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ANALYSIS OF *NLRP3* ACTIVATION IN HUMAN CONJUNCTIVAL MYOFIBROBLASTS CULTIVATED UNDER *IN VITRO* DRY EYE CONDITIONS

Análisis de la activación del NLRP3 en miofibroblastos humanos cultivados bajo condiciones in vitro de ojo seco

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TÍTULO: **Analysis of *NLRP3* activation in human conjunctival myofibroblasts cultivated under *in vitro* dry eye conditions**

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ABSTRACT

Dry eye disease (DED) is a prevalent and multifactorial disorder of the ocular surface, characterised by tear film instability and chronic inflammation. Recent studies have implicated inflammasomes, particularly the NOD-like receptor family, pyrin domain containing 3 (NLRP3), in the pathogenesis of DED. In this study, we investigated the effects of hyperosmolar (500 mOsM) and inflammatory (25 ng/mL TNF-α) conditions on *NLRP3* gene expression in conjunctival epithelial cells and conjunctival fibroblasts, exposed to stimulated conjunctival epithelial cells secretome, using an *in vitro* model. *NLRP3* gene expression was analysed by RT-qPCR with relative mRNA levels assessed using the $2^{(-\Delta\Delta Ct)}$ method and statistical significance determined via parametric, unpaired Student's t-test with equal variance and a two-tailed distribution. Our results demonstrated that epithelial cells exposed to these stressors significantly increased *NLRP3* expression, suggesting activation of inflammatory signalling pathways. However, the secretome from stressed epithelial cells did not induce a comparable response in conjunctival fibroblasts, indicating that paracrine signalling alone may not be sufficient to activate *NLRP3* in these cells under the tested conditions. These findings highlight the need for further research into the epithelial–mesenchymal communication in DED, including protein-level analysis of the secretome and investigation of additional inflammasome components, to better understand the contribution of fibroblasts to chronic ocular surface inflammation.

Keywords:

Dry eye disease, ocular inflammation, NLRP3 inflammasome, conjunctiva, myofibroblasts.

RESUMEN

La enfermedad de ojo seco (EOS) es un trastorno prevalente y multifactorial de la superficie ocular, caracterizado por inestabilidad de la película lagrimal e inflamación crónica. Estudios recientes han implicado a los inflamasomas, en particular la familia de receptores tipo NOD con dominio de pirina, miembro 3 (NLRP3), en la fisiopatología de la EOS. En este estudio, investigamos los efectos de las condiciones hiperosmolares (500 mOsM) e inflamatorias (25 ng/mL TNF-α) sobre la expresión del gen *NLRP3* en células epiteliales conjuntivales y fibroblastos conjuntivales, expuestos al secretoma de las células epiteliales conjuntivales estimuladas, utilizando un modelo *in vitro*. La expresión del gen *NLRP3* fue analizada mediante RT-qPCR, evaluando los niveles relativos de ARNm mediante el método $2^{(-\Delta\Delta Ct)}$ y determinando la significancia estadística mediante una prueba t de Student paramétrica, no pareada, con varianzas iguales y distribución bilateral. Nuestros resultados mostraron que las células epiteliales expuestas a estos estímulos incrementan significativamente la expresión de *NLRP3*, lo que sugiere la activación de vías de señalización inflamatorias. Sin embargo, el secretoma procedente de células epiteliales sometidas a estrés no indujo una respuesta comparable en los fibroblastos conjuntivales, lo que indica que la señalización paracrina por sí sola podría no ser suficiente para activar el *NLRP3* en estas células bajo las condiciones evaluadas. Estos hallazgos subrayan la necesidad de profundizar en el estudio de la comunicación epitelio-mesenquimal en la EOS, incluyendo el análisis proteico del secretoma y la investigación de otros componentes del inflamasoma, para comprender mejor la contribución de los fibroblastos a la inflamación crónica de la superficie ocular.

Palabras clave:

Enfermedad de ojo seco, inflamación ocular, NLRP3 inflamasoma, conjuntiva, miofibroblastos.

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LIST OF ABBREVIATIONS

ADDE – Aqueous-deficient dry eye

ASC – Apoptosis-associated speck-like protein containing a caspase recruitment domain

α-SMA – Alpha-smooth muscle actin

CARD – Caspase recruitment domain

DED – Dry eye disease

DEWS – Dry Eye Workshop

DAMP – Damage-associated molecular patterns

EDE – Evaporative dry eye

HConF – Human conjunctival fibroblasts

IM-HConEpiC – Immortalized human conjunctival epithelial cells

IL-1α – Interleukin-1 alpha

IL-1β – Interleukin-1 beta

LFU – Lacrimal functional unit

MAPK – Mitogen-Activated Protein Kinase

MMP9 – Matrix Metalloproteinase 9

NFκB – Nuclear Factor kappa-light-chain-enhancer of activated B cells

NLR – Nucleotide-binding oligomerization domain-like receptor

NLRP3 – NOD-like receptor family, pyrin domain containing 3

NSDE – Non-Sjögren syndrome dry eye

PAMP – Pathogen-associated molecular patterns

RT-qPCR – Reverse transcription-quantitative polymerase chain reaction

SSDE – Sjögren syndrome dry eye

TFOS – Tear Film and Ocular Surface Society

TLRs – Toll-like receptors

TNF-α – Tumor Necrosis Factor alpha

1. INTRODUCTION

1.1. LACRIMAL FUNCTIONAL UNIT

The lacrimal functional unit (LFU) comprises the ocular surface, the main lacrimal glands, and the neuronal pathways that regulate their function. The ocular surface (Figure 1) includes the cornea, the conjunctiva, the accessory lacrimal glands, and the Meibomian glands (1).

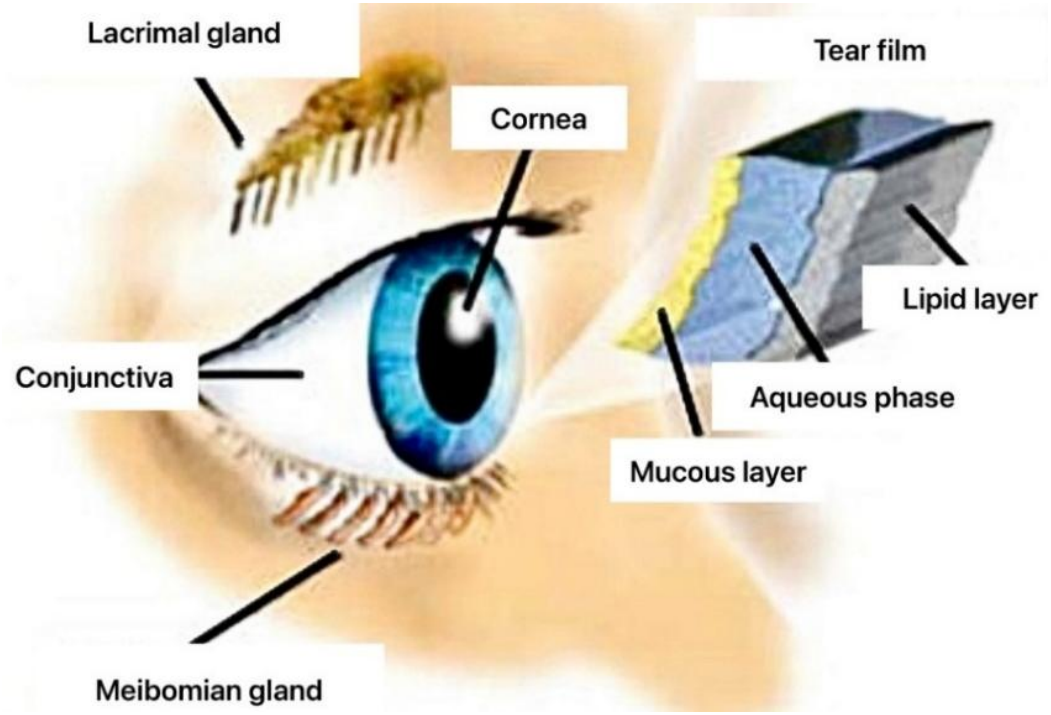

Figure 1. The ocular surface (modified from: Clinical Update on Dry Eye Disease for Non-Ophthalmologist Physicians (2)).

