/o prevención de la inflamación ocular.

Se entiende por “inflamación ocular” la inflamación producida en cualquier estructura ocular, incluidas todas las estructuras del segmento anterior, del segmento posterior del ojo y los anejos oculares, por

ejemplo, aunque sin limitarnos, en el nervio óptico, párpados, glándula lagrimal principal, conducto hialoideo, retina, coroides, esclera, musculatura ocular o en cualquier estructura que forme parte del segmento anterior, incluyendo la superficie ocular. Se entiende por "segmento anterior" cualquier estructura seleccionada de la lista que comprende: conjuntiva, cornea, limbo esclerocorneal, iris, pupila, 5 cristalino, zónula de Zinn, cuerpo ciliar, cámara anterior, humor acuoso, cámara posterior, glándulas de Meibomio, glándulas mucosas o aparato lagrimal (glándulas lagrimales, a excepción de la glándula lagrimal principal, conducto nasolagrimal y saco lagrimal). La inflamación ocular cursa con síntomas tales como por ejemplo, aunque sin limitarnos, dolor, enrojecimiento e hinchazón del tejido afectado, así como con la sobreexpresión de factores implicados en el proceso inflamatorio tales como, por ejemplo aunque 10 sin limitarnos, IL-6, IL-8, IP-10, VEGF, TNF-α, COX-1 o COX-2.

En una realización más preferida, el medicamento de la invención es para el tratamiento y/o prevención de la inflamación del segmento anterior y/o de la glándula lagrimal principal y/o párpados. En una realización aun más preferida, el medicamento de la invención es para el tratamiento y/o prevención de la 15 inflamación de la superficie ocular. Se entiende por "superficie ocular" cualquier estructura seleccionada de la lista que comprende: epitelios de la córnea, limbo esclero-corneal o conjuntiva, película lagrimal suprayacente o estroma subyacente a estas estructuras. En una realización aun más preferida, el medicamento de la invención es para el tratamiento y/o prevención de la inflamación del epitelio corneal.

20 La inflamación ocular está asociada a una diversidad de enfermedades y lesiones oculares, tales como por ejemplo, aunque sin limitarnos, Síndrome de Insuficiencia Límbica (SIL), SOS, blefaritis, disfunción de las glándulas de Meibomio, meibomitis, procesos alérgicos oculares, conjuntivitis, alteración de la superficie ocular, preferiblemente del epitelio corneal, provocada por el uso de lentes de contacto y de sus sistemas de limpieza y mantenimiento, enfermedades autoinmunes, preferiblemente que afectan al 25 segmento anterior del ojo, como por ejemplo aunque sin limitarnos, síndrome de Sjögren, postcirugía, preferiblemente del segmento anterior del ojo, quemaduras (tanto químicas o causticaciones, como térmicas) o lesiones o patologías oculares producidas por radiación ultravioleta. Por ello, el medicamento de la invención es de utilidad para el tratamiento y/o prevención de enfermedades o lesiones que cursan con inflamación ocular, preferiblemente, de las indicadas en este párrafo.

30

En otra realización preferida, el medicamento de la invención es para el tratamiento y/o prevención del estrés oxidativo ocular.

Se entiende por "estrés oxidativo ocular" la condición de citotoxicidad que es consecuencia de un 35 desequilibrio entre la producción de radicales libres y la capacidad de la célula de defenderse contra ellos, por lo que está causada por un incremento en la formación de dichos radicales libres o por una disminución de los agentes que actúan como antioxidantes, o por ambos motivos conjuntamente. El estrés oxidativo ocular puede producirse en cualquier estructura ocular, incluidas todas las estructuras del segmento anterior, del segmento posterior del ojo y los anejos oculares, por ejemplo, aunque sin 40 limitarnos, en el nervio óptico, glándula lagrimal principal, conducto hialoideo, retina, coroides, esclera, musculatura ocular o en cualquier estructura que forme parte del segmento anterior, incluyendo la superficie ocular. En una realización más preferida, el medicamento de la invención es para el tratamiento y/o prevención del estrés oxidativo del segmento anterior. En una realización aun más preferida, el medicamento de la invención es para el tratamiento y/o prevención del estrés oxidativo de la superficie 45 ocular. En una realización aun más preferida, el medicamento de la invención es para el tratamiento y/o prevención del estrés oxidativo del epitelio corneal.

El estrés oxidativo ocular está asociado a una diversidad de lesiones y enfermedades oculares, tales como por ejemplo, aunque sin limitarnos, pterigium, cataratas, distrofias corneales, glaucoma, retinopatía 50 diabética, degeneración macular, SOS, SIL, blefaritis, disfunción de las glándulas de Meibomio, meibomitis, procesos alérgicos oculares, conjuntivitis, alteración de la superficie ocular, preferiblemente del epitelio corneal, provocada por el uso de lentes de contacto y de sus sistemas de limpieza y mantenimiento, enfermedades autoinmunes, preferiblemente que afectan al segmento anterior del ojo, como por ejemplo aunque sin limitarnos, síndrome de Sjögren, postcirugía, preferiblemente del segmento 55 anterior del ojo, quemaduras (tanto químicas o causticaciones, como térmicas) o lesiones o patologías oculares producidas por radiación ultravioleta o por cualquier otro factor endógeno, como por ejemplo aunque sin limitarnos, reacciones inflamatorias, o exógeno, como por ejemplo aunque sin limitarnos, humo del tabaco o contaminantes ambientales, capaz de inducir formación de especies radicalarias. Por ello, el medicamento de la invención es de utilidad para el tratamiento y/o prevención de enfermedades o 60 lesiones que cursan con estrés oxidativo ocular, preferiblemente, de las indicadas en este párrafo.

En otra realización preferida, el medicamento de la invención es para el tratamiento y/o prevención de la neovascularización ocular.

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Se entiende por “neovascularización ocular” la formación de nuevos vasos sanguíneos en las estructuras oculares, por ejemplo aunque sin limitarnos, en la retina, papila óptica, coroides o en cualquier estructura que forme parte del segmento anterior, incluyendo la superficie ocular, preferiblemente en córnea, conjuntiva, limbo esclerocorneal o iris. En una realización más preferida, el medicamento de la invención
5 es para el tratamiento y/o prevención de la neovascularización del segmento anterior. En una realización aun más preferida, el medicamento de la invención es para el tratamiento y/o prevención de la neovascularización de la superficie ocular. En una realización aun más preferida, el medicamento de la invención es para el tratamiento y/o prevención de la neovascularización de la córnea.

10 La neovascularización ocular está asociada a una diversidad de lesiones y enfermedades oculares, tales como por ejemplo, aunque sin limitarnos, conjuntivitis, alteración de la superficie ocular, preferiblemente del epitelio corneal, provocada por el uso de lentes de contacto y de sus sistemas de limpieza y mantenimiento, enfermedades autoinmunes (como síndrome de Stevens-Johnson, Lyell, penfigoide de las membranas mucosas, etc.), preferiblemente que afectan al segmento anterior, postcirugía,
15 preferiblemente del segmento anterior, quemaduras (tanto químicas o causticaciones, como térmicas) o lesiones o patologías oculares producidas por radiación ultravioleta. Por ello, el medicamento de la invención es de utilidad para el tratamiento y/o prevención de enfermedades o lesiones que cursan con neovascularización ocular, preferiblemente, de las indicadas en este párrafo.

