INTRODUCTION

Glaucoma is a complex neurodegenerative disease with several target tissues and a relevant molecular complexity [2]. It is considered as the most common cause of blindness worldwide, which affects more than 70 million people in the world, a figure set to rise in the coming years [1].

Until today, therapeutic approaches have been mainly targeted to counteract this disease by lowering intraocular pressure (IOP) by antihypertensive drugs or surgery, because IOP has been proven to play a critical role in the progressive death of RGC. However, these approaches often fail because they do not offer complete protection towards sight deterioration [3,4]. Hence, there is a growing need to find both new molecular targets and new experimental approaches [5-7] to improve glaucoma knowledge. As known, trabecular meshwork (TM), the tissue responsible for aqueous humor outflow from the anterior chamber of the eye, plays a crucial role in IOP regulation [8,9]. Indeed, in high tension glaucoma, TM is considered the most important tissue of the anterior chamber because it is involved in the first events that lead to glaucoma [8]. TM changes, including its cellularity reduction [4], accumulation of proinflammatory cytokine [10], the loss of ECM turnover [11] and so on, are known to be relevant glaucoma features. In particular, several previous studies reported that oxidative stress (OS) plays a pivotal role in promoting both TM dysfunction and therefore the IOP [10,12-14]. Furthermore, Saccè et al. (2016) hypothesized that the proapoptotic signals, which lead to RGC death, start from the suffering cells of the trabecular meshwork [15]. Hence, TM alterations induce aqueous humor proteome changes [16] that, reaching the posterior segment, take on the functions of apoptosis activators for RGC [15]. Therefore, from a molecular point of view, the anterior segment of the eye, of which TM is...
the main tissue, plays a central role because it takes part in all the events leading to glaucoma [8].

This evidence suggests that TM integrity maintenance could be a promising therapeutic target.

Different types of polyphenols are proposed both as preventative and adjuvant treatments towards the OS damage-related [10,17,18]. As known, polyphenols include a heterogeneous group of molecules able to reduce the adverse outcomes of chronic degenerative diseases such as cardiovascular diseases, cancer, neurodegenerative disorders, and so on [19–21]. Thus, polyphenols modulate several cell-signaling pathways, although their health effects depend on the consumed amount and their bioavailability [18]. In ocular diseases, the topical use of polyphenols allows to their better bio-availability protecting all tissues, including TM, by oxidative stress in a more efficient way.

Recently we assessed an innovative 3D-human TM cells (HTMC) model based on millifluidic technology, that lead to improve both cell growth and cell-to-cell contact, compared to conventional methods of 3D cell culture in static conditions, in order to develop a useful tool to check therapeutic strategies for glaucoma [22]. For this purpose, in this study we analysed the anti-inflammatory and anti-oxidant potential effects of iTRAB®2, a patented formulation of polyphenols in high concentration and fatty acids, on 3D-dynamic HTMC cultures after OS-induced damage.

**MATERIAL AND METHODS**

**Cell Culture**

HTMC and Trabecular Meshwork Growth Medium (TMGM) were acquired from Cell APPLICATION INC. (San Diego, CA, USA). The proof of the presence of the HTMC phenotype, also after Dexamethasone treatment, was officially given by the Cell APPLICATION laboratory (see: https://www.cellapplications.com/human-trabecular-meshwork-cells-htmc).

HTMCs were grown in TMGM and were maintained at 37°C in a humidified atmosphere containing 5% CO₂. However, before performing experimental treatments, in order to reduce any Fetal Bovine Serum (FBS) interference on proliferation, HTMCs were cultured in low and high glucose DMEM (1:1 mix), 2mM L-glutamine, 0.5% gentamicin and 100 μg/ml streptomycin, w/o (FBS)-according to Keller et al. [23].

All cell cultures were found to be mycoplasma-free during regular checks with the Reagent Set Mycoplasma Euroclone (Euroclone® Milan, Italy).

3D-HTMCs were obtained by suspending 5x10⁴ cells in 200 μl Corning® Matrigel® Matrix (Corning Life Sciences, Tewksbury, MA USA), and quickly seeded in bioreactor LiveBox1 (LB1) (IVTech S.r.l. - Massarosa, Italy) culture chambers [7]. After polymerization at 37°C, 1 ml of culture media was added and then replaced with fresh medium 24 hrs later. The cells were then maintained under dynamic conditions for 72 hrs.