The cornea is composed of cellular elements: epithelial cells on the surface, keratocytes (corneal fibroblasts) located beneath the epithelium within the stroma, and endothelial cells lining the innermost layer; and acellular components primarily consist of collagen and glycosaminoglycans (3). Corneal fibroblasts are the most abundant stromal cells in the cornea and are responsible for the production of extracellular matrix components. Corneal injuries are known to promote their differentiation into myofibroblasts (4). These cells increase the secretion of aberrant extracellular matrix components and proinflammatory mediators, which exacerbates damage to the corneal stroma (5).

The conjunctiva (Figure 2) is a thin, transparent mucosa consisting of two parts: the palpebral conjunctiva and the bulbar conjunctiva, with the joining area called the fornix (6). It has two layers: the conjunctival epithelium and the subepithelial stroma where we can find the fibroblasts (7). The conjunctiva contributes to the tear film by secreting fluids, electrolytes, and mucins (6).

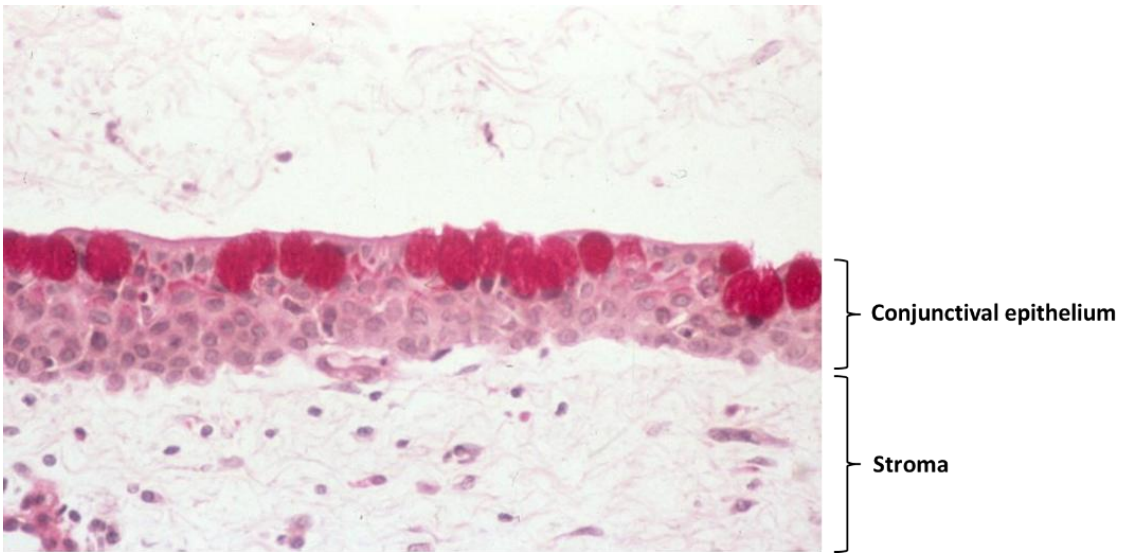

Figure 2. Histology of the conjunctiva (image provided by the Ocular Pathology Laboratory, Institute of Applied Ophthalmobiology (IOBA), University of Valladolid).

Stimulation of corneal nerves sends signals that activate the lacrimal glands, triggering the production of the tear film, which protects and lubricates the eye (8). Structurally, the tear film consists of three layers: a thin mucous layer, an aqueous phase, and a superficial lipid layer (9). The balanced production of these components is essential for maintaining clear vision. Any dysfunction of the ocular surface or tear film can disrupt the homeostasis and lead to dry eye disease (DED) (8).

1.2. GENERAL CONTEXT OF DED

According to the Tear Film and Ocular Surface Society (TFOS) Dry Eye Workshop (DEWS) II, DED is "a multifactorial disease of the ocular surface characterized by a loss of homeostasis of the tear film, and accompanied by ocular symptoms, in which tear film instability and hyperosmolarity, ocular surface inflammation and damage, and neurosensory abnormalities play etiological roles" (10).

This prevalent condition affects between 5% to 50% of the global population, with an increasing incidence due to aging, environmental changes, and lifestyle factors such as air conditioning, pollution, and prolonged screen exposure (11,12).

The prevalence of DED is higher in women, and geographical location plays an important role, with rates being less than 10% in Caucasians and over 60% in Asians (11,12). DED has a considerable economic impact. In the United States, the average cost per patient in 2008 was $11.302, and $55.4 billion nationwide (13). In Spain, the total annual expenditure increased from €4.9 million in 1997 to €30.3 million in 2015, with an average cost of €7,379 per patient. This increase reflects the rising incidence of the disease and its growing economic burden (14).

DED significantly impacts daily activities such as reading, driving, and using digital devices, reducing the quality of life of patients and increasing their risk of anxiety and depression (15,16).

The most common symptoms are dryness, discomfort, soreness, light sensitivity, and itching (17). The risk factors associated with this disease are shown in Table 1 (12,18).

Table 1. Risk factors associated with DED.

Non-modifiable	Modifiable
Ageing Female gender Asian ethnicity Meibomian gland dysfunction Connective tissue diseases Sjögren syndrome Autoimmune diseases	Androgen deficiency Computer use Contact lens wear Hormone replacement therapy Hematopoietic stem cell transplantation Reduced blinking Lifestyle factors: alcohol consumption and cigarette smoking Environment: pollution, low humidity, sick building syndrome Medications: antihistamines, antidepressants, anxiolytics, isotretinoin

Information obtained from references (12,18)

1.3. CLASSIFICATION OF DED

DED is primarily classified into two subtypes: aqueous deficient and evaporative (Figure 3) (10). However, mixed cases also exist (19).