20 En una realización aun más preferida, el medicamento de la invención es para el tratamiento y/o prevención de SOS, blefaritis, disfunción de las glándulas de Meibomio, meibomitis, procesos alérgicos oculares, distrofias corneales, conjuntivitis, alteración de la superficie ocular por el uso de lentes de contacto, enfermedades autoinmunes que afectan al segmento anterior del ojo, postcirugía del segmento anterior, quemaduras (tanto químicas o “causticaciones” como térmicas) o lesiones o patologías oculares
25 producidas por radiación ultravioleta. Aun más preferiblemente, el medicamento de la invención es para el tratamiento y/o prevención de SOS (queratoconjuntivitis seca, queratitis seca o xeroftalmia), el cual se ha definido como una enfermedad multifactorial de la superficie ocular, incluyendo la película lagrimal, que causa síntomas de incomodidad, perturbación visual e inestabilidad lagrimal, con un daño potencial a la superficie ocular, acompañado de un aumento de la osmolaridad de la película lagrimal e infamación de la
30 superficie ocular. Dicho síndrome se puede diagnosticar por ejemplo, aunque sin limitarnos, mediante un examen con lámpara de hendidura de la película lagrimal, durante el cual se puede colocar un colorante en el ojo, como la fluoresceína, para hacer que dicha película sea más visible y así poder evaluar su estabilidad, o bien mediante la prueba del test de Schirmer, la cual mide la tasa de producción de lágrimas usando una tira de papel de filtro que se coloca en el extremo del párpado y mide la cantidad de lágrima
35 que produce el ojo.

Por otro lado, la limpieza y mantenimiento de las lentes de contacto es fundamental para evitar posibles patologías oculares derivadas de su uso. Los sistemas de limpieza y mantenimiento deben cumplir una serie de requisitos como son no alterar ni irritar los tejidos oculares, no alterar ni interferir en la fisiología
40 normal del ojo, no alterar ni dañar las lentes de contacto, evitar la contaminación por microorganismos de las lentes de contacto y mantener éstas lo más limpias posible. Tanto la QCT sola como la composición de la invención cumplen todos estos requisitos, y además presentan las características que debe cumplir un agente desinfectante de este tipo: pH y tonicidad similares a la lágrima, bacteriostático y/o bactericida, soluble en agua y estable en solución acuosa y en frascos de plástico.

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Por todo ello, otro aspecto de la invención se refiere al uso de la composición de la invención como agente antimicrobiano, antioxidante y antiinflamatorio en soluciones de limpieza y mantenimiento de lentes de contacto. Otro aspecto de la invención se refiere a una solución para la limpieza y mantenimiento de lentes de contacto que comprende la composición de la invención.

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Un “agente antimicrobiano” es aquel compuesto químico, o mezcla de compuestos, que inhibe el crecimiento o mata a los microorganismos. El agente antimicrobiano, tal y como se entiende en la presente invención, puede ser, aunque sin limitarnos, antibacteriano (dirigido contra bacterias), antifúngico (dirigido contra hongos) o antivírico (dirigido contra virus) y puede ser estático, que inhibe el crecimiento
55 del microorganismo sin llegar a provocar su muerte, por ejemplo, bacteriostático o fungistático, o puede destruir a los microorganismos, por ejemplo, bactericida o fungicida.

Dentro de las “soluciones de limpieza y mantenimiento de lentes de contacto” se incluyen, aunque sin limitarnos, las soluciones limpiadoras, las soluciones conservadoras o humectantes, cuya función es
60 guardar y almacenar las lentes de contacto cuando no están en uso, las soluciones acondicionadoras, los sistemas de peróxidos y los sistemas de solución única, que realizan una limpieza mecánica y desinfectante y a su vez actúan como conservantes y humectantes de las lentes.

Tal y como se utiliza en la presente invención, el término "lentes de contacto" se refiere tanto a las lentes de contacto duras, incluyendo las rígidas y las gas permeable o semirrígidas, como a las blandas o hidrofílicas.

A lo largo de la descripción y las reivindicaciones la palabra "comprende" y sus variantes no pretenden excluir otras características técnicas, aditivos o componentes. Para los expertos en la materia, otros objetos, ventajas y características de la invención se desprenderán en parte de la descripción y en parte de la práctica de la invención. Los siguientes ejemplos y dibujos se proporcionan a modo de ilustración, y no se pretende que sean limitativos de la presente invención.

DESCRIPCIÓN DE LAS FIGURAS

Fig. 1. Muestra la cantidad de IL-6 producida en células de epitelio corneal humano (HCE) estimuladas con 25 ng/mL de TNF-α y tratadas con vehículo (0,5% de etanol (EtOH)), 50 µM de resveratrol (RES), 25 µM de quercetina (QCT), 25 µM de RES + 25 µM de QCT o 50 µM de RES + 25 µM de QCT, durante 24 horas. NOTA: las células fueron tratadas previamente a su estimulación durante 2 horas con el tratamiento correspondiente. Control: células de HCE no estimuladas con TNF-α y tratadas con vehículo. *: p<0,05.

Fig. 2. Muestra la cantidad de IL-8 producida en células de epitelio corneal humano (HCE) estimuladas con 25 ng/mL de TNF-α y tratadas con vehículo (0,5% de etanol (EtOH)), 50 µM de resveratrol (RES), 25 µM de quercetina (QCT), 25 µM de RES + 25 µM de QCT o 50 µM de RES + 25 µM de QCT, durante 24 horas. NOTA: las células fueron tratadas previamente a su estimulación durante 2 horas con el tratamiento correspondiente. Control: células de HCE no estimuladas con TNF-α y tratadas con vehículo. *: p<0,05.

Fig. 3. Muestra la cantidad de IP-10 producida en células de epitelio corneal humano (HCE) estimuladas con 25 ng/mL de TNF-α y tratadas con vehículo (0,5% de etanol (EtOH)), 50 µM de resveratrol (RES), 25 µM de quercetina (QCT), 25 µM de RES + 25 µM de QCT o 50 µM de RES + 25 µM de QCT, durante 24 horas. NOTA: las células fueron tratadas previamente a su estimulación durante 2 horas con el tratamiento correspondiente. Control: células de HCE no estimuladas con TNF-α y tratadas con vehículo. *: p<0,05.

Fig. 4. Muestra la cantidad de VEGF producida en células de epitelio corneal humano (HCE) estimuladas con 25 ng/mL de TNF-α y tratadas con vehículo (0,5% de etanol (EtOH)), 50 µM de resveratrol (RES), 25 µM de quercetina (QCT), 25 µM de RES + 25 µM de QCT o 50 µM de RES + 25 µM de QCT, durante 24 horas. NOTA: las células fueron tratadas previamente a su estimulación durante 2 horas con el tratamiento correspondiente. Control: células de HCE no estimuladas con TNF-α y tratadas con vehículo. *: p<0,05.

Fig. 5. Muestra la cantidad de ciclooxigenasa 2 (COX-2), en unidades normalizadas, producida en células de epitelio corneal humano (HCE) estimuladas con 25 ng/mL de TNF-α y tratadas con vehículo (0,5% de etanol (EtOH)), 50 µM de resveratrol (RES), 25 µM de quercetina (QCT), 25 µM de RES + 25 µM de QCT o 50 µM de RES + 25 µM de QCT, durante 24 horas. NOTA: las células fueron tratadas previamente a su estimulación durante 2 horas con el tratamiento correspondiente. Control: células de HCE no estimuladas con TNF-α y tratadas con vehículo. *: p<0,05.