The dynamic culture conditions were obtained from a sophisticated model of milli-scaled multi-organ devices (IVTech, srl) in a single flow configuration (LB1, IVTech srl) [24]. This device is described elsewhere [22].

After 24 hours following seeding, 3D HTMCs were connected to the complete circuit (please see Fig. 1 in Sacch et al. 2020 [22] for the complete circuit diagram). The cell medium within the LB1 was replaced daily, while the amount of cell medium in the mixing bottle was adequately filled to avoid any nutrient depletion during the 72hrs of experimental conditions. Moreover, the medium flow was maintained at the constant rate of 70μl/ml to promote both cell survival and uniform cell distribution and to overcome Matrigel® degradation over time.

**Experimental conditions**

The effects of iTRAB®, a concentrated mixture of polyphenols (≥2.5%) from Perilla frutescens[17], on 3D-HTMC were investigated after prolonged oxidative stress conditions.

iTRAB® was dissolved directly in growth medium w/o FBS and 1% DMSO (v/v) at the same concentration (0.15% m/v) of commercial DRAIN drops®.

3D-HTMC was exposed to static conditions, once a day for 2 hrs to 500μM H₂O₂ and parallel cultures were treated with 0.15% iTRAB® (m/v) for another 2 hrs. Then, HTMCs were cultured under dynamic conditions for 20hrs to promote cell recovery [25,26]. These experimental conditions were reiterating carried on for 72hrs.

At each end point, 3D HTMCs were freed from the Matrigel® by Corning Cell Recovery (Corning Life Sciences), according to the manufacturer’s instructions.

**DCF Assay**

The antioxidant efficacy of iTRAB® was evaluated on 3D HTMCs treated as above mentioned, by dichlorofluorescein (DCF) assay in terms of ROS production.

In short, 3D HTMCs were exposed to non-fluorescent 2’,7’-dichlorodihydrofluoresceindiacetate (H2DCFDA, Thermo Fisher Scientific Inc.), which is able to permeate into the plasma membrane and is reduced to the highly fluorescent 2’,7’-dichlorofluorescein [27]. The experiments were performed as described elsewhere [7] and each condition was analyzed 6 times. DCF emission was recorded at 2hrs on a fluorescent plate reader at excitation and emission wavelengths of 485 and 520 nm, respectively. The fluorescence intensity was extrapolated after subtracting the blank (Matrigel plus medium plus DCF) and data were expressed as percentages of relative fluorescence units of treated vs. untreated HTMC cultures.

**Alamar Blue Assay**

During experimental procedures, the healthy state of 3D-HTMCs was monitored daily by Alamar Blue (AB) assay (InvitrogenTM, Thermo Fisher Scientific Inc., Monza, Italy) within the last 4 hrs of incubation of the 20hrs of recovery time according to the manufacturer’s instructions. In short, 10% AB solution (v/v) was added to the 3D-culture after 4 hrs of incubation and then the resazurin reduction was quantified spectrophotometrically at wavelengths of 570 and 630 nm. The results were expressed as number-fold of viability index changes of treated vs. untreated 3D-HTMC.
**Western Blotting**

Cell lysates were collected using RIPA buffer (Sigma Aldrich S.r.l., Milan, Italy) plus protease inhibitor cocktail (Complete Tablets, Roche Diagnostic GmbH, Mannheim Germany) and sonicated until solubilized. Samples were mixed with 4x Laemmli loading buffer (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and loaded onto 4-20% Tris-Glycine Gels (Bio-rad), and electrophoresis was performed for 50 minutes at 200 V. Protein was transferred to a polyvinylidene difluoride membrane (Thermo Scientific, Rockford, USA) and probed overnight with primary antibodies phospho-NF-κB p65, Ser 536 (Cell Signaling Technology, Santa Cruz, CA, USA) and obtained from Amersham Life Science, Milan, Italy). The proteins were detected by Western Bright™ ECL (Advansta, CA, USA), exposed to film and analysed using a BIORAD Geldoc 2000. The data presented were calculated according to normalization with GAPDH. Densitometrical data obtained from Quantity One software (Bio-Rad Laboratories, Inc., Hercules, CA, USA), exposed to film and analysed using a BIORAD Geldoc 2000 and each protein spot was normalized against Positive Control Spots printed on each membrane.