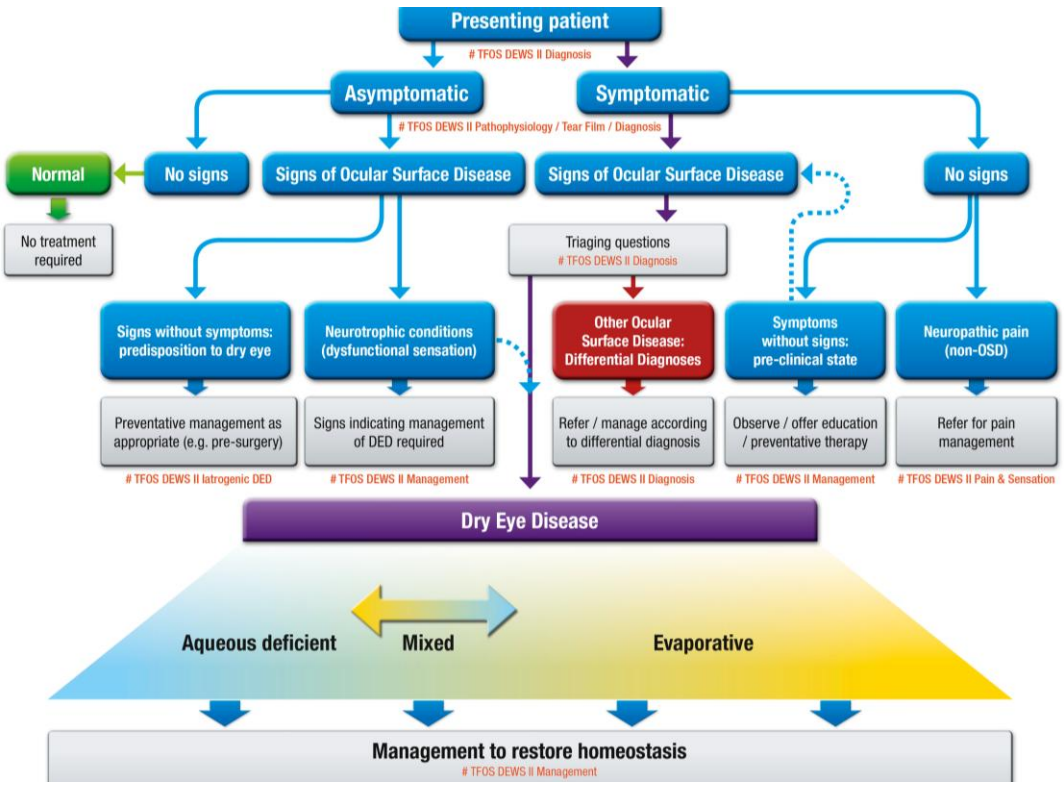

Figure 3. Dry eye etiopathogenic classification (reproduced from TFOS DEWS II Definition and Classification Report (10)).

Aqueous-deficient dry eye (ADDE) is caused by reduced tear secretion with normal tear evaporation. ADDE is subdivided into Sjögren syndrome dry eye (SSDE) and non-Sjögren syndrome dry eye (NSDE), both characterised by dysfunction of the lacrimal glands (19–23). SSDE is an autoimmune inflammatory disease that affects the exocrine glands (salivary and lacrimal glands), leading to dry eyes and a dry mouth. It can be divided into a primary disease, in which the symptoms occur alone, and a secondary disease, which is associated with another systemic autoimmune disease such as rheumatoid arthritis, systemic lupus erythematosus, or systemic sclerosis (19–23).

Evaporative dry eye (EDE) is due to excessive water loss from the exposed ocular surface with normal tear secretory function. There are intrinsic causes, which can be structural (e.g. abnormalities of the eyelids) or functional (e.g. dysfunction of the meibomian glands), and extrinsic causes (e.g. contact lens wear) (19,23).

1.4. PATHOPHYSIOLOGY OF DED

Impaired tear film quality results in hyperosmolarity and instability. Tear hyperosmolarity damages the ocular surface both directly and through the activation of inflammatory pathways (19). It triggers a cascade of events in ocular surface epithelial cells, involving the Mitogen-Activated Protein Kinase (MAPK) and Nuclear Factor kappa-light-chain-enhancer of activated B cells (NFκB) signalling pathways (24). This leads to the production of inflammatory cytokines such as Interleukin-1 alpha (IL-1α), Interleukin-1 beta (IL-1β), Tumor Necrosis Factor alpha (TNF-α), and proteases such as Matrix Metalloproteinase 9 (MMP9), which recruit additional inflammatory cells (25). The combined effects of inflammation and hyperosmolarity alter the glycocalyx mucins, impairing ocular surface lubrication and accelerating tear film breakup. This perpetuates hyperosmolarity, establishing a vicious cycle that sustains disease progression (19).

1.5. INFLAMMASOME COMPLEX AND ITS ROLE IN DED

In vitro studies have shown that corneal epithelial cells incubated under hyperosmolar conditions activate the inflammasome complex (26).

Inflammasomes are multiprotein complexes that contain a pattern recognition receptor, typically a nucleotide-binding oligomerization domain-like receptor (NLR). Upon activation by pathogen- or damage-associated molecular patterns (PAMP or DAMP), these receptors oligomerize and recruit the adaptor protein ASC (apoptosis-associated speck-like protein containing a caspase

recruitment domain, CARD) along with the cysteine protease procaspase-1, leading to the autocatalysis and activation of caspase-1 (27).

Several inflammasomes have been identified, including NLRP1, NLRP2, NLRP3, AIM2, and IPAF/NLRC4 (27). Among them, the NLRP3 inflammasome (Figure 4) is the most deeply studied and is composed of NLRP3, ASC and pro-caspase-1 (28). Cell stress and DAMP induce interaction between the pyrin domain of NLRP3 and the pyrin domain of ASC, activating assembly of the inflammasome in the cytoplasm (29). Once assembled, this complex promotes the proteolytic activation of the interleukins IL-1β and IL-18. Several studies suggest that dysregulation of the inflammasome through the processing of IL-1β contributes to the pathogenesis of chronic pain in various diseases (30). In the context of DED, NLRP3 activation by DAMP may promote immune cell infiltration into the corneal stroma, leading to persistent epithelial damage and ocular surface pain (31,32).

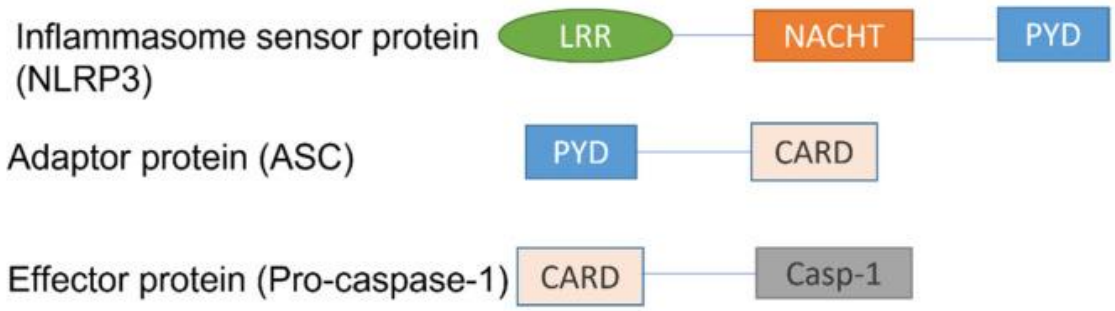

Figure 4. Structure of the NLRP3 inflammasome complex (reproduced from NLRP3 Inflammasome as a potential Therapeutic Target in Dry Eye Disease (31)).