Fig. 6. Muestra la cantidad de fluorescencia en unidades normalizadas a la cantidad total de proteína de la sonda H2DCF-DA oxidada, como medida de la generación intracelular de especies relativas de oxígeno, en células de epitelio corneal humano (HCE) expuestas a radiación UVB y tratadas con vehículo (0,5% de etanol (EtOH)), 50 µM de resveratrol (RES), 25 µM de quercetina (QCT), 25 µM de RES + 25 µM de QCT o 50 µM de RES + 25 µM de QCT, durante una hora. NOTA: las células fueron tratadas previamente a su estimulación durante 1 hora con el tratamiento correspondiente. Control: células de HCE no irradiadas con UVB y tratadas con vehículo. *: p<0,05.

Fig. 7. Muestra el promedio de células T CD4 + en la conjuntiva de ratones C57BL/6 en un modelo de SOS en ratón (Dursun *et al.*, 2002, *Invest Ophthalmol Vis Sci.*; 43(3):632-638) y tratados con vehículo, 0,01% resveratrol (RES), 0,01% quercetina (QCT) y 0,01% QCT + 0,01% RES. Control: ratones no expuestos a ojo seco inducido (OSI) y no tratados tópicamente. Sin tratamiento: ratones expuestos a OSI y no tratados tópicamente. *: p<0,05.

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EJEMPLOS DE REALIZACIÓN

A continuación se ilustrará la invención mediante unos ensayos realizados por los inventores, que ponen de manifiesto la efectividad de la composición de la invención, así como de la quercetina (QCT) y del 5 resveratrol (RES) individualmente, en la prevención y/o tratamiento de la inflamación, del estrés oxidativo y de la neovascularización ocular. Estos ejemplos específicos que se proporcionan sirven para ilustrar la naturaleza de la presente invención y se incluyen solamente con fines ilustrativos, por lo que no han de ser interpretados como limitaciones a la invención que aquí se reivindica. Por tanto, los ejemplos descritos más adelante ilustran la invención sin limitar el campo de aplicación de la misma.

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EJEMPLO 1. Efecto antiinflamatorio de la quercetina (QCT), del resveratrol (RES) y de su mezcla sobre una línea celular de epitelio corneal humano estimulada con TNF-α.

Cultivo celular

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Para todos los experimentos se utilizó una línea celular (HCE) derivada de epitelio corneal humano. Las células fueron cultivadas en medio DMEM/F12 suplementado con 15% de suero fetal bovino (FBS), 0,5% de DMSO, 0,1 mg/mL de toxina colérica, 10 ng/mL de EGF, 5 mg/mL de insulina obtenida de páncreas bovino y antibióticos (100 U/mL de penicilina y 0,1 mg/mL de estreptomicina). Para la realización de los 20 experimentos, se reemplazó el medio de cultivo por medio DMEM libre de $NaHCO_3$, rojo fenol y piruvato.

RES, QCT y su mezcla se disolvieron en etanol (EtOH). Las disoluciones stock fueron preparadas para tener una concentración constante de vehículo en todos los pocillos.

25 Estimulación celular con TNF-α

Las células se plantaron en placas de cultivo de 24 pocillos y se dejaron crecer hasta preconfluencia. Posteriormente, se pretrataron con 50 μM de RES, 25 μM de QCT, 25 μM de RES + 25 μM de QCT o 50 μM de RES + 25 μM de QCT, durante 2 horas. Pasado este tiempo, se eliminaron los sobrenadantes y se 30 estimularon las células durante 24 horas con 25 ng/mL de TNF-α en presencia de RES, QCT o QCT+RES (se volvieron a añadir). Como controles se utilizaron células no estimuladas con TNF-α y células estimuladas y tratadas ambas solo con vehículo. Una vez transcurrido el tiempo de incubación, los sobrenadantes se recogieron, se centrifugaron y se congelaron a -80 ºC para su posterior análisis. Las células adheridas a las placas de cultivo también se congelaron a -80 ºC para su posterior análisis.

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Determinación de la producción de citoquinas y quimioquinas

Se llevó a cabo el análisis de la producción de las citoquinas IL-6 y VEGF y de las quimioquinas IL-8 e IP-10 en los sobrenadantes recogidos tras la estimulación con TNF-α. Este análisis se llevó a cabo mediante 40 tecnología X-MAP en un Luminex IS-100 con un ensayo multianalito comercial (Milliplex, Millipore), siguiendo las indicaciones del fabricante. Los valores de producción, en pg/mL, de citoquinas/quimioquinas (obtenidos tras la interpolación de los valores de fluorescencia en curvas estándares generadas en el ensayo) se normalizaron posteriormente respecto a la cantidad de proteína total correspondiente en cada muestra, determinada mediante un ensayo de BCA comercial (Pierce, USA) 45 en las células adheridas al pocillo.

Determinación de la producción de ciclooxigenasa 2 (COX-2)

Para este ensayo se utilizó un ensayo comercial "Cell-based ELISA" (R&D, USA) siguiendo las 50 indicaciones del fabricante. En este ensayo, se determinan simultáneamente las cantidades de COX-2 y de GAPDH de cada pocillo. Las células se plantaron en la placa del ensayo y se trataron y estimularon de la misma manera que se describió anteriormente. Finalizado el tiempo de incubación, se midieron los valores de fluorescencia correspondientes a la cantidad de COX-2 y de GAPDH en un espectrofotómetro (SpectraMAX M5, Molecular Devices, Inc. USA). Los valores de COX-2 en cada pocillo se normalizaron a 55 la cantidad de GAPDH correspondiente.

Resultados de la producción de citoquinas y quimioquinas

La figura 1 muestra la cantidad de IL-6 estimulada con TNF-α y su variación respecto a los tratamientos 60 con QCT, RES y QCT+RES. Se puede ver cómo la estimulación de las células HCE con TNF-α aumentó significativamente la producción de IL-6 ($p<0,05$). QCT, RES y QCT+RES disminuyeron drásticamente los valores de IL-6 ($p<0,05$). Los compuestos estudiados y su vehículo, en las concentraciones testadas, no fueron citotóxicos.

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La figura 2 muestra la cantidad de IL-8 estimulada con TNF-α y su variación respecto a los tratamientos con QCT, RES y QCT+RES. La estimulación de las células HCE con TNF-α aumentó significativamente la producción de IL-8 ($p<0,05$). QCT, RES y QCT+RES disminuyeron significativamente los valores de IL-8 ($p<0,05$).

La figura 3 muestra la cantidad de IP-10 estimulada con TNF-α y su variación respecto a los tratamientos con QCT, RES y QCT+RES. La estimulación de las células HCE con TNF-α aumentó significativamente la producción de IP-10 ($p<0,05$). QCT, RES y QCT+RES disminuyeron significativamente los valores de IP-10 hasta niveles basales ($p<0,05$).

La figura 4 muestra la cantidad de VEGF estimulada con TNF-α y su variación respecto a los tratamientos con QCT, RES y QCT+RES. La estimulación de las células HCE con TNF-α aumentó significativamente la producción de VEGF ($p<0,05$). QCT, RES y QCT+RES disminuyeron significativamente los valores de VEGF hasta niveles basales ($p<0,05$).

