

# Investigation of Conjunctival Fibrosis Response Using a 3D Glaucoma Tenon's Capsule + Conjunctival Model

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**PURPOSE.** Surgical techniques such as trabeculectomy aim to treat glaucoma by making an incision into the scleral tissue, to create an alternative drainage pathway for aqueous to flow into the sub-Tenon's/subconjunctival space. However, tissue fibrosis and wound healing occurring after the procedures can reduce the success rate. This study aims to investigate the synergistic effects of aqueous humor in combination with shear stress on the fibrosis response occurring in Tenon's capsule and conjunctival tissue (TCCT) after glaucoma surgery.

**METHODS.** Two-dimensional (2D) and 3D in vitro TCCT models were constructed by seeding porcine Tenon's capsule + conjunctival fibroblasts in collagen gel. These were used to investigate key growth factors (singular and natural form) with shear stress, which are believed to influence tissue fibrosis after glaucoma surgery. In addition to cell proliferation assessments, a nondestructive assay to quantify neocollagen synthesis in TCCT models, in response to these factors, has been applied up to 14 days.

**RESULTS.** TCCT fibroblast proliferation increased significantly with doses of TGF- $\beta$ , TNF- $\alpha$ , and VEGF, in comparison with the control. Furthermore, fibroblasts exposed to 50% aqueous humor had significantly increased proliferation and actin expression. Shear stress-induced mechanotransduction was also found to promote metabolic activity across experimental conditions. Neocollagen labeling cross validated the fibrosis process.

**CONCLUSIONS.** Shear stress appeared to enhance the influence of key growth factors and further promoted fibrotic response within the model. These findings offer a useful insight for further study into the wound-healing response triggered by aqueous fluid outflow after glaucoma surgery.

Keywords: conjunctiva, Tenon's capsule, fibrosis, glaucoma, 3D model

Glaucoma is a major cause of vision loss worldwide characterized by optic nerve damage and retinal ganglion cell death, with IOP the main modifiable risk factor.<sup>1</sup> For the treatment of early-stage glaucoma, eye drops containing ingredients such as beta blockers and prostaglandin analogues can be administered to modulate aqueous production or increase aqueous humor outflow, thereby reducing IOP. However, for advanced-stage glaucoma that can no longer be controlled using eye drops, the most common surgical treatment is trabeculectomy. A trabeculectomy involves making an incision into the sclera and carefully placing sutures, to allow aqueous to drain into the sub-Tenon's/conjunctival space forming a bleb. The fluid is then absorbed by capillaries in the subconjunctival/episcleral tissue leading to the reduction of IOP. However, excessive wound healing and fibrosis of the Tenon's capsule + conjunctival tissue (TCCT) following surgery can cause the created opening to heal up again and become blocked, significantly reducing the surgery success rate to just 55%.<sup>2</sup>

Tissue fibrosis is characterized by the disproportionate increase and deposition of extracellular matrix (ECM), a complex mixture of molecules secreted by cells to provide their surrounding structural and biochemical support.<sup>3</sup> The

fibrous protein present within Tenon's and conjunctival ECM is primarily collagen.<sup>4</sup> Excessive ECM synthesis following a tissue injury often leads to scar tissue formation and abnormalities in tissue function. Equally, the glaucoma surgery wound stimulates overexpression of cytokines such as TGF- $\beta$ , TNF- $\alpha$ , VEGF, and IL-1,<sup>5,6</sup> which increases proliferation of Tenon's capsule and conjunctival fibroblasts. Activation of fibroblasts subsequently modulates the formation of collagen, leading to the excessive accumulation of ECM and scar tissue that causes the surgically created opening to heal up again and become blocked. This increase in fibroblast activity may be further influenced by the flow of aqueous humor fluid, which is thought to contain growth factors such as TGF- $\beta$  and VEGF.<sup>7,8</sup> In addition, the continuous drainage of aqueous fluid through the new drainage pathway is likely to exert shear stress to the TCCT, triggering mechanotransduction and the overexpression of cytokines such as TGF- $\beta$ .<sup>9</sup>

Current antimetabolite drugs used in an attempt to reduce this fibroblast proliferation include mitomycin C and 5-fluorouracil, which cause apoptosis of fibroblasts via disruption of DNA synthesis.<sup>10</sup> However, these drugs are evidently highly toxic and can lead to further complications, such as subconjunctival bleb thinning and leakage, cytotoxicity, and destruc-



tion of other tissues (e.g., cornea).<sup>11,12</sup> A better understanding of the fibrosis response after glaucoma surgery is therefore required to develop safer and more efficient anti-inflammatory agents.

This study aimed to use reliable two-dimensional (2D) + three-dimensional (3D) TCCT models for the investigation of factors influencing fibrotic response after glaucoma surgery. The quantifiable influence of shear stress-induced mechanotransduction on TCCT fibrosis remains unclear. It would therefore be beneficial to assess the synergistic effects of shear stress in the presence of aqueous humor and key growth factors separately. We demonstrate a clinically relevant method to successfully isolate fibroblasts from the Tenon's capsule and conjunctival membrane, for the quantitative assessment of fibrotic response. Furthermore, we apply a neocollagen fluorescent labeling technique<sup>13</sup> to image the collagen production of TCCT fibroblasts within the model and assess the extent of fibrosis continuously, in addition to cell proliferation assessment. We hypothesize that shear stress-induced mechanotransduction may further enhance the fibrotic influence of key growth factors (TGF- $\beta$ , TNF- $\alpha$ , and VEGF) within the TCCT model.

## MATERIALS AND METHODS

### Porcine Aqueous Humor + TCCT Fibroblast Isolation

Porcine eyes were obtained from a local abattoir within a few hours of slaughter and subsequently rinsed in PBS. Aqueous humor fluid was isolated from each eye by inserting a 23-gauge needle into the anterior chamber and drawing the fluid out. After removal of extraneous tissue from each eye, pieces of the Tenon's capsule tissue and bulbar conjunctival membrane were cut away and washed consecutively three times in 10x antibiotic solution containing penicillin-streptomycin and gentamicin. The dissected tissue was then digested enzymatically using 3 mg/mL Dispase (Sigma-Aldrich, Dorset, UK) followed by 3 mg/mL collagenase (Sigma-Aldrich). Obtained cells were then cultured in high-glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 1% L-glutamine, and 1% antibiotics (penicillin and streptomycin) at 37°C, 5% CO<sub>2</sub> and 95% humidity.

### TCCT Fibroblast Model Construction

**Two-dimensional Model.** The 2D TCCT fibroblast model was set up by seeding fibroblasts at passage 2 into a 48-well culture plate at a density of  $1 \times 10^4$  cells per well; 200  $\mu$ L of above DMEM supplemented medium was added to each well for culture up to 7 days. Medium was changed every 3 days.