**Statistical analysis**

Apoptosis pathway analysis was performed as reported earlier [7] by Human Apoptosis Antibody Array C1 (RayBio®, Norcross, GA), to detect the difference between the 43 human protein expression patterns in tested cells (Table 2).

The intensity of protein array signals was analyzed using a BIORAD Geldoc 2000 and each protein spot was normalized against Positive Control Spots printed on each membrane.

The data analysis was conducted according to the Protocol instructions of Human Apoptosis Array C1, and the relative protein expression on different arrays was extrapolated by using the algorithm, according to Human Apoptosis Array C1 protocol.

**qPCR**

Primers and probes for human IL1α, IL1β, IL6, TNFa, TGFβ, SPARC, MMP1, MMP3 and HPRT-1 were designed using the Beacon Designer 7.0 software (Premier Biosoft International, Palo Alto CA, USA) and obtained from TibMolBiol (Genova, Italy). The sequences of PCR primers are listed in Table 1.

**Table 1** Primer sequences used for real time quantitative polymerase chain reaction analysis.

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<thead>
<tr>
<th>Gene</th>
<th>GenBank</th>
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**Table 2** The mini map of Human Apoptosis Array C1 (according to RayBio® manufacturer manual)

<table>
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<th>C</th>
<th>D</th>
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<td>IGFBP3</td>
<td>IGFBP4</td>
<td>IGFBP5</td>
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<td>IGFB-1R</td>
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Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Milan, Italy) according to the manufacturer’s instructions. Nano Drop spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) was used to quantify the RNA. Then, 150 ng per sample of cDNA was synthesized by using Super Script™ III First Strand Synthesis System (Thermo Fisher Scientific). Each PCR reaction was performed as described elsewhere [7].

Data analyses were obtained using the DNA Engine Opticon® 3 Real-Time Detection System Software program (3.03 version) and, in order to calculate the relative gene expression compared to an untreated (control) calibrator sample, the comparative threshold Ct method [28] relative to that of HPTR (the internal control), was used within the Gene Expression Analysis for iCycler iQ Real Time Detection System software (Bio-Rad) [29].

**Statistical analysis**

Reported data are expressed as mean ± Standard Deviation (SD) and results were analyzed using two-way analysis of ANOVA for single comparison or two-way analysis of ANOVA variance followed by Bonferroni posttest for multiple comparisons. GraphPad Prism for Windows-version 5.03 and GraphPad Software, Inc., La Jolla, CA, USA) was used.
Statistically significant differences were set at p<0.05; p<0.01; p<0.001.

RESULTS

ROS production
The fluorometric DCF assay was performed on 3D HTMC in order to evaluate the potential capacity of the iTRAB® in counteracting oxidative stress. In 500µM H₂O₂ treated 3D HTMC, after 2 hrs exposure ROS production markedly increased up to 300% compared to untreated (U.T.) and to co-treated cultures (H₂O₂ plus iTRAB®) (Figure 1, Panel A).

Viability Index
After the 2nd exposure to 500µM H₂O₂, 3D-HTMC cultures evidenced a significant decrease of viability, in terms of their metabolic state as evaluated by Alamar blue assay, and this effect was maintained at similar levels also after a 3rd exposure to the stressor. Conversely, at the same time the exposure to iTRAB® in addition to H₂O₂ induced a time-dependent cell viability restoration. (Figure 1, panel B)

Figure 1 Ros production and viability. Panel A: ROS production. DCF assays were performed after 2 hrs of experimental procedures. Data is expressed as % of U.T. 3D-HTMC and represents the mean ± SD of 3 independent experiments. Panel B: Viability index. Viability indices were performed by Alamar blue assay and data are expressed as Arbitrary Units (AU). Each value represents the mean ± S.D of 3 experiments running in triplicate.

Apoptosis Pathway
To further determine the effects of iTRAB® on 3D HTMC following the 2nd day of experimental exposures, pro/anti protein apoptosis array was carried out by Human Apoptosis Antibody Array C1.