Resultados de la producción de COX-2

La figura 5 muestra la cantidad total de COX-2 (normalizada con GAPDH) estimulada con TNF-α y su variación respecto a los tratamientos con QCT, RES y QCT+RES. La estimulación de las células HCE con TNF-α aumentó significativamente la producción de COX-2 ($p<0,05$). QCT, RES y QCT+RES disminuyeron significativamente la cantidad de COX-2 estimulada por TNF- α ($p<0,05$).

EJEMPLO 2. Efecto antioxidante del resveratrol (RES), de la quercetina (QCT) y de su mezcla sobre una línea celular de epitelio corneal humano estimulada con radiación ultravioleta.

Cultivo celular

Como en el ejemplo anterior, se utilizó la línea celular HCE derivada de epitelio corneal humano. Las células fueron cultivadas en medio DMEM/F12 suplementado con 15% de suero fetal bovino (FBS), 0,5% de DMSO, 0,1 mg/mL de toxina colérica, 10 ng/mL de EGF, 5 mg/mL de insulina obtenida de páncreas bovino y antibióticos (100 U/mL de penicilina y 0,1 mg/mL de estreptomicina). Para la realización de los experimentos, se reemplazó el medio de cultivo por medio DMEM libre de $NaHCO_3$, rojo fenol y piruvato.

QCT, RES y su mezcla se disolvieron en etanol (EtOH). Las disoluciones stock fueron preparadas para tener una concentración constante de vehículo en todos los pocillos.

Estrés oxidativo intracelular provocado por estimulación con luz ultravioleta

Las células se plantaron en placas de cultivo de 24 pocillos y se dejaron crecer hasta preconfluencia. Posteriormente, se pretrataron con 50 μM de RES, 25 μM de QCT, 25 μM de RES + 25 μM de QCT o 50 μM de RES + 25 μM de QCT, durante 1 hora. Seguidamente, se cargaron las células con 10 μM de diacetato de 2',7'-diclorodihidrofluoresceina (H2DCF-DA) durante 30 minutos. A continuación, se trataron las células con QCT, RES o QCT+RES, se expusieron a luz UVB (302 nm; 107 mJ/cm^2) y se dejaron en incubación a 37 °C durante 1 hora. Como controles se utilizaron células no irradiadas y células irradiadas, y tratadas ambas solo con vehículo. Finalmente, se leyó la fluorescencia a 522 nm. Los valores de fluorescencia se normalizaron respecto a la cantidad de proteína total en cada muestra, determinada mediante un ensayo de BCA comercial (Pierce, USA) en las células adheridas al pocillo.

Resultados del estrés oxidativo provocado por radiación UVB

La figura 6 muestra la cantidad de fluorescencia relativa producida por la exposición a la radiación UVB y su variación respecto a los tratamientos con QCT, RES y QCT+RES. La estimulación de las células HCE con radiación UVB produjo un aumento significativo de especies oxidativas ($p<0,05$). QCT, RES y QCT+RES actuaron como antioxidantes disminuyendo los niveles de especies oxidativas generadas por la exposición a la radiación UVB, disminución que en el caso de la QCT y de la QCT+RES fue significativa ($p<0,05$).

EJEMPLO 3. Efecto antiinflamatorio de la quercetina (QCT), del resveratrol (RES) y de su mezcla, administrados en forma tópica, en un modelo animal de ojo seco.

Modelo animal de ojo seco

El ojo seco inducido (OSI) se provocó en ratones C57BL/6 mediante la inyección subcutánea de 200 µL de escopolamina (5mg/mL) tres veces al día durante 10 días, y la exposición a un flujo de aire constante durante 24 horas. La humedad en la sala se mantuvo entre el 30% y 35%, a una temperatura constante de 25 ºC (Dursun et al. *Invest Ophthalmol Vis Sci.* 2002; 43(3):632-8).

Ratones y tratamientos tópicos

Los ratones C57BL/6 se dividieron en 6 grupos de acuerdo con el tratamiento seguido:

a) Grupo control: ratones no expuestos a OSI y no tratados tópicamente.
b) Grupo OSI: ratones expuestos a OSI y no tratados tópicamente.
c) Grupo OSI+vehículo: ratones expuestos a OSI y tratados tópicamente con el vehículo.
d) Grupo OSI+RES: ratones expuestos a OSI y tratados tópicamente con 0,01% RES.
e) Grupo OSI+QCT: ratones expuestos a OSI y tratados tópicamente con 0,01% QCT.
f) Grupo OSI+QCT+RES: ratones expuestos a OSI y tratados tópicamente con 0,01% QCT + 0,01% RES.

Los tratamientos tópicos comenzaron 1 día antes de inducir el ojo seco, instilando en ambos ojos 5 µL del tratamiento correspondiente para cada grupo tres veces al día.

Determinación de linfocitos T CD4+ en conjuntiva

Los ojos embebidos en OCT se seccionaron en un criostato. Las muestras se fijaron con acetona fría a -20 ºC. Posteriormente, se bloqueó la peroxidasa endógena con peróxido de hidrógeno. Después, se bloquearon las uniones no específicas con suero y se incubaron las muestras con el anticuerpo primario a temperatura ambiente. Consecutivamente, se utilizó un anticuerpo secundario biotinilado seguido de reactivo ABC. Finalmente, se utilizó el cromógeno vector NovaRed para colorear el marcaje de la IMQ, seguido de contratinción nuclear con hematoxilina. Se examinaron tres secciones de cada ratón al microscopio óptico y los resultados se expresaron como el promedio de células T CD4+ en conjuntiva.

Resultados de las propiedades antiinflamatorias de QCT, RES y su mezcla en un modelo animal de ojo seco

La figura 7 muestra el promedio de linfocitos T CD4+ en conjuntiva respecto a los tratamientos tópicos con vehículo, QCT, RES y QCT+RES. El modelo de ojo seco produjo un aumento en la cantidad de células CD4+ en conjuntiva respecto al grupo control ($p<0,05$). RES mostró una disminución no significativa ($p>0,05$) de linfocitos CD4+, comparada con el grupo OSI+vehículo. QCT y QCT+RES disminuyeron significativamente la cantidad de células CD4+ en conjuntiva producida por OSI en ratones, comparado con el grupo OSI+vehículo ($p<0,05$).

REIVINDICACIONES

1. Composición que comprende quercetina, o cualquiera de sus sales o derivados, y al menos otro compuesto estilbenoide, o cualquiera de sus sales o derivados.

2. Composición según la reivindicación 1 donde el otro compuesto estilbenoide es resveratrol, o cualquiera de sus sales o derivados.

3. Composición según cualquiera de las reivindicaciones 1 ó 2 que además comprende un vehículo farmacéuticamente aceptable.

4. Uso de la composición según cualquiera de las reivindicaciones 1 a 3 para la elaboración de un medicamento.

5. Uso de la composición según la reivindicación 4 donde el medicamento está formulado para su administración oftálmica.

6. Uso de la composición según cualquiera de las reivindicaciones 4 ó 5 donde el medicamento es para el tratamiento y/o prevención de la inflamación ocular.

7. Uso de la composición según la reivindicación 6 donde el medicamento es para el tratamiento y/o prevención de la inflamación del segmento anterior y/o de la glándula lagrimal principal.