**Three-dimensional Model.** The 3D TCCT membrane model was constructed by incorporating fibroblasts at passage 2 into 3 mg/mL collagen hydrogels (Fisher Scientific, Loughborough, UK) at a density of  $4 \times 10^4$  cells per 50  $\mu$ L gel. After the hydrogels were set, 200  $\mu$ L culture medium was added for each gel and kept in culture up to 14 days. Medium was changed every 3 days.

### Stimulators to TCCT Fibroblast Model

**Growth Factors, Aqueous Humor, and Conditional Medium.** Growth factors, aqueous humor, and conditional medium were administered at 24 hours after construction of the 2D and 3D models. For the conditional medium study, spent medium at the day 7 time point with 80% cell confluency was collected from 48-well plates and frozen (-80°C) for later

use. This included conditional medium from stationary (control), shear stress-treated, TGF- $\beta$ -treated, and TGF- $\beta$  + shear stress-treated cultures. Growth factors TGF- $\beta$ , TNF- $\alpha$ , and VEGF were administered via addition to the culture medium at concentrations of 20 ng/mL for each well in 2D experiments and 50 ng/mL for each sample in 3D experiments. Aqueous humor fluid and conditional medium were added to culture medium at a 50% concentration.

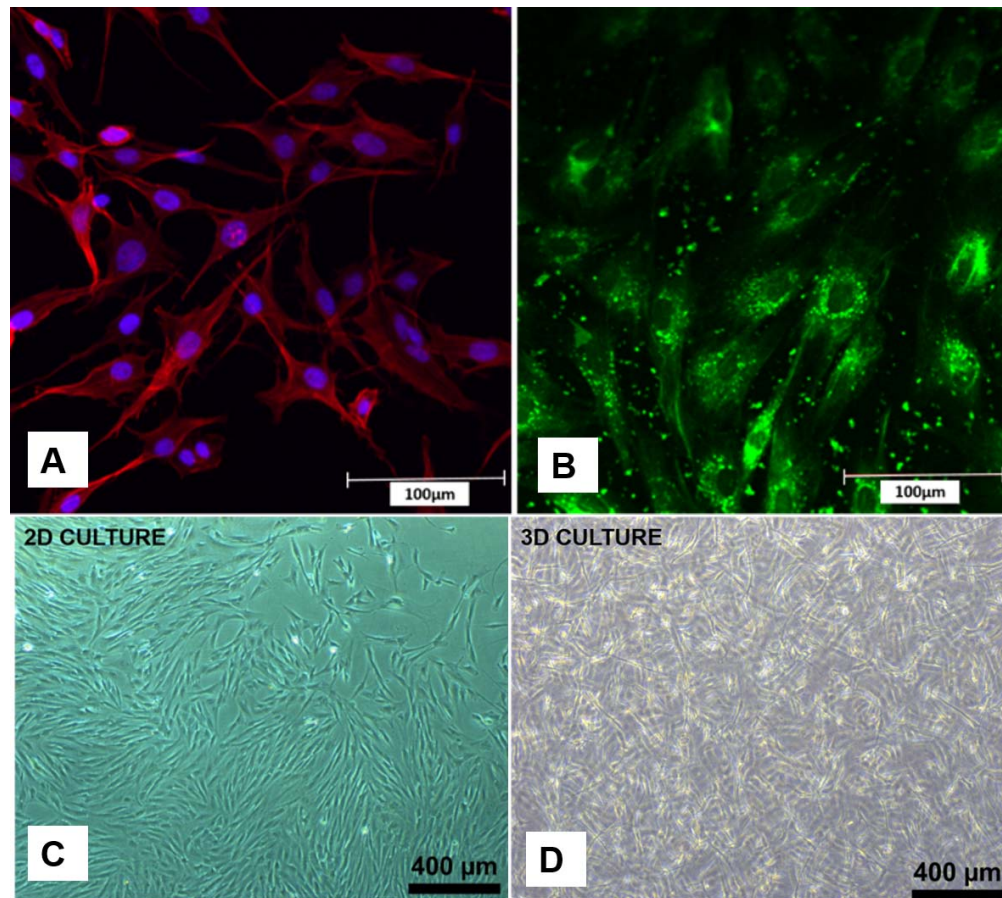
**Shear Stress.** The effect of shear stress on fibrosis was tested using a see-saw motion rocker (Cole-Parmer, Saint Neots, UK) stored inside an incubator at 37°C, 5% CO<sub>2</sub>, and 95% humidity. Subjected samples were placed on the rocker for 1 hour per day at a speed of 5 rpm.

## Characterization of Cellular Responses

**Cellular Proliferation.** Cell proliferation and viability was quantified by Alamar Blue assay (Bio-Rad, Hercules, CA, USA) at days 3, 5, and 7 for 2D experiments and additionally day 14 for 3D experiments. Culture medium was firstly removed and 200  $\mu$ L of fresh medium containing 10% Alamar Blue reagent was added and incubated with the samples for 2 hours at 37°C. After incubation, the solution was taken from each sample and the fluorescence measured at 530/590 nm (excitation/emission, Synergy II; BioTek, Winooski, VT, USA). Following quantification, samples were washed thoroughly three times in PBS to remove any residual Alamar Blue reagent for continuous culture and measurement. All results were compared against a control of blank collagen hydrogels without cells, to ensure that any residual Alamar Blue absorbed by the gels did not influence experimental results.

**Collagen Synthesis.** The quantification and semiquantification of neocollagen synthesis in TCCT models was conducted using an azide-modified proline in culture medium, with subsequent detection of fluorescent alkyne that corresponded to the newly synthesized collagen, following a protocol previously established in our laboratory.<sup>13</sup> Deprotected azido-L-proline (Iris Biotech GmbH, Marktredwitz, Germany) was added to cell culture medium every 2 days at a concentration of 36  $\mu$ g/mL. Three-dimensional samples were imaged for neocollagen detection periodically at days 3, 7, and 14. For imaging, each sample first had all culture medium removed and was washed in PBS to remove any residual culture media before the Click-IT reaction. Serum-free culture medium was then added to samples before the addition of 10  $\mu$ M Click-IT Alexa Fluor 594 (ThermoFisher Scientific, Winsford, UK) to each sample. Samples were incubated with the agent for 1 hour at 37°C while protected from light. Samples were then washed four times in PBS and placed back into the serum-containing culture medium. Confocal microscopy (Olympus, Southend-on-Sea, UK) was used to image the fluorescently tagged collagen. Individual imaging parameters were kept the same across groups and throughout the experiments.

**Immunohistochemistry.** For cellular characterization, morphology, and actin expression assessments, obtained cells were seeded in 2D culture at a density of 7500 cells/cm<sup>2</sup>. Samples were fixed in 4% paraformaldehyde. For F-actin staining, samples were immersed in phalloidin actin working stain solution (1:50) (Sigma-Aldrich) and incubated for 1 hour at room temperature before imaging. For Vimentin immunostaining, samples were immersed in Vimentin goat polyclonal primary antibody (1:100 in 1% BSA) and incubated at 4°C overnight. The samples were then washed with PBS three times and immersed in anti-goat IgM-FITC secondary antibody (1:100 in 1% BSA) for 1 hour before imaging. Confocal microscopy (Olympus) was used to view the outcome of staining.