Fibroblasts, essential for tissue repair and extracellular matrix maintenance under physiological conditions, become activated during chronic inflammation, where they contribute to sustained inflammatory responses and facilitate the transition from acute to chronic disease (33). Injuries are known to promote their differentiation into myofibroblasts (4). Numerous studies have described that human myofibroblasts express a broad repertoire of innate immune receptors, such as Toll-like receptors (TLRs) and NLRs, which recognize DAMP and participate in inflammasome activation (34,35). Additionally, recent studies suggests that their activation contributes to the degeneration of corneal nerve endings (32,36).

1.6. RATIONALE OF THE STUDY

The mechanisms involved in the development of inflammation associated with DED are not fully understood, limiting the availability of effective treatments (37,38). Inflammation associated with the ocular surface was initially detected in the lacrimal glands of patients with DED. It is now recognised that this inflammatory response also extends to the conjunctiva (31).

Some previous studies have shown that NLRP3 is expressed in human corneal and conjunctival epithelial cells, and its activation is implicated in the pathophysiology of DED patients (37,38). However, in the context of DED, the interaction between the inflammasome and conjunctival fibroblasts or myofibroblasts remains unclear.

During chronic inflammation, fibroblasts undergo activation and play a pivotal role in sustaining the inflammatory milieu, thereby promoting the progression from an acute to a chronic disease state (33). A previous study demonstrated that salivary gland fibroblasts respond to TNF-α stimulation by upregulating pro-inflammatory mediators such as IL-6, IL-8, CCL20, and ICAM-1. Notably, no significant differences were observed between salivary gland fibroblasts from Sjögren's syndrome patients and healthy controls, although high interindividual variability was reported. These findings highlight the intrinsic pro-inflammatory potential of fibroblasts under immune stimulation, which may be extrapolated to fibroblasts from other tissues, including the conjunctiva (33).

This project aims to investigate inflammasome activation in human conjunctival epithelial cells and in conjunctival fibroblasts cultured with the secretome of conjunctival epithelial cells under *in vitro* dry eye conditions (inflammation and hyperosmolarity). The findings will contribute to characterising the molecular mechanisms induced in ocular surface cells during DED, potentially identifying therapeutic targets to mitigate DED progression.

2. HYPOTHESIS AND OBJECTIVES

2.1. HYPOTHESIS

"The secretome of conjunctival epithelial cells cultured under hyperosmolar or inflammatory conditions, simulating dry eye disease, activates inflammasome, by increasing the gene expression of *NLRP3* in conjunctival myofibroblasts."

2.2. GENERAL OBJECTIVE

To determine the gene expression of *NLRP3* in human conjunctival epithelial cells and conjunctival myofibroblasts treated with the secretome of human conjunctival epithelial cells under *in vitro* dry eye conditions.

2.3. SPECIFIC OBJECTIVES

1. To incubate conjunctival epithelial cells *in vitro* under hyperosmolar and inflammatory conditions resembling DED and collect their secretome.
2. To stimulate conjunctival fibroblasts *in vitro* with the secretome of conjunctival epithelial cells previously incubated under hyperosmolar and inflammatory conditions.
3. To statistically analyse and compare the gene expression of *NLRP3* in conjunctival epithelial cells and conjunctival myofibroblasts under the previously described conditions.
4. To draw conclusions from the obtained results, identify their potential limitations, and suggest future complementary research directions.

3. MATERIALS AND METHODS

3.1. MATERIALS

The materials used are listed in a table in Appendix 1.

3.2. METHODS

3.2.1 Cell lines and culture conditions

To perform the *in vitro* experiments (Figure 5), two commercial cell lines from the ocular surface were used: immortalized human conjunctival epithelial cells (IM-HConEpiC, Innoprot P10870-IM) (39) and human conjunctival fibroblasts (HConF, primary culture, Innoprot P10876). IM-HConEpiC cells were cultured in DMEM + GlutaMax supplemented with fetal bovine serum (10%), human epithelial growth factor (10 ng/mL), insulin (1µg/mL), penicillin (5000 U/mL) and streptomycin (5000 µg/mL). The passages used were from 11 to 13. HConF cells were thawed in Fibroblast Medium: 500 mL of fibroblast basal medium, 10 mL of fetal bovine serum, 5 mL of fibroblast growth supplement and 5 mL of penicillin/streptomycin solution and subsequently cultured in DMEM + GlutaMax supplemented with fetal bovine serum (10%), penicillin (5000 U/mL) and streptomycin (5000 µg/mL). The passages used were from 4 to 6.

Cell cultures were incubated at 37 ºC under 5% CO2 and 95% humidity. The medium was changed every second day, and daily observations were performed using a phase-contrast microscope.

IM-HConEpiC cells were seeded in 25 cm² flasks (300,000 cells/flask). Once the culture reached 80% confluence, the cells were cultured in serum-free-medium without any additives for 24 hours. The cells were then incubated for 4 hours under basal (300 mOsM), inflammatory (25 ng/mL TNF-α), or hyperosmolar (500 mOsM) conditions by adding NaCl (100 mM), to mimic dry eye conditions. After this treatment, the media were retired and fresh, basal medium was added. After 24 hours, the supernatants with their secretome were collected. A portion was immediately used to stimulate HConF cells, and 1 mL was frozen at -80°C. Adherent cells were lysed with RLT buffer containing 1% β-mercaptoethanol and stored at −80°C for total RNA isolation.

HConF cells were seeded in 24-well plates (12,000 cells/well). When the cells reached 80% confluence, they were cultured in serum-free medium without any additives for 24 hours. Subsequently, the medium was carefully removed, and 1 mL of previously collected 24-hour secretomes from treated IM-HConEpiC cells was added to each well. Four wells were used for each condition (basal, inflammatory, or hyperosmolar conditions), and the cells were then incubated for 6 hours. HConF cells, maintained in serum-free DMEM were used as controls. After

incubation, the supernatants were discarded, and adherent cells were lysed with RLT buffer containing 1% β-mercaptoethanol and stored at −80°C for total RNA isolation.

Five independent experiments were performed.