8. Uso de la composición según la reivindicación 7 donde el medicamento es para el tratamiento y/o prevención de la inflamación de la superficie ocular.

9. Uso de la composición según cualquiera de las reivindicaciones 4 ó 5 donde el medicamento es para el tratamiento y/o prevención del estrés oxidativo ocular.

10. Uso de la composición según la reivindicación 9 donde el medicamento es para el tratamiento y/o prevención del estrés oxidativo del segmento anterior.

11. Uso de la composición según la reivindicación 10 donde el medicamento es para el tratamiento y/o prevención del estrés oxidativo de la superficie ocular.

12. Uso de la composición según cualquiera de las reivindicaciones 4 ó 5 donde el medicamento es para el tratamiento y/o prevención de la neovascularización ocular.

13. Uso de la composición según la reivindicación 12 donde el medicamento es para el tratamiento y/o prevención de la neovascularización del segmento anterior.

14. Uso de la composición según la reivindicación 13 donde el medicamento es para el tratamiento y/o prevención de la neovascularización de la córnea.

15. Uso de la composición según cualquiera de las reivindicaciones 6 a 14 donde el medicamento es para el tratamiento y/o prevención de síndrome de ojo seco, blefaritis, disfunción de las glándulas de Meibomio, meibomitis, procesos alérgicos oculares, distrofias corneales, conjuntivitis, alteración de la superficie ocular por el uso de lentes de contacto, enfermedades autoinmunes que afecten al segmento anterior del ojo, postcirugía del segmento anterior, quemaduras o lesiones o patologías oculares producidas por radiación ultravioleta.