**FIGURE 1.** Tenon's capsule + bulbar conjunctival fibroblast characterization and morphology. (A) Fibroblasts stained with phalloidin (*red*) to label F-actin and 4',6-diamidino-2-phenylindole (DAPI) (*blue*) to label cell nuclei. (B) Fibroblasts immunostained (*green*) for Vimentin marker. (C) P1 TCCT fibroblasts growing in cell culture flask (2D culture) before seeding into collagen gels. (D) P2 fibroblasts growing within 3 mg/mL concentrated collagen hydrogel (3D culture) 24 hours after seeding.

### Enzyme-Linked Immunosorbent Assay (ELISA)

Commercially available ELISA kits were used to quantify TNF- $\alpha$  (Invitrogen, Carlsbad, CA, USA) and TGF- $\beta$  (R&D Systems, Minneapolis, MN, USA) cytokine levels in porcine aqueous humor. Briefly, 50  $\mu$ L of incubation buffer was added to each antibody-coated well, followed by 100  $\mu$ L of the standards or samples and incubated at room temperature for 3 hours (control blank wells remained empty for this step). The wells were then fully aspirated and washed four times with wash buffer, before the addition of 100  $\mu$ L TNF- $\alpha$ /TGF- $\beta$  biotin conjugate solution into each well. After a further 1 hour of incubation at room temperature, the wells were then fully aspirated again and washed four times with wash buffer, followed by the addition of 100  $\mu$ L substrate solution to each well. After another 30-minute incubation at room temperature (protected from light), 100  $\mu$ L Stop Solution was then added to each well. The optical density of each well was then read using a microplate reader (BioTek Synergy II) at 450-nm wavelength.

### Statistical Analysis

All experiments were run with three replicates in each group and values stated are the representative mean and standard error of observations from three independent experiments. Analysis of statistical significance was performed using a two-tailed Student's *t*-test. Results were considered statistically

different at three levels with graphic expressions: \* $P \leq 0.05$ , \*\* $P \leq 0.01$ , and \*\*\* $P \leq 0.001$ , respectively.

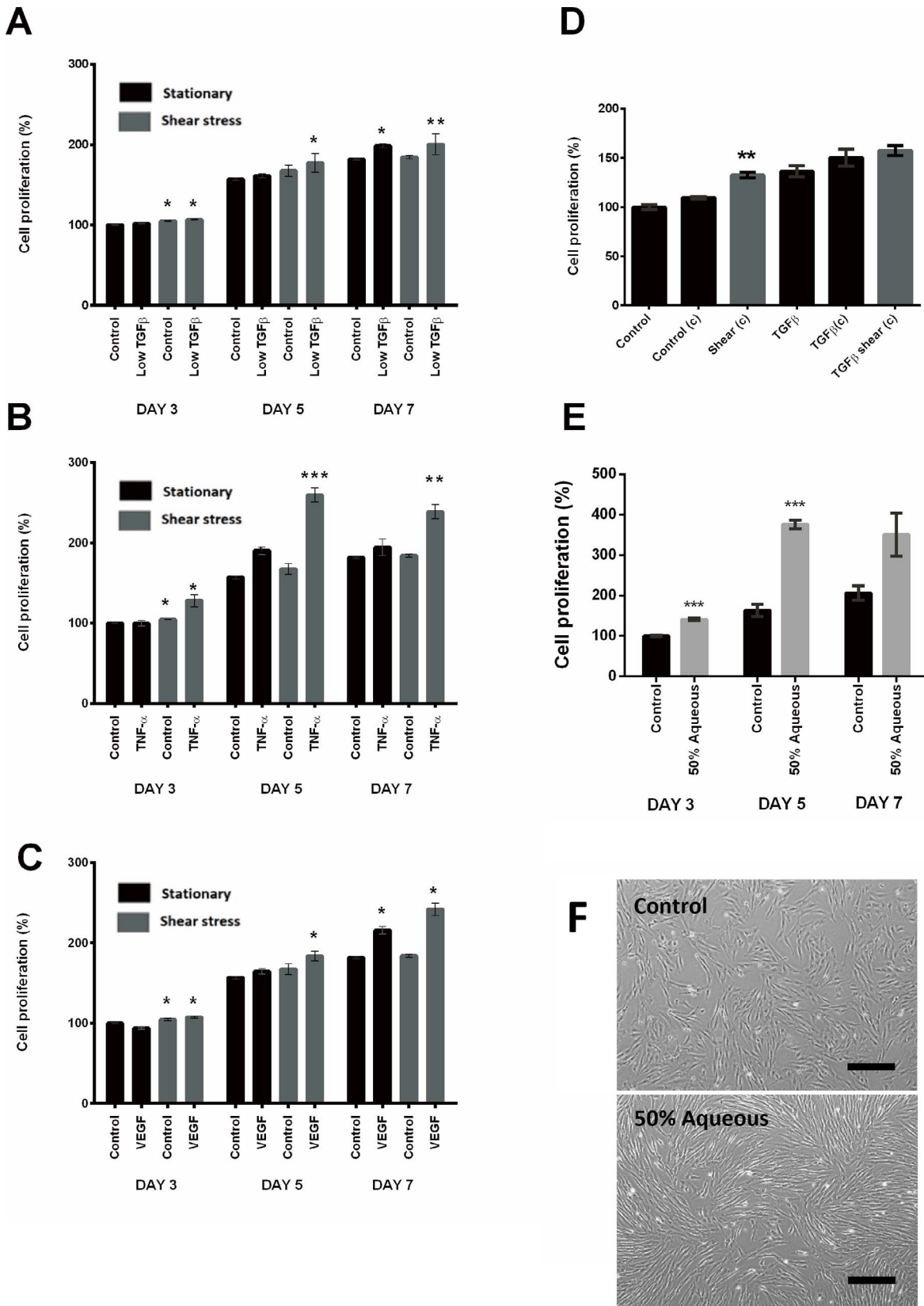
## RESULTS

### Characterization of TCCT Fibroblasts and TCCT Model

Tissue pieces from the Tenon's capsule and bulbar conjunctival anatomical regions were isolated from porcine eyes. As shown in Figures 1A and 1B, fibroblasts obtained from the Tenon's capsule and bulbar conjunctival location stained positively for both F-actin and Vimentin ( $n = 3$ ). Their morphology was characteristic of healthy, spindle-shaped fibroblasts (Fig. 1C). When seeding the fibroblasts in 3D culture within 3 mg/mL collagen hydrogel to form 3D TCCT models, the fibroblasts maintained this spindle-shaped morphology (Fig. 1D). The stretched cell morphology was preserved for the culture duration with a slight reduction in thickness of the gel samples.