As shown in Figure 2 panel A and B, the analysis of pro/anti-apoptotic protein expressions revealed significant differences between H₂O₂-treated and co-treated with iTRAB® and H₂O₂ -3D HTMC cultures.

A significant overexpression of protein involved in the apoptotic pathway, including p53, BAD, BAX, BID, BIM, SMAC, TNFa and TNFβ, was observed in H₂O₂-treated 3D HTMC compared to untreated cultures. Instead, coexposure to iTRAB® and H₂O₂ counteracted the apoptosis response efficiently by decreasing levels of the H₂O₂-induced pro-apoptotic proteins. Indeed, increasing xIAP, survivin, IGFR, IGF 1 and cIAP anti-apoptotic protein levels showed a significant increase, while IGF2 and BCL2 expressions were decreased in a significant way compared to observed H₂O₂-induced levels.

For the anti-apoptotic pattern, only data related to those proteins that evidenced significant different levels compared to UT and H₂O₂-treated cultures was reported. As shown in Fig. 2 panel B, co-treated HTMC evidenced a significant increase of analysed antiapoptotic proteins.

Western Blot
In our 3D HTMC culture model, NF-kB was activated by chronic oxidative stress starting from the 3rd exposure, based on our previous reported data [7,22]. Therefore, the effects of iTRAB® on the phospho-NF-kB p65 subunit decrease were monitored after 500µM H₂O₂ addition during this time-frame (Figure 3 Panel A). Indeed, a remarkable NF-kB activation occurred in 3D-HTMC after the 3rd exposure to only 500µM H₂O₂, while co-treated 3D-HTMC resulted in a significant decrease of NF-kB activation.

qPCR
In order to clarify the molecular mechanism underlying the effects of iTRAB® on 3D HTMC, we further analysed the differences in gene expression of pro inflammatory and pro fibrotic markers, as well as metalloproteases (MMP1 and 3).
As shown in Figure 3 panels B, C and D, 500µM H2O2 alone affected the trigger of the inflammation pathway as evidenced by qPCR analysis. Indeed, a significant up-regulation of pro-inflammatory IL1β, pro fibrotic TGFβ, as well as MMP1 and MMP3 was detectable already after the 2nd H2O2 exposure. However, after the 3rd H2O2 exposure, an increase in the other pro-inflammatory cytokines evaluated (IL1α, IL6 and TNFα) was observed, compared both to untreated and co-treated cultures. In spite of the increase of TGFβ, SPARC expression did not show significant modulation by H2O2-treatment.

Co-treated 3D HTMC resulted in a significant down-regulation of the above-mentioned markers, starting from 2nd exposure.

![Figure 3. western blot and qPCR analysis. (Panel A) NF-kBp65 activation was evaluated in 3D HTMC cells after 3rd exposure of 500µM H2O2 only and co-treatment with iTRAB® and 500µM H2O2. The analysis was performed by immunoblotting and the bars represent the ratio of phospho-NF-kBp65/NF-kBp65, and are expressed as fold vs. untreated HTMC cultures. The blue dotted line represents the ratio of phosphoNF-kBp65/NF-kBp65 of untreated 3D HTMC. Data represent the mean ± S.D. of 3 independent experiments. Quantitative PCR gene expression analysis was performed on 3D-HTMC’s after the 2nd and 3rd exposure of 500µM H2O2 only and co-treatment with iTRAB® and 500µM H2O2. (Panel B) IL1α, IL1β, IL6, TNFα (Panel C) TGFβ and SPARC; (Panel D) MMP1 and MMP3. Data are expressed as fold-increase relative to untreated cultures at the same endpoint and normalized to HPRT1 housekeeping gene expression. The blue dotted line represents the gene level of untreated 3D HTMC for each gene examined. Each bar represents the mean ± S.D. of three independent experiments performed in triplicate. *p<0.05; bp<0.01 vs. untreated cultures (U.T.); b<0.05; bp<0.001 vs. H2O2+iTRAB® (Two-way ANOVA followed by Bonferroni’s test).