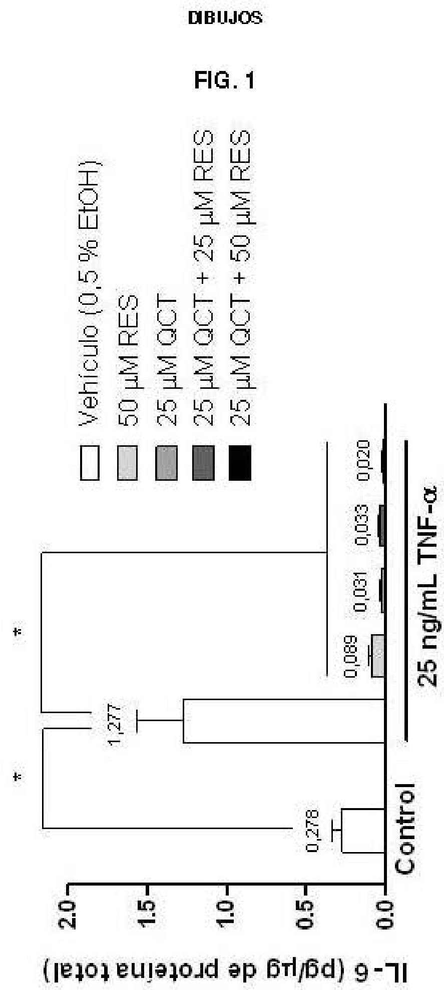

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FIG. 2

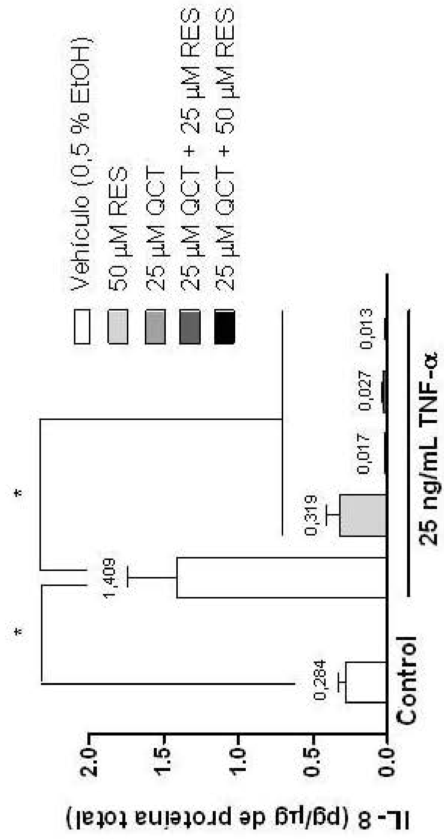

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FIG. 3

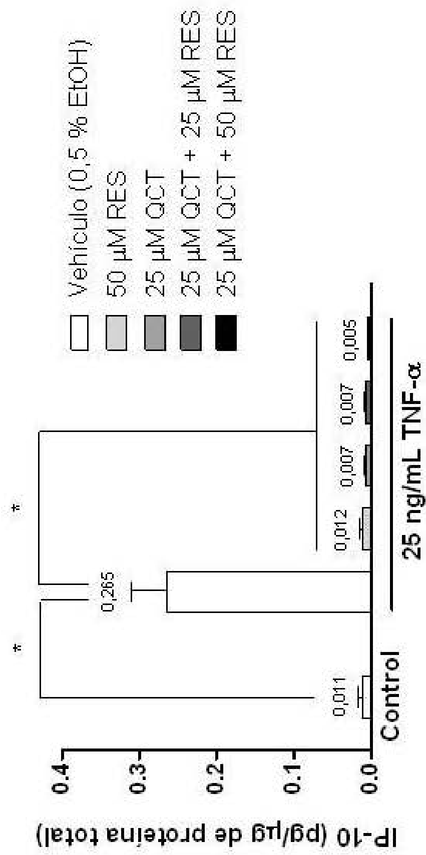

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FIG. 4

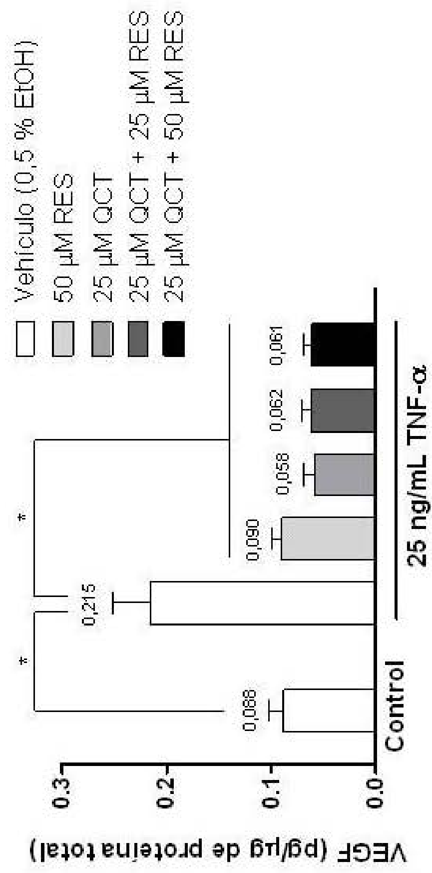

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FIG. 5

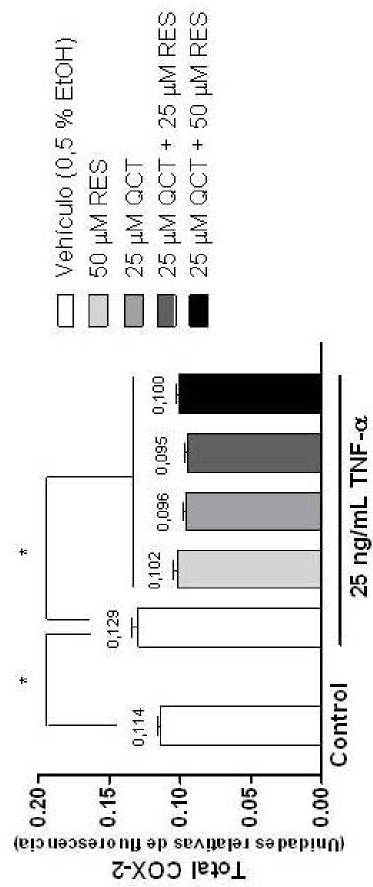

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FIG. 6

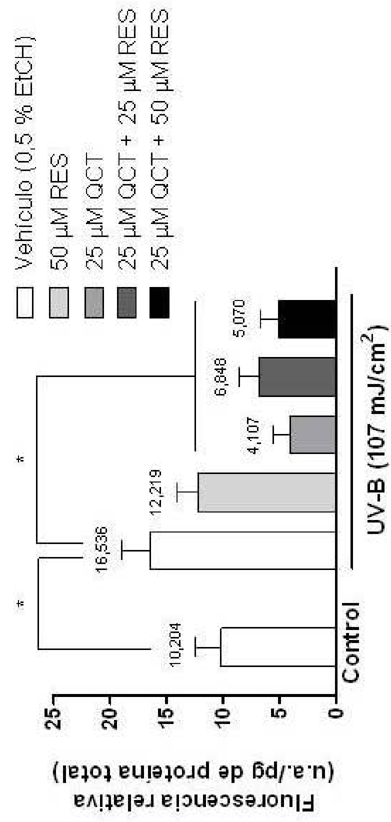

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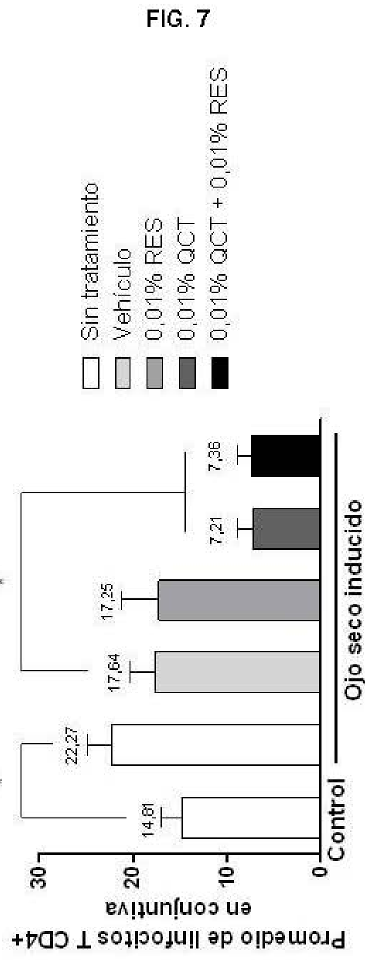
FIG. 7



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RESUMEN

COMPOSICIÓN PARA SU USO EN EL TRATAMIENTO Y/O PREVENCIÓN DE LA INFLAMACIÓN, EL ESTRÉS OXIDATIVO Y LA NEOVASCULARIZACIÓN OCULAR.

5

La presente invención se refiere al uso de la quercetina (QCT) o de una composición que comprende QCT y al menos otro compuesto polifenólico, preferiblemente resveratrol (RES), para el tratamiento y/o prevención de lesiones o enfermedades que cursan con inflamación, estrés oxidativo y/o neovascularización ocular, preferiblemente del segmento anterior, más preferiblemente de la superficie
10 ocular y aun más preferiblemente del epitelio corneal. La invención también se refiere al uso de la QCT y de dicha composición como agente antimicrobiano, antioxidante y antiinflamatorio en soluciones de limpieza y mantenimiento de lentes de contacto.

XIII. Appendix II

A preliminary study of the effect of QCT and RES on a normal conjunctival epithelial cell line exposed to UV-B radiation

This appendix describes briefly the results from a preliminary study regarding the effect of QCT and RES in UV-B-induced apoptosis in IOBA-NHC cells. These experiments were carried out in Allergan headquarter (Irvine, CA, USA) under the supervision of Dr. Michael E. Stern in 2013. This section has been divided into five parts: I) brief introduction describing the molecular mechanism underlying apoptosis, II) methodology, III) results and IV) conclusions.

Introduction

Apoptosis is the process of programmed cell death that occurs in autoimmune and inflammatory diseases. DED is characterised by an increased apoptotic cell death in the ocular resident epithelium of the LFU,[83] suggesting that this process contributes to pathogenesis of DED.

Apoptosis can be triggered by an extrinsic mechanism mediated by death receptors (members of the TNF receptor gene superfamily) located in the outer membrane of cells, or by an intrinsic mechanism mediated by mitochondria.

Focusing on the intrinsic mechanism, this is an early apoptotic process that involves an increase of the outer mitochondrial membrane permeability that results in the loss of mitochondrial membrane potential ($\Delta\Psi$), finally leading to rupture of the outer mitochondrial membrane. This process is called the mitochondrial membrane permeability transition (MPT). Thus, the MPT refers to the massive swelling and depolarisation of mitochondria that occurs under some conditions, most notably as a result of calcium overload and oxidative stress. The cause of the MPT is the opening of a non-specific pore in the inner mitochondrial membrane, known as the mitochondrial permeability transition pore (MPTP).[390] As a result, proteins from the intermembrane space are released into the cytoplasm, including apoptogenic molecules such as cytochrome C or Smac/Diablo, triggering a cascade of caspase activation that propagates apoptotic signals.[391]

A previous experiment from this thesis demonstrated that UV-B increases oxidative stress and QCT and RES can reduce UV-B-induced ROS in IOBA-NHC cells. UV-B radiation is also a pro-inflammatory stimulus[233,280] that can cause apoptosis.[392]

It has been described that inflammatory stimuli such as TNF-α and IFN-γ produces aberrant MPTP opening resulting in an increased apoptosis in IOBA-NHC cells.[393] Thus, the aim of this work was to investigate the role of MPTP and the effect of

QCT and RES on inflammatory apoptosis of human conjunctival epithelial cells exposed to UV-B radiation.

Materials and methods

Cell line and culture conditions

IOBA-NHC cells were used for this experiment. Cells were culture as described in section V.1.3. All experiments were carried out using the CM also described in section V.1.3.

UV-B exposure

Cells were seeded in 75 cm^2 flasks at $2x10^6$ cells per flask to confluence. Then, culture medium was replaced with CM and cells were exposed to UV-B light (9.5 mW/cm^2) for 1 min. After light exposure, cells were cultured for 1h and then collected by trypsin for further experiments.

Polyphenol treatments

QCT and RES were dissolved in EtOH. Fresh stock solutions of QCT and RES were firstly prepared and serial dilutions were carried out to achieve final concentrations of 1, 10 and 25 µM QCT; 5, 50 and 100 µM RES; and 0.5 % EtOH (vehicle). Polyphenol treatments were added 1 h before UV-B.

Detection of MPTP opening

MPTP opening was determined using the MitoProbe™ Transition Pore Assay Kit (Life Technologies), as previously described by Gao et al.[393] Briefly, UV-B-exposed cells were collected and then washed with PBS without $MgCl_2$ and $CaCl_2$. After that, cells were loaded with 2 µM calcein acetoxymethyl (AM) and 80 mM $CoCl_2$. Ionomycin (10 nM), an inductor of apoptosis, was added as a positive control. Cells were then incubated for 15 min, washed and analysed using a flow cytometer (BD).