### Effect of Additives and Shear Stress on Metabolic Function (Proliferation) in 2D Model

Individual key growth factors and cytokines, TGF- $\beta$ , VEGF, TNF- $\alpha$ , aqueous humor, and the secretome of fibroblasts (conditional media) were tested via addition to culture medium. The metabolic function, an indicator of proliferation



**FIGURE 2.** Alamar Blue assay to assess the effect of culture conditions on TCCT fibroblast proliferation in the 2D TCCT model. Addition of growth factors TGF- $\beta$  (20 ng/mL) (A), TNF- $\alpha$  (20 ng/mL) (B), and VEGF (20 ng/mL) (C) under stationary and shear stress conditions; addition of shear stress and TGF- $\beta$  conditioned medium (c) (D); addition of 50% aqueous humor (E). Data are expressed as mean  $\pm$  SE ( $n = 3$ ). \* Statistical significance in comparison with corresponding controls. (F) Phase-contrast images of TCCT fibroblasts at day 3 of culture with 50% aqueous humor and without (control). Scale bar: 400  $\mu$ m. \* $P \leq 0.05$ , \*\* $P \leq 0.01$ , and \*\*\* $P \leq 0.001$ , respectively.

for the fibroblasts has been assessed. As shown in Figure 2A, TCCT fibroblasts treated with 20 ng/mL TGF- $\beta$  showed a 2% increase in cell number in comparison with the control at day 3, a 4% increase at day 5, and 17% increase at day 7 ( $P=0.017$ ). This increase was more significant with additional exposure to shear stress, with increases of 7% at day 3 ( $P=0.04$ ), 20% at day 5 ( $P=0.012$ ), and 18% ( $P=0.008$ ) at day 7 in comparison with the control at each time point. As shown in Figure 2B, TCCT fibroblasts treated with 20 ng/mL TNF- $\alpha$  interestingly did not show much of an increase in cell number in comparison with the control at day 3, but had a 33% increase at day 5 ( $P=0.037$ ) and a 13% increase at day 7. This increase was also more significant with additional exposure to shear stress, with increases of 7% at day 3 ( $P=0.016$ ), 103% at day 5 ( $P=0.0006$ ), and 57% at day 7 ( $P=0.003$ ) in comparison with the controls. Figure 2C indicates that TCCT fibroblasts treated with 20 ng/mL VEGF also did not show much of an increase in cell number in comparison with the control at day 3, but had a 7% increase at day 5 and 34% increase at day 7 ( $P=0.016$ ). This increase was also more significant with additional exposure to shear stress, with increases of 8% at day 3 ( $P=0.04$ ), 27% at day 5 ( $P=0.015$ ), and 60% at day 7 ( $P=0.012$ ) in comparison with the controls.

Results were cross validated by the 2D conditional medium study presented in Figure 2D, where TCCT fibroblasts were treated with conditioned medium that contained the secretome of fibroblasts subjected to stationary (control), shear stress-treated, TGF- $\beta$ -treated, and TGF- $\beta$  + shear stress-treated cultures. Interestingly, fibroblasts cultured in conditional medium from the shear stress-treated group had a significant increase in cell number by 9% in comparison with the control ( $P=0.001$ ).

As indicated in Figures 2E and 2F, 2D TCCT cultures treated with 50% aqueous humor had a significant increase in cell number in comparison with the control. Cell number increased by a further 41.0% in comparison with the control group at day 3 ( $P=0.0003$ ), 212.7% at day 5 ( $P=0.0003$ ), and 144.0% at day 7. Batch-to-batch variability of the primary cell cultures did present some variation in fibroblast growth rate during experiments. Dependent on this variability, some 2D cultures tended to reach confluency by day 7 and hyperconfluency would likely explain for any decrease in cell number observed at the final time points.

### Effect of Additives and Shear Stress on Metabolic Function (Proliferation) in 3D Model

In consideration of increased cell number in the 3D TCCT model, the dosage of growth factors TGF- $\beta$ , TNF- $\alpha$ , and VEGF was increased to 50 ng/mL. This appeared to be the maximum dosage suitable for the 3D TCCT model before a detrimental reduction in gel thickness due to contraction. As shown in the Alamar Blue results presented in Figure 3, 3D samples treated with TGF- $\beta$  showed a significant increase in TCCT fibroblast number, for example by 36% in comparison with the control at day 5 of culture ( $P=0.010$ ) and 60% at day 7 ( $P=0.004$ ). A slight increase was detected when additionally exposed to shear stress, with increases of 19% at day 3 ( $P=0.03$ ), 42% at day 5 ( $P=0.004$ ) and 60% at day 7 ( $P=0.004$ ) in comparison with the control at each time point. Three-dimensional samples treated with TNF- $\alpha$  also showed a significant increase in TCCT fibroblast number, for example by 9% in comparison with the control at day 5 of culture and 58% at day 7 ( $P=0.003$ ). This increase was more significant with additional exposure to shear stress, with increases of 25% at day 3 ( $P=0.04$ ), 21% at day 5 ( $P=0.009$ ), and 69% at day 7 ( $P=0.004$ ) in comparison with the controls. In addition, 3D samples treated with VEGF showed a

significant increase in cell number, for example by 9% in comparison with the control at day 5 of culture and 4% by day 7. This increase was significant when additionally exposed to shear stress, with increases of 39% at day 3 ( $P=0.0009$ ), 26% at day 5 ( $P=0.007$ ), and 12% at day 7 ( $P=0.03$ ) in comparison with the controls.

As shown in Figure 3D, 3D TCCT model constructs treated with aqueous humor also had a significant 24% increase in cell number in comparison with the control by day 5 ( $P=0.004$ ) and 45% by day 7 ( $P=0.0006$ ). This increase was more significant with additional exposure to shear stress, with increases of 36% at day 5 ( $P=0.008$ ) and 55% at day 7 ( $P=0.0003$ ) in comparison with the controls.