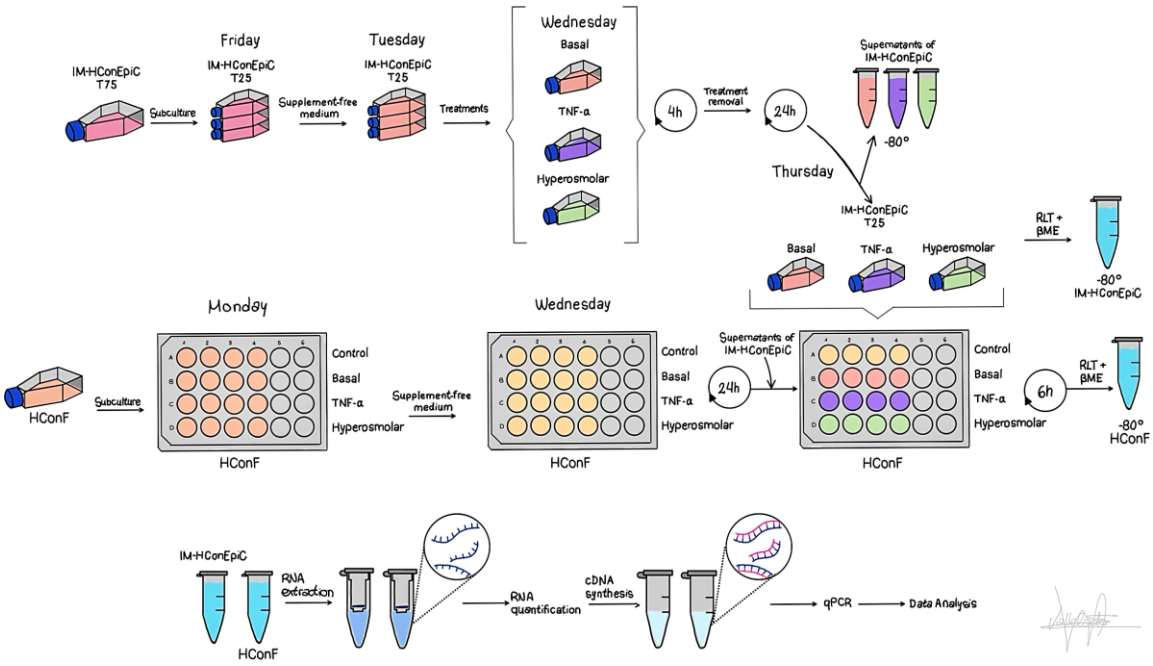

Figure 5. Experimental design. Created by the author. IM-HConEpiC: immortalized human conjunctival epithelial cells; HConF: human conjunctival fibroblasts. RLT + βME: RLT buffer and β-Mercaptoethanol.

3.2.2 RNA extraction

Total RNA from IM-HConEpiC and HConF cells cultured *in vitro* was extracted using the commercial RNeasy Micro Kit following the manufacturer's protocol. (Appendix 2)

3.2.3 RNA quantification

RNA concentrations from IM-HConEpiC and HConF cells were measured using the Qubit RNA HS Assay Kit and the Qubit 4 fluorometer following the manufacturer's instructions. (Appendix 3)

3.2.4 NLRP3 gene expression analysis

RT-PCR

Reverse transcription was performed using 65 ng of RNA with the commercial iScript cDNA Synthesis Kit, following the manufacturer's instructions. (Appendix 4)

RT-qPCR

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was performed using 1 ng of cDNA with the SsoAdvanced Universal SYBR Green Supermix and specific primers in a 20 µL reaction volume. The primers used were *NLRP3* (qHsaCID0036694) and *GAPDH* (qHsaCED0038674), which was used as housekeeping gene. The reaction was carried out on a QuantStudio 5 system following the manufacturer's protocol. (Appendix 5)

3.2.5 Statistical analysis

NLRP3 gene expression was studied using the Ct method, where the target fold = 2−ΔΔCt (User Bulletin, No. 2, P/N 4303859; Applied Biosystems) was used to assess relative differences in specific mRNA levels between the groups of interest. It was calculated by dividing the normalised gene expression in the test sample (2^ (- ΔC_T)) by that in the control sample (2^ (- ΔC_T)). Fold-regulation was used to biologically interpret these values, indicating overexpression when the fold-change was greater than one and repression when it was less than one.

Parametric, unpaired Student's t-test with equal variance and a two-tailed distribution was conducted in GraphPad Prism version 8.0.2 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com, to compare the replicated 2^ (- ΔCT) values between the control and treatment groups, considering *p*-values lower than 0.05 as statistically significant.

4. RESULTS

4.1. *NLRP3* GENE EXPRESSION BY CONJUNCTIVAL EPITHELIAL CELLS UNDER *IN VITRO* DED CONDITIONS

IM-HConEpiC cells incubated for 24 hours, after the 4 hours under inflammatory (TNF-α at 25 ng/mL) or hyperosmolar (500 mOsM) conditions, exhibited an increased expression of *NLRP3* compared with basal condition (Figure 6). *NLRP3* gene expression, either with inflammatory ($p < 0.001$) or hyperosmolar ($p < 0.01$) conditions, was significantly increased (Table 2). Individual CT values are shown in Table 3.

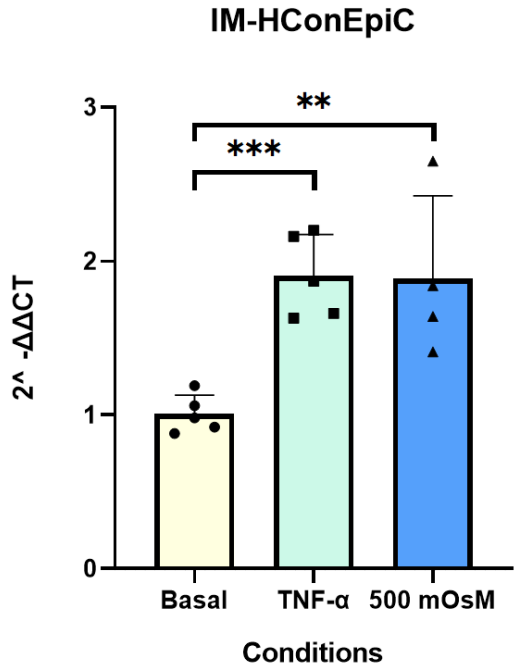

Figure 6. Effect of inflammatory (25 ng/mL TNF-α) and hyperosmolar (500mOsM) treatments on *NLRP3* gene expression by IM-HConEpiC. Data are presented as gene relative expression ± standard deviation over basal condition (300 mOsM without TNF-α). *GAPDH* expression was used as a housekeeping gene. N = five independent experiments. ** Denotes $p < 0.01$, *** denotes $p < 0.001$. IM-HConEpiC: immortalized human conjunctival epithelial cells.

Table 2. *NLRP3* expression fold change in IM-HConEpiC cells exposed to inflammatory and hyperosmolar treatments relative to the basal group.

IM-HConEpiC	**25 ng/mL TNF-α**		**500 mOsM**	
Gene Symbol	**Fold Change**	***p* value**	**Fold Change**	***p* value**
NLRP3	1.89	**0.000150**	1.83	**0.008850**

IM-HConEpiC: immortalized human conjunctival epithelial cells. 25 ng/mL TNF-α: inflammatory condition; 500 mOsM: hyperosmolar condition.

Table 3. *NLRP3* Ct values detected by real time-PCR in IM-HConEpiC cells.

Gene	Basal	25 ng/mL TNF-α	500 mOsM
***NLRP3* Ct**	32.21 ± 0.30	31.76 ± 1.10	31.29 ± 0.34
***GAPDH* Ct**	18.79 ± 0.37	19.26 ± 1.09	18.75 ± 0.58

Data are presented as Ct (cycle threshold) mean ± standard deviation. 25 ng/mL TNF-α: inflammatory condition; 500 mOsM: hyperosmolar condition. IM-HConEpiC: immortalized human conjunctival epithelial cells.

4.2. *NLRP3* GENE EXPRESSION BY CONJUNCTIVAL FIBROBLASTS UNDER *IN VITRO* DED CONDITIONS

HConF cells exposed to the secretome of the IM-HConEpiC cells for 6 hours increased gene expression of *NLRP3* compared with basal conditions (Figure 7). However, no significant differences were observed in either condition (Table 4). Individual CT values are shown in Table 5.