Calcein AM is a fluorescent dye that passively diffuses into cells. Upon entering the cells, intracellular esterases cleave the acetoxymethyl (AM) ester group, yielding the membrane-impermeable calcein fluorescent dye. In the absence of $CoCl_2$ and ionomycin, fluorescent calcein is present in the cytosol as well as the mitochondria, resulting in a bright signal. In the presence of $CoCl_2$, calcein in the mitochondria emits a signal, but the cytosolic calcein fluorescence is quenched; the overall fluorescence is reduced compared to calcein alone. When ionomycin (a calcium ionophore) and $CoCl_2$

are added to cells at the same time as calcein AM, the fluorescence signals from both cytosol and mitochondria are largely abolished.

Apoptosis

Apoptosis was determined using a FITC annexin V apoptosis detection kit (BD), following manufacturer's instructions. Briefly, collected UV-B-exposed cells were washed with PBS and resuspended in 1X binding buffer at 10^6 cells/mL. Then, 5 µL of FITC Annexin V and 5 µL PI were added to 100 µL of cell suspension and incubated for 15 min at room temperature in the dark. Finally, 400 µL of 1X binding buffer were added and analysed by flow cytometry. Percentage of positive annexin V and positive PI cells were obtained using the CellQuest Pro software (BD).

Statistical analysis

All data were expressed as mean ± SEM. Statistics were analysed using the SPSS software package (SPSS version 15.0 for Windows, SPSS Inc., Chicago, IL, USA). Homogeneity of variances were analysed using Levene's test. Data were analysed using the *t*-test or *t*-test with Welch correction. Two different experiments were performed in duplicate.

Results

Figure 1 shows the effect of QCT and RES on MPTP opening (Figure 1A) and late apoptosis (Figure 1B) in IOBA-NHC cells. MPTP opening was induced in the normal IOBA-NHC cells by ionomycin ($p<0.001$) as a positive control. UV-B radiation (570 mJ/cm2) induced MPTP opening in IOBA-NHC cells after 1 h. QCT at 1, 10 and 25 µM (all $p<0.01$) doses and RES at 5 µM ($p<0.01$) and 50 µM ($p<0.05$) doses significantly inhibited MPTP opening (Figure 1A) without affecting apoptosis (Figure 1B).

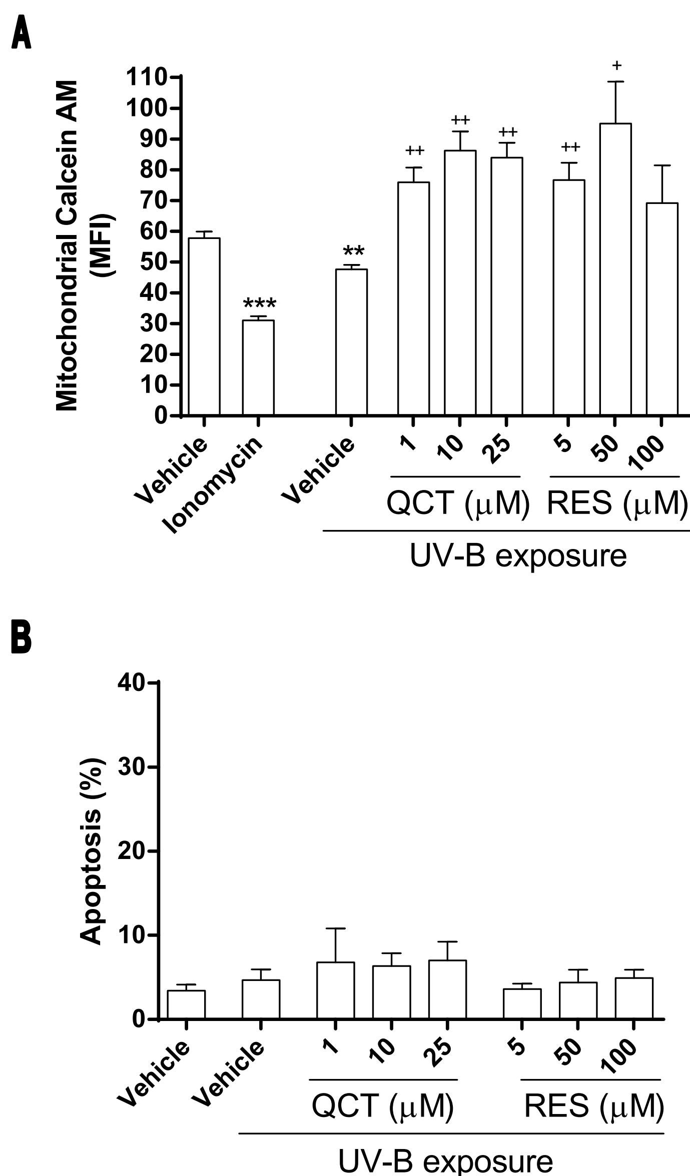
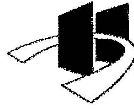


Figure 1. Effect of quercetin (QCT) and resveratrol (RES) on MPTP (A) and late apoptosis (B) on UV-B-exposed IOBA-NHC cell. $^{**}p<0.01$, $^{***}p<0.001$, compared to unexposed cells treated with vehicle; $^{+}p<0.05$, $^{++}p<0.01$, compared to vehicle-treated UV-B-exposed cells (0.5 % EtOH). N=2 for both experiments.

Conclusions

a) UV-B radiation (570 mJ/cm^2) promoted MPTP opening in IOBA-NHC cells.
b) UV-B radiation (570 mJ/cm^2) did not induce apoptosis after 1 h.
c) QCT prevented MPTP opening at all concentrations tested (1, 10 and 25 µM) in UV-B-exposed IOBA-NHC cells and did not induce apoptosis.
d) Concentrations of 5 and 50 µM RES prevented MPTP opening, without affecting apoptosis.

XIV. Appendix III



COMITÉ ÉTICO DE INVESTIGACIÓN CLÍNICA
ÁREA DE SALUD VALLADOLID – ESTE (CEIC-VA-ESTE-HCUV)

Valladolid a 23 de Mayo de 2013

En la reunión del CEIC ÁREA DE SALUD VALLADOLID – ESTE del 23 de Mayo de 2013, se procedió a la evaluación de los aspectos éticos del siguiente proyecto de investigación

A continuación les señalo los acuerdos tomados por el CEIC ÁREA DE SALUD VALLADOLID – ESTE en relación a dicho Proyecto de Investigación:

PI-13-82	"EFECTOS DE COMPUESTOS CXCLL-1 Y CXCLL-2 SOBRE CÉLULAS MONONUCLEARES PROCEDENTES DE SANGRE PEROFÉRICA HUMANA"	IOBA Investigador principal: Dra. AMALIA ENRIQUEZ DE SALAMANCA (BIOQUIMICA) Equipo investigador: ANTONIO ABENGÓZAR VELA (QUÍMICO, ÓPTICO-OPTOMETRISTA), CARMEN GARCÍA VÁZQUEZ (TÉCNICO DE LABORATORIO) Y Mª JESUS GONZÁLEZ GARCÍA (ÓPTICO.OPTOMETRISTA) Recibido: 07-05-2013

Considerando que el Proyecto contempla los Convenios y Normas establecidos en la legislación española en el ámbito de la investigación biomédica, la protección de datos de carácter personal y la bioética, se hace constar el **informe favorable** y la **aceptación** del Comité Ético de Investigación Clínica del Área de Salud Valladolid Este para que sea llevado a efecto dicho Proyecto de Investigación.