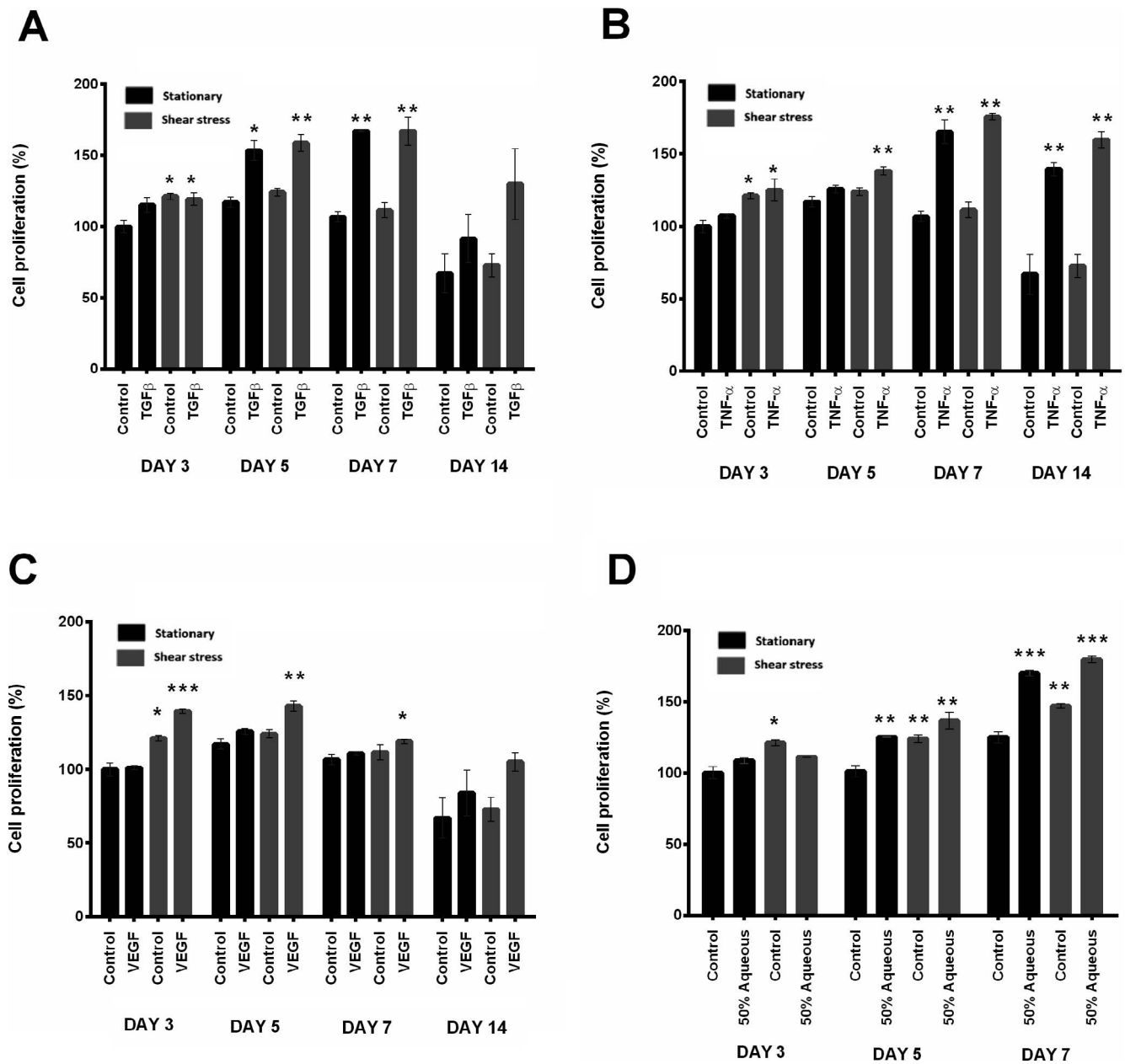
When comparing the 2D and 3D model responses, 2D control samples appeared to increase gradually up to day 7. Samples treated with growth factors followed a similar pattern, apart from TNF- $\alpha$ , which appeared to peak at day 5 and decline slightly by day 7. The greatest proliferation rate appeared to occur between days 3 and 5 for samples treated with TGF- $\beta$  and TNF- $\alpha$ . Interestingly, proliferation was slower for 2D samples treated with VEGF, which had the most proliferation between days 5 and 7. Samples treated with TNF- $\alpha$  appeared to have the greatest proliferation rate when combined with the synergistic effect of shear stress, reaching the highest overall proliferation (260% at day 5) among the 2D samples. In the 3D model, proliferation in control samples appeared to increase gradually up to day 5 before declining. Samples treated with the growth factors TGF- $\beta$  and TNF- $\alpha$  appeared to proliferate for longer, with most proliferation occurring between days 5 and 7 before a decline after day 7. Three-dimensional samples treated with aqueous humor also appeared to show this pattern. Interestingly, proliferation occurred slightly faster for 3D samples treated with VEGF, which had the most proliferation between days 3 and 5, but had the lowest overall proliferation among 3D samples. In the 3D model, TGF- $\beta$  appeared to stimulate the greatest increase in proliferation, reaching the highest overall percentage (167% at day 7) among the stationary samples. Similar to the 2D model, samples treated with TNF- $\alpha$  appeared to proliferate the most when combined with the synergistic effect of shear stress, reaching the highest overall proliferation (176% at day 7) among the 3D samples.

### Assessment of Neocollagen Synthesis Continuously

To determine new collagen synthesis by TCCT fibroblasts, azido-L-proline was added to the medium of experimental samples during culture and subsequently detected after incorporation into the neocollagen using Click-IT Alex Fluor 594 DIBO Alkyne stain. Figures 4 and 5 indicate the imaged and semiquantified neocollagen of 3D TCCT fibroblasts at days 3, 7, and 14 of culture. Samples treated with growth factors TGF- $\beta$ , TNF- $\alpha$ , VEGF, and 50% aqueous humor evidently presented more neocollagen than control samples, with TGF- $\beta$  and 50% aqueous humor-treated samples presenting the most labeled neocollagen. Furthermore, samples exposed to shear stress presented a slight increase in labeled neocollagen in comparison with the stationary conditions.

### F-actin Expression of Fibroblasts in TCCT Models

Cellular morphology and actin expression also appeared to differ in the presence of TGF- $\beta$  and aqueous humor, as shown in Figure 6. Three-dimensional TCCT samples cultured with TGF- $\beta$  and 50% aqueous humor showed an evident increase in actin expression in comparison with the control group. The F-actin expression appeared to increase even more



**FIGURE 3.** Alamar Blue assay to assess the effect of growth factors TGF-β (50 ng/mL) (A), TNF-α (50 ng/mL) (B), VEGF (50 ng/mL) (C), and 50% aqueous humor (D) under stationary and shear stress conditions on TCCT fibroblast proliferation in the 3D TCCT model. Data are expressed as mean ± SE (n = 3). \* Statistical significance in comparison with the corresponding control group at each time point. \*P ≤ 0.05, \*\*P ≤ 0.01, and \*\*\*P ≤ 0.001, respectively.

dramatically with exposure to shear stress. The cellular morphology also appeared to change, with fibroblast shape becoming more defined and elongated with exposure to TGF-β and aqueous humor, as well as shear stress. Fibroblasts exposed to shear stress also appeared to have a slightly stretched morphology, most likely caused by the fluid movement.

**ELISA Quantification of Porcine Aqueous Humor**

The TNF-α and TGF-β content of aqueous humor obtained from porcine eyes was quantified with ELISA, as shown in Figure 7. The average TNF-α content measured at approximately 63.88 pg/mL, with an upper limit of 97.18 pg/mL and lower limit of 22.59 pg/mL. The average TGF-β content measured at

approximately 177.46 pg/mL, with an upper limit of 192.37 pg/mL and lower limit of 163.54 pg/mL.