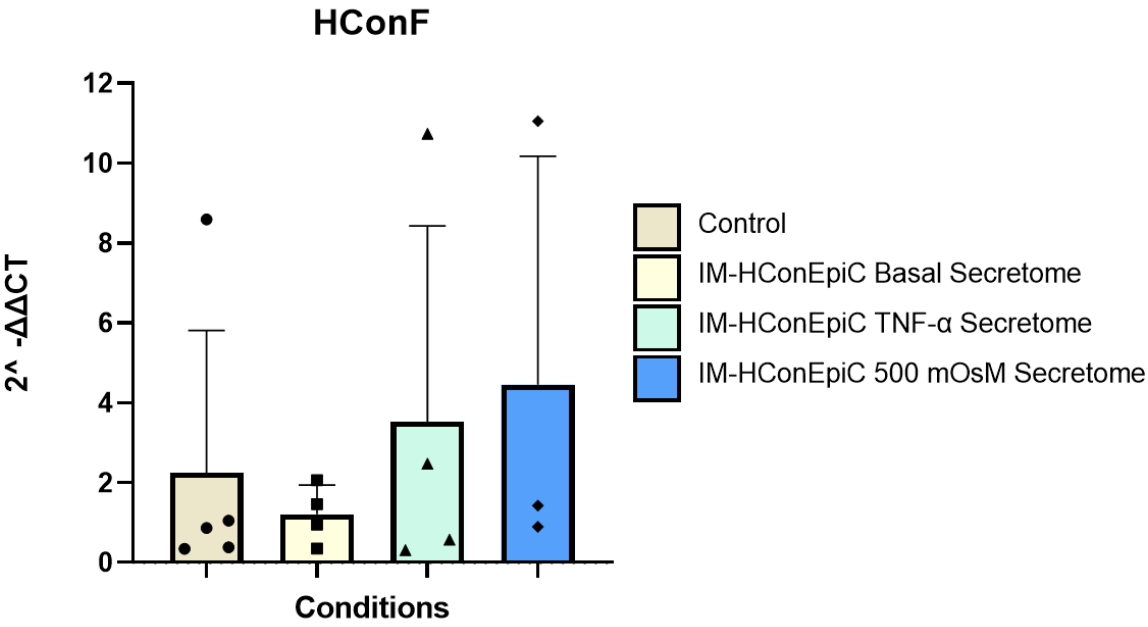

Figure 7. Effect of IM-HConEpiC inflammatory (25 ng/mL TNF-α) and hyperosmolar (500mOsM) secretome on *NLRP3* gene expression by HConF cells. Data are presented as gene relative expression ± standard deviation over basal condition (300 mOsM without TNF-α). *GAPDH* expression was used as a housekeeping gene. N = five independent experiments. Control: fibroblasts cultured with their own cell culture medium. IM-HConEpiC: immortalized human conjunctival epithelial cells; HConF: human conjunctival fibroblasts.

Table 4. *NLRP3* expression fold change in HConF cells exposed to inflammatory and hyperosmolar secretomes relative to the basal group.

HConF	**25 ng/mL TNF-α**		**500 mOsM**	
Gene	**Fold Change**	***p* value**	**Fold Change**	***p* value**
NLRP3	3.52	0.373447	4.27	0.297916

HConF: human conjunctival fibroblasts. 25 ng/mL TNF-α: inflammatory condition; 500 mOsM: hyperosmolar condition.

Table 5. *NLRP3* Ct values detected by real time-PCR in HConF cells.

Gene	**Basal**	**25 ng/mL TNF-α**	**500 mOsM**
***NLRP3* Ct**	33.20 ± 1.38	33.86 ± 1.91	33.18 ± 0.65
***GAPDH* Ct**	18.87 ± 0.86	19.92 ± 2.52	20.28 ± 2.96

Data are presented as Ct (cycle threshold) mean ± standard deviation. 25 ng/mL TNF-α: inflammatory condition; 500 mOsM: hyperosmolar condition. HConF: human conjunctival fibroblasts.

5. DISCUSSION

Over the past decade, the NLRP3 inflammasome has been increasingly implicated in the pathogenesis of DED, and previous studies have shown that desiccating stress and hyperosmolarity (450 mOsM) induce the activation of the NLRP12 and NLRC4 inflammasomes, in models *in vivo* and *in vitro*, in the corneal epithelium in the context of DED (26,40).

In this study, we evaluated the effects of inflammatory and hyperosmolar conditions (two main etiopathogenic factors of DED) on conjunctival epithelial cells and conjunctival fibroblasts, with a focus on the expression of the *NLRP3* gene, a key component of the inflammasome pathway.

Our *in vitro* experiments demonstrated that conjunctival epithelial cells exposed to these stressors exhibited a significant upregulation of *NLRP3* gene expression, suggesting activation of inflammatory signalling under such conditions. This agrees with previous studies by other authors conducted in both primary and immortalized human corneal epithelial cells, as well as in mouse and rabbit corneal epithelial cells (41–43). In those studies, cells were stimulated with hyperosmotic media at 500 mOsM for a period of 4 hours, after which RNA was immediately collected for PCR analysis (41–43). Similarly, in our study, immortalized human conjunctival epithelial cells were also stimulated for 4 hours; however, we measured the epithelial cell response 24 hours after stimulation, allowing us to assess a later phase of gene expression changes. These studies reported significant increases in the gene expression levels of *NLRP3* compared to isotonic conditions. Moreover, elevated protein expression levels of these inflammatory mediators were also observed, indicating functional activation of the NLRP3 inflammasome in response to hyperosmotic stress (41–43). Similarly, an *in vivo* study in a murine model of DED demonstrated increased NLRP3 production in both the corneal and conjunctival epithelia (42). These findings are further supported by additional studies reporting enhanced NLRP3 inflammasome activation in the conjunctiva, cornea, and lacrimal glands across multiple DED models of varying aetiology (44). Consistently, our findings indicate that this same pattern of *NLRP3* upregulation under hyperosmolar and inflammatory stress is also observed in conjunctival epithelial cells in our experimental model.

Our hypothesis was that the secretome released by conjunctival epithelial cells cultured under hyperosmolar or inflammatory conditions could induce inflammasome activation in conjunctival myofibroblasts. Specifically, we expected an increase in *NLRP3* gene expression in these target cells upon exposure to the secretome of the conjunctival epithelial cells. However, the results obtained in the study did not provide clear evidence supporting this hypothesis.

Although we observed an increase in *NLRP3* gene expression in conjunctival epithelial cells under both inflammatory and hyperosmolar conditions, their secretome produced 24 hours later did not

show a significant effect on *NLRP3* gene expression in conjunctival fibroblasts after 6 hours of stimulation.

Currently, data on *NLRP3* expression in conjunctival fibroblasts are limited. A previous study analysed gene expression in fibroblasts treated with TNF-α (1, 3, 6, and 24 hours; 10 ng/mL), and found that the cells responded to stimulation by upregulating the expression of cytokines, chemokines, and adhesion molecules (33). However, *NLRP3* expression was not assessed in that study, and unlike our experimental design, the fibroblasts were directly stimulated with TNF-α.