Un cordial saludo.

F. Javier Álvarez

Dr. F. Javier Álvarez.
CEIC Área de Salud Valladolid Este - Hospital Clínico Universitario de Valladolid
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Junta de Castilla y León

HOJA DE INFORMACIÓN AL PACIENTE Y CONSENTIMIENTO INFORMADO

Título del estudio:

Efecto de los compuestos CXCLL-1 y CXCLL-2 sobre células mononucleares procedentes de sangre periférica humana.

Investigador principal: Amalia Enríquez de Salamanca Aladro

Centro: IOBA, Universidad de Valladolid

1. Introducción.

Nos dirigimos a usted para informarle sobre el desarrollo del estudio en el que se le propone participar. Su participación en el estudio consiste en donar una muestra de sangre para aislar células mononucleares (linfocitos T) y estudiar sobre ellas las propiedades antiinflamatorias de ciertos compuestos de origen natural.

Nuestra intención es que usted reciba la información correcta y suficiente para que pueda evaluar y juzgar si quiere o no participar en este estudio. Para ello, lea esta hoja informativa con atención y nosotros le aclararemos las dudas que le puedan surgir después de la explicación.

2. Objetivos y justificación del estudio.

El síndrome de ojo seco es una enfermedad inflamatoria de la superficie ocular que afecta aproximadamente al 15 % de la población mundial. Uno de los problemas de la terapia médica es la falta de tratamientos efectivos para el síndrome de ojo seco; siendo el uso de lágrimas artificiales para reducir la sintomatología el único tratamiento hasta el momento disponible en España.

El objetivo final del estudio es desarrollar un tratamiento efectivo basado en compuestos de origen natural para el síndrome de ojo seco. Para ello, se pretende estudiar si los compuestos naturales objeto de estudio son capaces de disminuir la proliferación y el tipo de células mononucleares estimuladas en experimentos *in vitro*.

3. Participación voluntaria.

Debe saber que su participación en este estudio es voluntaria y que puede decidir no participar o cambiar su decisión y retirar el consentimiento en cualquier momento, sin que por ello se altere la relación con el centro. En caso de retirar el consentimiento para participar en el estudio, ningún dato nuevo será añadido a la base de datos y puede exigir la destrucción de todas las muestras identificables previamente retenidas para evitar la realización de nuevos análisis.

4. Descripción general del estudio. Procedimiento.

Para este procedimiento no se le administrará tratamiento extraordinario alguno. Si acepta la participación mediante la firma de este documento, se le realizará una extracción de sangre venosa de aproximadamente 40 mL (volumen máximo). La sangre se procesará para aislar las células mononucleares y éstas se utilizarán para determinar el efecto de los compuestos objeto de estudio, que tienen propiedades antiinflamatorias.

Usted deberá notificar al responsable del estudio si en el momento de la misma padece alguna enfermedad (muy especialmente si padece de algún tipo de alergia o proceso inflamatorio crónico) y/o está tomando algún tipo de medicación, bien sea bajo prescripción o no.

En el caso de requerirse una nueva extracción por imposibilidad de obtener los datos necesarios de la muestra ya extraída para la realización del estudio, se le requerirá para que realice una visita para realizar otra extracción de sangre.

5. Manejo de las muestras.

Las muestras obtenidas no se etiquetarán con ningún dato que permita revelar la identidad del donante. Las muestras obtenidas se recogerán en diferentes tipos de tubos para su procesamiento y serán destruidas una vez terminen los experimentos.

6. Confidencialidad.

Los datos recogidos para el estudio estarán identificados mediante un código y solo el investigador principal del estudio, y sus colaboradores, podrán relacionar dichos datos con usted. Por lo tanto, su identidad no será revelada a persona alguna ajena a los procedimientos aquí descritos.

Todos los datos recogidos para el estudio, procedentes de su Historia Clínica o facilitados por usted mismo, serán tratados con las medidas de seguridad establecidas en cumplimiento de la Ley Orgánica 15/1999 de Protección de Datos de carácter personal. Debe saber que tiene derecho de acceso, rectificación y cancelación de los mismos en cualquier momento. Los datos recogidos para el estudio estarán identificados mediante un código y solo el investigador principal/colaboradores podrán relacionar dichos datos con usted y con su historia clínica.

Todas las muestras obtenidas serán utilizadas exclusivamente para los fines descritos en este documento y serán destruidas al finalizar el estudio.

7. Otra información relevante.

Si usted decide retirar el consentimiento para participar en este estudio, ningún dato nuevo será añadido a la base de datos y, puede exigir la destrucción de todas las muestras identificables previamente retenidas para evitar la realización de nuevos análisis. También debe saber que puede ser excluido del programa si los responsables del estudio lo consideran oportuno.

En caso de necesitar cualquier información o por cualquier otro motivo no dude en contactar con los investigadores principales del estudio en el teléfono 983184761.

8. Riesgos.

La extracción de sangre tiene como principal inconveniente la percepción de una molestia mínima derivada de la extracción de sangre. Muy infrecuentemente se pueden producir un hematoma leve, o una hemorragia leve local.

Dado a que no es esperable que aparezcan acontecimientos adversos en lo que concierne a los sujetos que participan en la investigación, no se han tomado especiales medidas orientadas a su tratamiento.

CONSENTIMIENTO INFORMADO: Efecto de los compuestos CXCLL-1 y CXCLL-2 sobre células mononucleares procedentes de sangre periférica humana.

Promotor: IOBA

Responsables del proyecto: Amalia Enríquez de Salamanca Aladro, Instituto de Oftalmobiología Aplicada (IOBA), Paseo de Belén 17; 47011 Valladolid.

Centro donde se realiza la recogida de muestra:

Yo, (nombre y apellidos):

He leído la Hoja de Información que se me ha entregado, he podido hacer preguntas sobre el Programa y he recibido suficiente información sobre el estudio.

He hablado con, (nombre y apellidos del investigador):

Comprendo que mi participación es voluntaria y que puedo retirarme del estudio cuando quiera, sin tener que dar explicaciones y sin que esto repercuta en mis cuidados médicos.

Presto libremente mi conformidad para participar en el proyecto y doy mi consentimiento para el acceso y utilización de mis datos en las condiciones detalladas en la hoja de información.

Firma del paciente:	Firma del investigador:
Nombre:	Nombre:
Fecha:	Fecha:

Copia para el paciente

CONSENTIMIENTO INFORMADO: Efecto de los compuestos CXCLL-1 y CXCLL-2 sobre células mononucleares procedentes de sangre periférica humana.

Promotor: IOBA

Responsables del proyecto: Amalia Enríquez de Salamanca Aladro, Instituto de Oftalmobiología Aplicada (IOBA), Paseo de Belén 17; 47011 Valladolid.

Centro donde se realiza la recogida de muestra:

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Firma del paciente:	Firma del investigador:
Nombre:	Nombre:
Fecha:	Fecha:

Copia para el centro

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