**DISCUSSION**

The fibrosis and scar tissue formation occurring within TCCT after glaucoma surgery can significantly reduce the treatment success rate to just 55%.<sup>2</sup> This study uses a reliable 2D and 3D in vitro TCCT model to investigate the factors that may influence fibrosis after glaucoma surgery. Initially, isolated porcine TCCT cells were characterized using F-actin and Vimentin expression; two markers acknowledged in previous literature for their ability to distinguish TCCT fibroblasts.<sup>14</sup> The cells from Tenon capsule and bulbar conjunctival tissues did

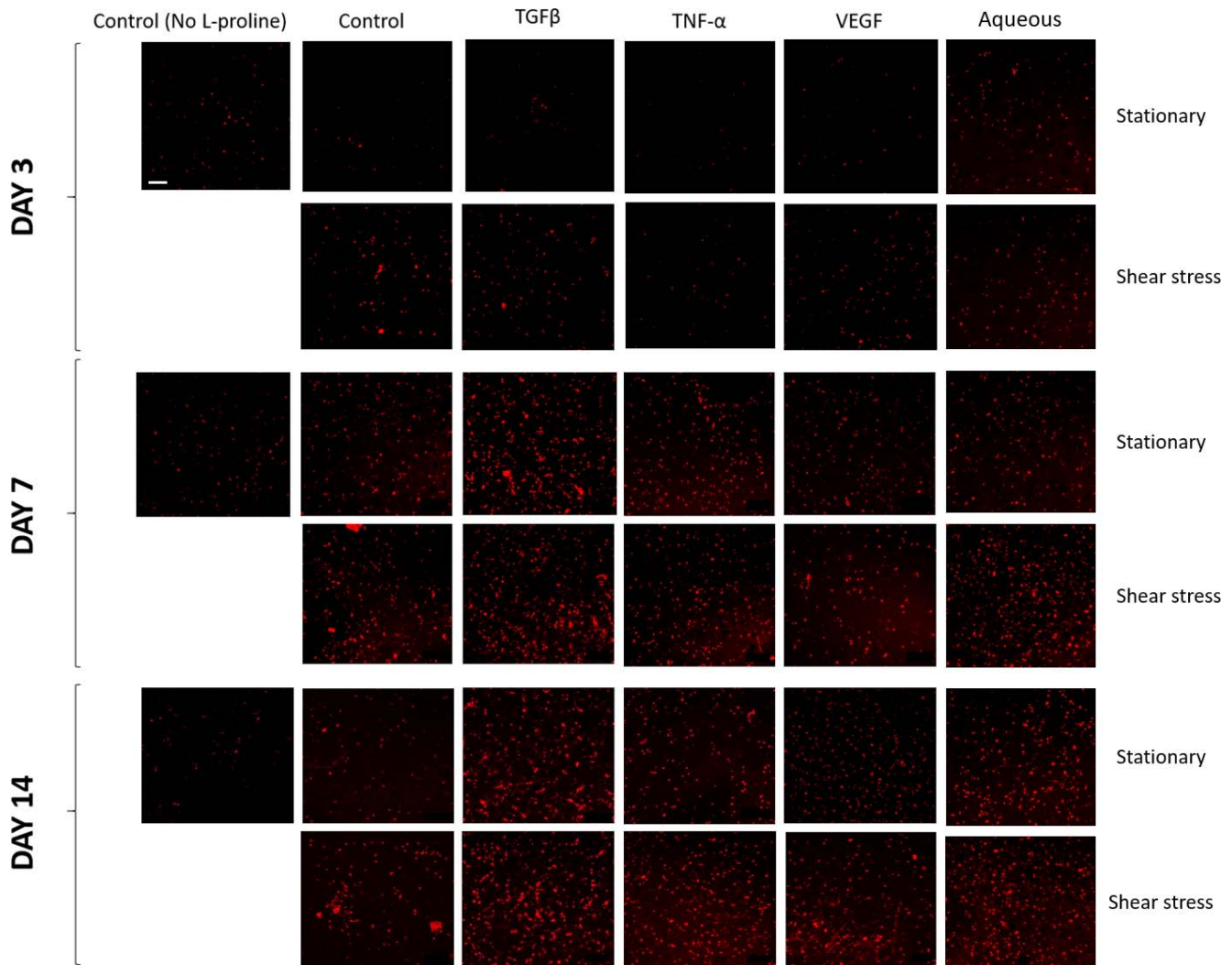


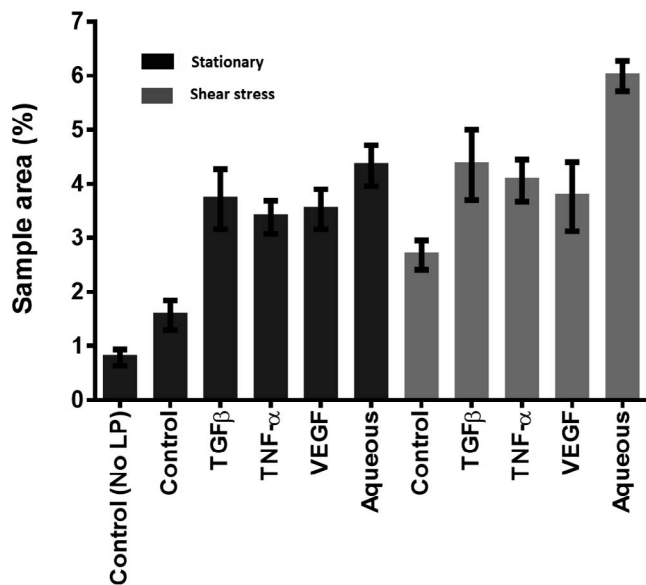
FIGURE 4. Neocollagen synthesis (red) monitored by Click-IT Alexa Fluor 594 DIBO Alkyne reaction with azido-L-proline, which was incorporated in the culture of 3D TCCT models with the additives TGF- $\beta$ , TNF- $\alpha$ , VEGF (50 ng/mL, respectively), and 50% aqueous humor under stationary and shear stress conditions. Negative control without adding of azido-L-proline (No L-proline) is included. Scale bar: 150  $\mu$ m.

not show different staining of the two markers. The mixture of the fibroblasts from the two locations was used to build 2D and 3D TCCT in vitro models. The fibrous protein present within TCCT ECM is believed to be primarily type I collagen.<sup>4</sup> Three-dimensional cultures were therefore seeded within a type I collagen hydrogel with the aim to mimic native TCCT in a controlled, quantifiable way (Fig. 1D).