**DISCUSSION**

Glaucoma is an optic neuropathy characterized by a progressive loss of sight. In particular, the high tension glaucoma pathogenesis includes several features such as elevated IOP, oxidative stress, inflammation, increase of ECM deposition in the outflow pathway, TM senescence, and the loss of RGC [8,9,12,30].

Currently, glaucoma therapy aims to lower IOP without actually providing a complete protection from blindness [4]. Moreover, except for Timolol and Dorzolamide, which have antioxidant properties [31], the IOP-reduction with medication neither restricts nor improves the oxidative stress rate [32]. Therefore, there is a need for new compound identification which is able to act in a therapeutic way on the several mechanisms involved in this disease.

As known, OS is involved in glaucoma pathogenesis [12] because it leads to an inflammatory condition which promotes TM damage and outflow dysfunction [33]. The antioxidant compounds (i.e. polyphenols, carotenoids, resveratrol, vitamin E and N-acetylcysteine) used to prevent or counteract glaucoma have been previously studied [4,17,34-36]. In particular, polyphenols properties are exploited to protect TM from free radical attack in order to reduce its progressive OS-related damage and provide a long term visual function [18]. However, the bioavailability of dietary polyphenols (i.e. flavonoids) is usually very low and, above all, their protective effects are exerted in the gastrointestinal tract only [37]. In the field of ophthalmology, their topical application is therefore recommended in order to increase the therapeutic efficacy of polyphenols.

A previous study had already demonstrated the antioxidant activity of iTRAB®, the active principle of DRAIN Drops®, after chronic OS exposure on 2D TM model cultured under static conditions [17].

Here we evaluated the effects of iTRAB® in counteracting prolonged OS on HTMC cultured in a 3D-dynamic model, that we have recently performed to better mimic physiological cell behavior [22].

3D HTMC co-treated with iTRAB® and 500µM H2O2 showed a significant decrease of ROS production (Figure 1 Panel A), compared to the 3D HTMC exposed to 500µM H2O2 only, highlighting iTRAB® anti-oxidant activity [17,34,35]. Moreover, co-treated 3D HTMC showed a significant increase in the viability index already after the 2nd exposure, compared to 500µM H2O2 exposed 3D HTMC (Figure 1 Panel B). Besides, the addition of iTRAB® also hindered the apoptotic pathway by both a significant decrease in pro-apoptotic protein levels and by an increase in anti-apoptotic proteins (Figure 2).

The cytokine levels of IL-1α, IL-1β, IL-6, and TNF-α were analyzed because their increase has been associated with a pathogenic role in glaucoma in the literature [38,39].

In Our 3D Model, the TM cells, as a results of OS condition, evidenced an increase in pro-inflammatory and pro fibrotic cytokine and MMPs gene expression [38-41] (Figure 3 Panels B, C and D).

The pro-inflammatory cytokine ILα is considered a glaucoma risk factor [41], since it acts in synergism with TNFα in TM, and is involved in outflow regulation.

Indeed, in glaucoma, TNFα is described as a pro-inflammatory cytokine which could be rapidly up-regulated after brain injury by determining RGC death [42] or after TM damage by promoting both MMP3 and IL6 expressions, at the same time as IL1α up-regulation [43,44].

The role of IL6 in glaucoma is not completely understood because it has both physiological and pathological functions. Indeed, if on one hand short-term activation of IL6 protects neurons from excitotoxicity and RGC from microgli- and IOP-derived pro-apoptotic signals and contributes to IOP regulation [40,45], on the other its chronic activation contributes to worsen the trabecular tissue motility [36]. In particular, TM subjected to prolonged OS-exposure reacts with chronic activation of pro-inflammatory cytokines such as IL6 and IL1α and this behavior contributes to glaucoma progression [47].

In our model, after the 2nd exposure to OS, IL-1β mRNA expression was just significantly increased. Conversely, other
analysed pro-inflammatory cytokine raised their gene expression at significant levels only after the 3rd H2O2 exposure. These findings suggested the triggering of adaptive mechanisms similar to those found in vivo to counter early OS-related damage \[12,29\]. This assumption was supported also by the increase in phospho-NF-kB p65 subunit levels (Figure 3 Panel A) after the 3rd exposure to H2O2, according to our previously reported data \[7,25\].