In our case, it is possible that the duration and/or intensity of the stimulation (either for conjunctival epithelial cells or conjunctival fibroblasts) was insufficient to induce a detectable transcriptional response in conjunctival fibroblasts. Previous studies used direct stimulation, which may elicit a more robust or rapid activation (33,35). Alternatively, our time point might have missed the peak of *NLRP3* expression, which can be transient. Moreover, the induction of NLRP3 specifically in conjunctival fibroblasts in response to epithelial secretomes remains poorly characterised. Our experimental design was based on previous experience from our group which showed us that 4-6 hours stimulation time was enough for *NLPR3* epithelial conjunctival cell gene expression stimulation; however, this might not be the optimal exposure time for conjunctival fibroblast. It is also possible that our experimental design, based on sequential culture and media transfer, did not adequately mimic the physiological cell-cell interactions required for inflammasome activation in conjunctival fibroblasts. Future experiments using more dynamic co-culture systems, such as transwell models, may provide greater insight.

Additionally, while mRNA expression is a widely used indicator of activation, it does not always correlate with functional protein levels. The absence of transcriptional changes does not rule out the possibility that NLRP3 protein is activated through post-transcriptional mechanisms. Therefore, our findings should be complemented by protein-level analyses such as Western blotting or immunofluorescence microscopy to confirm NLRP3 expression and localisation in conjunctival fibroblasts and epithelial cells under dry eye–like conditions. These studies are currently ongoing in our laboratory.

This study has some limitations that should be considered when interpreting the results. Firstly, the experiments were conducted *in vitro* using cell lines and primary cultures that may not fully mimic the complex microenvironment of the ocular surface *in vivo*. The lack of systemic and neuronal regulatory factors, tear dynamics and interactions between immune cells limits the translational relevance of the results.

Secondly, due to the data distribution observed in the conjunctival fibroblast's response, additional replicates are required to reduce variability and confirm the consistency of the

response. Increasing the sample size would enhance the statistical power and help clarify whether the observed tendencies reflect true biological responses or are the result of experimental variability for this gene under these conditions.

Thirdly, only specific doses and treatment durations were tested. It is possible that NLRP3 activation in conjunctival fibroblasts may require different experimental conditions, such as the use of combined pro-inflammatory cytokines (e.g., TNF-α and IFN-γ), increased stimulus concentrations, or alternative exposure durations. Moreover, only the expression of the *NLRP3* gene was assessed, making it possible that fibroblast activation is occurring through alternative pathways independent of NLRP3. Consequently, other signalling mechanisms that were not examined in this study may be involved.

It is also conceivable that the time point used missed the RNA expression peak, either because activation occurs earlier and was not sustained, or because a longer stimulation period was needed. Future studies should investigate a broader spectrum of stimuli and time points to better define the conditions that elicit inflammasome activation in this cell type.

Finally, it should be noted that we are assuming the presence of myofibroblasts in our experimental model. It is well-established that fibroblasts can differentiate into myofibroblasts in response to various inflammatory stimuli, and we have assumed that treatment with the stimulated secretome would promote this differentiation (4). However, we did not assess the expression of alpha-smooth muscle actin (α-SMA), a key marker of myofibroblast differentiation, and therefore cannot confirm that this phenotypic transition occurred.

6. FUTURE STUDIES

Further investigations will be necessary to elucidate the mechanisms underlying inflammasome activation on the ocular surface. For instance, complementary techniques such as Western blotting or immunofluorescence microscopy could be employed to assess protein-level expression of NLRP3 and related inflammasome components, which may not always correlate directly with gene expression.

Additionally, the scope of the inflammasome analysis was restricted to *NLRP3*. Other inflammasome components or related NLRs, such as NLRC4 and NLRP12, which have been previously associated with DED, were not analysed in this study. A more comprehensive profiling of inflammasome pathways would provide a fuller understanding of the inflammatory responses elicited by hyperosmolar and inflammatory stimuli on the ocular surface.

Another promising approach for future research involves the detailed characterisation of the secretome of conjunctival epithelial cells under different stress conditions. Efforts are currently underway in our research group to analyse the cytokine profiles of these secretomes, which could help identify key soluble mediators responsible for paracrine signalling to myofibroblasts. Such studies may provide insight into the complex intercellular communication that regulates inflammatory responses in the ocular surface environment.

7. CONCLUSIONS

This study contributes to the growing evidence of inflammasome activation in the pathogenesis of DED. Our results show that conjunctival epithelial cells respond to hyperosmolar and inflammatory stress by upregulating *NLRP3* gene expression, suggesting the inflammasome activation under these conditions. However, *NLRP3* expression in conjunctival fibroblasts was not significantly increased when treated with the conditioned media derived from these stressed epithelial cells, suggesting that the paracrine signalling mechanisms involved may be more complex or dependent on additional factors not captured in our *in vitro* model.

These findings highlight the importance of further exploring the interaction between epithelial cells and fibroblasts in DED, especially through expanded inflammasome analysis and proteomic characterisation of epithelial secretomes. Such approaches could clarify the contribution of fibroblasts to chronic ocular surface inflammation and reveal new targets for therapeutic intervention.

CONCLUSIONES

Este estudio contribuye a la creciente evidencia sobre la activación del inflamasoma en la patogénesis de la EOS. Nuestros resultados muestran que las células epiteliales conjuntivales responden al estrés hiperosmolar e inflamatorio mediante la sobreexpresión del gen *NLRP3*, lo que sugiere la activación del inflamasoma bajo estas condiciones. Sin embargo, la expresión de *NLRP3* en los fibroblastos conjuntivales no se incrementó significativamente al ser tratados con el medio condicionado derivado de estas células epiteliales estresadas, lo que sugiere que los mecanismos de señalización paracrina involucrados podrían ser más complejos o depender de factores adicionales que no están representados en nuestro modelo *in vitro*.

Estos hallazgos resaltan la importancia de seguir explorando la interacción entre las células epiteliales y los fibroblastos en la EOS, especialmente mediante un análisis ampliado del inflamasoma y la caracterización proteómica del secretoma epitelial. Estos enfoques podrían aclarar la contribución de los fibroblastos a la inflamación crónica de la superficie ocular y revelar nuevas dianas para la intervención terapéutica.

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APPENDIX 1: Materials

Category	Material	Manufacturer/ Source	Reference
Cells	Immortalized Human Conjunctival Epithelial Cell Line (IM-HConEpiC)	Innoprot	P10870-IM
	Human Conjunctival Fibroblasts (HConF)	Innoprot	P10876
Cell culture reagents	DMEM, low glucose, GlutaMAX Supplement, pyruvate	Gibco	21885025
	Fibroblast Medium (FM)	Innoprot	P60108
	Fetal Bovine Serum (FBS)	Gibco	10270-106
	Epidermal Growth Factor	PeproTech	AF-100-15-500UG
	Insulin, Human Recombinant Zinc Solution	Gibco	12585014
	Penicillin-Streptomycin Solution	Corning	30-001-CI
	DPBS (1X)	Gibco	14190144
	Trypsin-EDTA (0,25%), phenol red	Gibco	25200072
	RLT buffer	Qiagen	74004
	2-Mercaptoethanol	Sigma-Aldrich	60-24-2
	Trypan Blue solution	Sigma-Aldrich	T8154-100ML
Reagents for cell culture treatments	Human TNF-α	PeproTech	300-01A-10UG
	Hyperosmolarity Solution (500 mOsM – 100mM NaCl)	------	------
Other cell culture materials	Multiplates	Thermo Scientific	142485
	T25 Cell Culture Flask	Sigma-Aldrich	SIAL0639
	T75 EasYFlask	Thermo Scientific	156499
	5 mL Serological Pipets	Fisherbrand	1367811D
	10 mL Serological Pipets	Fisherbrand	1367811E
	25 mL Serological Pipets	Corning	4489
	Micropoint Pipette Tips, Universal Fit, Non-Filtered (0.1 to 10 µL)	Fisherbrand	02707441
	Micropoint Pipette Tips, Universal Fit, Non-Filtered (5 to 300 µL)	Fisherbrand	02707410
	Micropoint Pipette Tips, Universal Fit, Non-Filtered (100 to 1250 µL)	Fisherbrand	02707407