Aqueous humor is a transparent fluid present in the anterior chamber of the eye with a range of functions including the removal of metabolic waste products, supply of nutrients, transport of neurotransmitters, and stabilization of ocular structure.<sup>15</sup> The evidence of increase in fibroblast activity seen in TCCT after conjunctival filtration surgery may be partly influenced by the presence of aqueous humor as it drains via the new pathway created during glaucoma surgery biochemically and biomechanically. It was therefore of interest to test aqueous humor on the in vitro TCCT model. TGF- $\beta$  and VEGF include some of the key growth factors previously found to be present in aqueous humor.<sup>7,8</sup> As shown in Figure 2E, the presence of aqueous humor significantly increased the proliferation rate of TCCT fibroblasts in both 2D and 3D model constructs. This increase was also significantly greater in comparison with fibroblasts cultured in the presence of

singular growth factors (TGF- $\beta$  and VEGF) at a concentration of 20 ng/mL and 50 ng/mL, respectively. The presence of aqueous humor also appeared to cause an increase in actin expression within TCCT fibroblasts (Fig. 6), which further confirms the presence of aqueous humor as a strong contributor to the fibrosis response in TCCT following glaucoma surgery.

Our ELISA quantification (Fig. 7) confirmed the presence of TNF- $\alpha$  and TGF- $\beta$  in porcine aqueous humor. However, previous studies have quantified the levels of multiple growth factors in human aqueous humor, such as hepatocyte growth factor at 12.22 ng/mL and glial cell-line-derived neurotrophic factor at 114.42 pg/mL, in addition to TGF- $\beta$  and TNF- $\alpha$ .<sup>16,17</sup> The quantity of TGF- $\beta$  in human aqueous humor has also been found to vary based on race and age, with healthy African American patients having an average of 165.69 pg/mL and Caucasian American patients having an average of 145.54 pg/mL TGF- $\beta$  in their aqueous humor, which increased with age.<sup>18</sup> Although higher dosages of TGF- $\beta$  and TNF- $\alpha$  were used in our model than the quantities found naturally in aqueous humor, our results showed that the aqueous humor triggered a far stronger proliferation rate on cultured TCCT fibroblasts. This further confirms the presence of many growth factors in

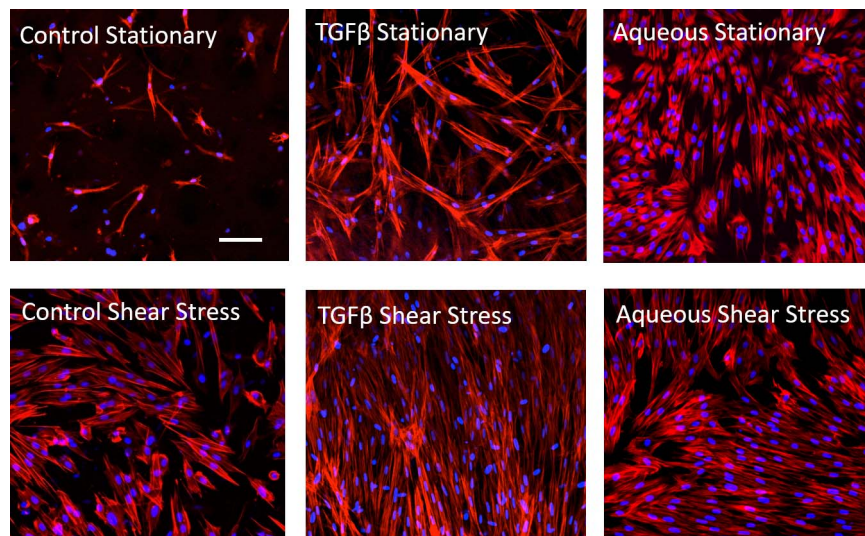


**FIGURE 5.** Semiquantification of neocollagen synthesis monitored by Click-IT Alexa Fluor 594 DIBO Alkyne reaction with azido-L-proline, which was incorporated in the culture of 3D TCCT models with the additives TGF- $\beta$ , TNF- $\alpha$ , VEGF (50 ng/mL, respectively), and 50% aqueous humor under stationary and shear stress conditions. Confocal microscopy images were quantified using ImageJ software (<http://imagej.nih.gov/ij/>; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA).

aqueous humor with variable quantities, which in combination may synergistically cause the significant increase in fibroblast proliferation observed in this study, outweighing the influence of individual cytokines TGF- $\beta$  or TNF- $\alpha$ .

The continued flow of aqueous fluid drainage across TCCT after glaucoma surgery may further influence the fibrosis response via shear stress that induced mechanotransduction. Shear stress-induced mechanotransduction has been identified to promote the overexpression of cytokines such as TGF- $\beta$ <sup>9</sup> and may therefore influence the increase in fibroblast activity seen in TCCT after glaucoma surgery. The quantified influence

of shear stress-induced mechanotransduction on TCCT fibrosis remains unclear. It was therefore of interest to assess the synergistic effects of shear stress in the presence of aqueous humor and key growth factors in vitro. We used 2D and 3D models and tested individual growth factor/cytokine or combinatory stimulators, aqueous humor, and conditional media in stationary and shear stress culture conditions. A “see-saw” motion rocker was used to apply shear stress, because it is an established method used in numerous credible studies to mimic fluid shear stress,<sup>19–22</sup> including use with 3D culture.<sup>23</sup> The method enables a high throughput, which was advantageous for this study, allowing us to easily expose many samples at once to 1 hour per day of shear stress during culture in 48-well plates. The method also has a low cost and is easy to establish. Because the 3D samples were thin (approximately 150  $\mu$ m) with a low collagen concentration (3 mg/mL), we can be confident that shear stress would have passed through sample pores, previously estimated to measure between 2.2 and 1.1  $\mu$ m for 1- to 4-mg/mL collagen hydrogels.<sup>24</sup> Puwanun et al.<sup>25</sup> previously stimulated bone cells in rigid polycaprolactone (PCL) electrospinning scaffolds (width 15 mm  $\times$  length 35 mm  $\times$  thickness 390  $\mu$ m) and found enhancement of mineralization due to the shear stress exerted by a see-saw motion rocker. This occurred throughout the full thickness of the scaffold, as evidenced by micro computed tomography scan images.<sup>23</sup> Furthermore, surface shear stress has previously been applied via fluid movement across the top of 3 mg/mL collagen hydrogels successfully, in addition to interstitial flow, using microfluidic perfusion systems.<sup>25,26</sup> The fluid shear stress generated in our 3D model is calculated as previously described<sup>19</sup> to be 0.065 Pa, based on fluid viscosity ( $10^{-3}$  Pa s), a maximum 7° flip angle, 1.6-mm fluid depth, 11-mm well length, and 12-second cycle (5 rpm). The significant increase of proliferation rate observed in our see-saw motion-treated group in comparison with the control group confirmed the shear stress influence on cells in our 3D hydrogel samples. However, there is a limitation that cells at the top of each gel would have been exposed to more fluid shear stress than those at the bottom by using a see-saw motion rocker. Conversely it could be argued that this mimics the in vivo environment more realistically. Fibroblasts located more closely to the created opening in conjunctival tissue after glaucoma surgery would be



**FIGURE 6.** F-actin expression in fibroblasts of 3D TCCT model; DAPI (blue) and actin (red) to indicate differences in cellular morphology and actin expression between TCCT fibroblasts, cultured with the additives TGF- $\beta$  (50 ng/mL) and 50% aqueous humor under stationary and shear stress conditions. Scale bar: 150  $\mu$ m.