In contrast, the co-treatment H2O2 and iTRAB\(^\beta\) significantly reduced both the phospho-NF-kB p65 subunit levels and the pro-inflammatory cytokine expression (p<0.05), implying that iTRAB\(^\beta\)-anti-oxidant activity could be responsible for the observed decrease in inflammatory mediators.

Furthermore, TGF\(\beta\) and metallocproteinases (MMP) gene expressions were investigated due to their crucial role both in ECM metabolism impairment and in increase of aqueous outflow resistance in TM \[41,48,49\].

As known, an increase of TGF-\(\beta\) levels plays a role in primary open angle glaucoma (POAG) pathogenesis because it increases outflow resistance by altering both TM extracellular matrix homeostasis and its cell contractility \[50\]. In our platform we therefore observed an increase in TGF-\(\beta\) starting from the 2nd exposure to OS.

Moreover, matricellular proteins, such as SPARC, are known to be able to act as control for TGF-\(\beta\) activation but, in addition, they can be regulated by TGF-\(\beta\). Indeed, during H2O2 exposure, we observed that TGF-\(\beta\) gene showed a more marked increased expression than SPARC, but both markers behaved in the same way. Indeed, during exposure to H2O2 alone or in addition to iTRAB\(^\beta\) their mRNA decreased in a time dependent way, and it is to be underlined that iTRAB\(^\beta\) plus the oxidative stressor was able to reduce the gene levels of TGF-\(\beta\) as well of SPARC. So it can be argued that in our experimental conditions TGF-\(\beta\) could exert a role as a key regulator of the cell–matrix interactions \[51\]. In our platform TGF-\(\beta\) increased starting from the 2nd OS exposure, despite an increase of IL6 levels. This finding suggests that chronic activation of IL6 did not repress TGF-signaling \[40\] but, in turn, this effect resulted in an increase of outflow resistance with 3D HTMC-contractility alteration \[52\]. Indeed, as reported by in vivo studies, both chronic activation of TGF\(\beta\) due to mutation \[53\] and the high levels of TGF-\(\beta\) in aqueous humor are correlated with the ECM-component production in TM \[54\]. However, iTRAB\(^\beta\) co-treatment down-regulated, in 3D-HTMC, TGF\(\beta\) expression in a significant manner (p<0.001).

In addition to being involved in several processes such as immunity, angiogenesis, cell migration, inflammatory process \[55,56\], MMPs are involved in ECM turnover as a result of tissue alteration \[57\]. In particular, in our platform prolonged OS induced the increase both in MMP1 and MMP3, which are respectively a collagenase \[58\] and a proteolytic enzyme \[59\] important for ECM synthesis and its degradation.

Therefore, as a result of OS, the TM suffering led both to ECM alteration and its motility reduction, as observed in glaucoma.

Thus, the significant downregulation of TGF\(\beta\) and MMPs in 3D HTMC co-treated with iTRAB\(^\beta\) suggests that iTRAB\(^\beta\) has a prospective role in modulating several gene expressions of genes involved in OS-induced TM damage. Also the anti-apoptotic effects of iTRAB\(^\beta\) were demonstrated by protein array which showed a significant reduction of pro-apoptotic proteins, compared to 3D HTMC OS-treated. Moreover, its anti-inflammatory and anti-fibrotic effects were demonstrated by the significant reduction in IL-\(\alpha\), IL6, TNF-\(\alpha\), and TGF-\(\beta\) cytokine profile, compared to OS-treated 3D HTMC. Indeed, 3D HTMC co-treated with iTRAB\(^\beta\) and H2O2 significantly down-regulated the MMP expressions (p<0.001).

In conclusion, our in vitro 3D-advanced human model of TM, providing a precise control of experimental conditions to better simulate the cell in vivo microenvironment, allows the study of early OS-induced TM-alterations. Indeed, we demonstrated that prolonged OS-condition induced the 3D HTMC to activate the inflammatory pathway rather than apoptosis \[22\]. Thus, in this present work, we proposed the same in vitro platform as a useful tool to evaluate the anti-inflammatory and anti-oxidant effects of iTRAB\(^\beta\), as an important formulation to counteract OS-induced TM damage.

Further developments of this model will lead to a better characterization of glaucomatous damage.

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**Conflict of Interest**: none declared

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