	Dual-Chamber Cell Counting Slides	BioRad	1450011
	Cell Culture Dish	Corning	430166
	Sterile PES Syringe Filters, Pore: 0,22µm, Φ25mm	BRANCHIA	SFPE-22E-050
	10 mL Plastic Syringe	Fisherbrand	14955453
	Disposable Centrifuge Tube Sterile, Polypropylene 15mL Flat Cap	Fisherbrand	07200886
RNA extraction and quantification	RNeasy Micro Kit	Qiagen	74004
	RNase-Free DNasa Set	Qiagen	79254
	Qubit RNA HS Assay Kit	Invitrogen	Q32852
	Qubit Assay Tubes	Invitrogen	Q32856
RT-PCR	iScript cDNA Synthesis Kit	BioRad	1708891
	SsoAdvanced Universal SYBR Green Supermix	BioRad	1725274
	NLRP3 Primers	BioRad	10025636 qHsaCID0036694
	GAPDH Primers	BioRad	10025636 qHsaCED0038674
Instruments/ Equipment	Microfuge 16 Centrifuge	Beckman Coulter	A46473
	Qubit 4 Fluorometer	Invitrogen	15723679
	ZX3 Vortex Mixer	Fisherbrand	356119
	QuantStudio 5 Real-Time PCR Instrument (96-Well 0.2mL Block)	Applied Biosystems	A28134
	Eclipse TS100	NIKON	NI-TS100
	Allegra X-22R	Beckman Coulter	ALDO4CO24
	TC20 Automated Cell Counter	BioRad	508BR02695

APPENDIX 2: RNA Extraction Protocol – RNeasy Micro Kit

Materials

For each sample:

- 2 x 2 mL Collection Tubes
- 1 x Eppendorf Tube
- 1 x Spin Column
- 70% Ethanol (1:1)
- 80% Ethanol (500 µL)
- RW1 Buffer (700 µL): Wash buffer
- DNase I (RDD Buffer 70 µL + DNase I 10 µL)
- RPE Buffer (500 µL): contains 44 mL of ethanol in the bottle
- RNase-free water (14 µL)

Proportions

- Use the same amount of 70% ethanol as the volume of Buffer RLT added.
- DNase I: Add 550 µL of RNase-free water to the lyophilized enzyme. Make 30 µL aliquots of the enzyme.

Procedure

1. Add the same volume of 70% ethanol as the amount of Buffer RLT + B-ME (β-Mercaptoethanol) 1% to the sample.
2. Mix and add up to a maximum of 700 µL to the spin column.
3. Centrifuge for 15 seconds at 8,000 g and discard the liquid.
4. Add 350 µL of RW1 buffer, centrifuge for 15 seconds at 8,000 g, and discard the liquid.
5. Add 80 µL of DNase I and incubate for 15 minutes. Be careful when adding DNase I to ensure it falls onto the membrane and not on the sides of the tube.
6. Add 350 µL of RW1 buffer, centrifuge for 15 seconds at 8,000 g, and discard the tube with the liquid.
7. Place another tube, add 500 µL of RPE buffer, centrifuge for 15 seconds at 8,000 g, and discard the liquid.
8. Add 500 µL of 80% ethanol, centrifuge for 2 minutes at 8,000 g, and discard the tube with the liquid.
9. Place another tube and centrifuge for 5 minutes at the maximum g-force possible. Discard the tube with the liquid.
10. Place the Eppendorf tube, add 14 µL of RNase-free water to the column, and centrifuge for 2 minutes at maximum g-force.
11. Quantify the RNA and store at -80°C.

APPENDIX 3: RNA Quantification Protocol – Qubit RNA HS Assay Kit

Materials

- Qubit Tubes (Invitrogen)
- Standard 1: Qubit™ RNA HS Standard #1
- Standard 2: Qubit™ RNA HS Standard #2
- Qubit™ RNA HS Buffer
- Qubit™ RNA HS Reagent (fluorophore)

Proportions

- **1:200** → Reagent 1, Buffer 200 → Working Solution (WS)
- **Standard:** 190 µL WS + 10 µL standard
- **Sample:** 199 µL WS + 1 µL sample

Procedure

1. Prepare the working solution (WS).
2. Prepare the standards.
3. Prepare the samples.
4. Quantify RNA using the High Sensitivity (HS) Assay.
5. Store samples at -80°C until further use.

APPENDIX 4: cDNA Synthesis Protocol – iScript cDNA Synthesis Kit

Materials

- iScript Reaction Mix
- iScript Reverse Transcriptase
- RNase-free water
- RNA sample

Proportions

Total reaction volume: 20 µL

- 4 µL iScript Mix
- 1 µL Reverse Transcriptase (RT)
- 15 µL RNA + H_2O mixture (65 ng RNA in H_2O)

Reaction Conditions

1. **Priming:** 5 minutes at 25°C
2. **Reverse Transcription:** 20 minutes at 46°C
3. **Inactivation:** 1 minute at 95°C
4. **Optional:** Store at 4°C after completion

Procedure

1. Complete the Excel file with sample identification and determine the required µL of sample and water needed to obtain 65 ng of RNA per reaction.
2. Add the water to 0.2 mL tubes.
3. Add the RNA sample to the tubes.
4. Add 4 µL of iScript Mix to each tube.
5. Add 1 µL of Reverse Transcriptase (RT) to each tube.
6. Vortex briefly.
7. Set up the thermal cycler program and load the samples.
8. Start the reaction.
9. Upon completion, add 80 µL of RNase-free water to each tube.
10. Store the samples at -80°C.

APPENDIX 5: PCR Protocol – SsoAdvanced Universal SYBR Green Supermix

Materials

- SYBR Green Supermix
- Primers
- RNase-free water
- cDNA
- PCR plate and consumables (tubes, tips, etc.)

Proportions

For each reaction:

- 10 µL SYBR Green Supermix
- 4 µL RNase-free water
- 1 µL primers
- 5 µL cDNA (5 ng)

Reaction Conditions

1. **Hold Stage**
   - 2 minutes at 95°C
2. **PCR Stage (40 cycles)**
   - 5 seconds at 95°C
   - 35 seconds at 60°C
3. **Melt Curve Stage**
   - 15 seconds at 95°C
   - 1 minute at 60°C
   - 15 seconds at 95°C
   - 15 seconds at 60°C

Procedure

1. Prepare the SYBR Green Supermix mixture with RNase-free water.
2. Distribute the mixture into 0.2 mL tubes based on the number of genes to be identified.
3. Add the primers to each tube.
4. Transfer the mixture from the tubes into the appropriate wells on the PCR plate.
5. Add 5 µL of cDNA to each well.
6. Load the plate into the qPCR machine.
7. Start the reaction (approximately 1 hour).
8. Analyse the data obtained.