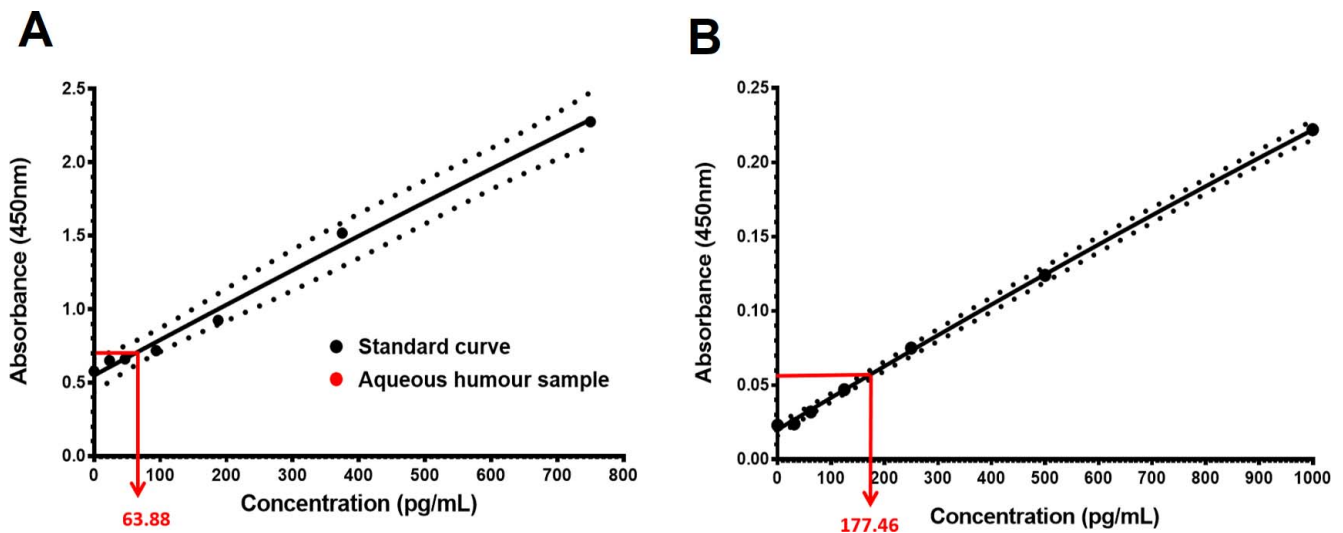


FIGURE 7. ELISA quantification of the TNF- $\alpha$  (A) and TGF- $\beta$  (B) content in porcine aqueous humor ( $n = 3$ ).

exposed to a greater amount of shear stress force than fibroblasts located more deeply inside the tissue.

It was confirmed that exposure to shear stress significantly increased the proliferation rate of 2D- and 3D-cultured TCCT fibroblasts, and the proliferative effect of aqueous humor appeared to be enhanced further by exposure to shear stress. These findings suggest that a combination of both aqueous humor and the shear stress induced by fluid outflow may synergistically contribute to the fibrosis response in TCCT after glaucoma surgery.

Results were cross validated by a 2D conditional medium study in which spent culture medium from TCCT fibroblasts previously exposed to shear stress was added to a new culture of TCCT fibroblasts. As shown in Figure 2D, TCCT fibroblasts treated with shear stress-conditioned medium had significantly increased proliferation in comparison with control samples. This result supports the idea that exposure to shear stress may cause an overexpression of cytokines in TCCT fibroblasts via mechanotransduction.

Similar increases were observed in the presence of singular growth factors (TGF- $\beta$ , TNF- $\alpha$ , and VEGF), where exposure to shear stress appeared to synergistically enhance the proliferative effect of singular cytokines in both 2D and 3D TCCT models (Figs. 2, 3). Generally, the proliferation occurred faster in 3D samples, with most growth occurring between days 0 and 7, in comparison with 2D samples in which most growth occurred between days 3 and 5. This may be explained by the fact that a collagen matrix offered protection to cells, making them less prone to initial degradation, in comparison with 2D culture in which cells were exposed to growth factors more directly. In both 2D and 3D, samples treated with TNF- $\alpha$  appeared to reach the greatest proliferation rate when combined with the synergistic effect of shear stress, closely followed by TGF- $\beta$ . These findings can be supported by previous literature that mentions TGF- $\beta$  and TNF- $\alpha$  as key growth factors that may stimulate fibrosis in the conjunctiva.<sup>5,6,27</sup>

In addition to the activation of proliferation, the subsequent formation of collagen by fibroblasts is another feature of the fibrosis that leads to excessive accumulation of ECM and scar tissue. To determine any influence that growth factors and shear stress may have on the collagen synthesis of TCCT fibroblasts, a nondestructive monitoring technique has been explored in this study. Because 2D cultures tended to reach confluency by day 7, they would have been too hyper-

confluent by day 14 for neocollagen assessment. Neocollagen data collection was therefore prioritized for the 3D response, because this was arguably of most interest for the study. Azido-L-proline was added to the medium of experimental 3D samples during culture and subsequently imaged after incorporation into neocollagen through the Click-IT Alex Fluor 594 DIBO Alkyne reaction with the azido-L-proline.<sup>13</sup> As shown in Figure 4, growth factors TGF- $\beta$ , TNF- $\alpha$ , and VEGF, plus 50% aqueous humor, all appeared to stimulate an increase in TCCT fibroblast collagen synthesis in comparison with the control, with TGF- $\beta$  and 50% aqueous humor having the greatest effect. Exposure to shear stress also appeared to stimulate an increase in collagen synthesis, although this difference was more subtle.

These findings confirm the influence that growth factors and shear stress are likely to have on TCCT ECM synthesis, in addition to an increase in fibroblast proliferation. Furthermore, we have applied a useful neocollagen fluorescent labeling technique for determining the fibrosis response of TCCT fibroblasts over a prolonged culture period.

## CONCLUSIONS

In summary, this study uses a reliable 2D and 3D TCCT model to investigate the factors influencing the fibrosis response after glaucoma surgery. Results confirm that a combination of both the growth factors present in aqueous humor, as well as the shear stress induced by aqueous fluid outflow, may synergistically trigger the fibrosis response in TCCT after glaucoma surgery. TGF- $\beta$  and TNF- $\alpha$  are two key growth factors influencing the fibrosis. To assess this response further, we applied a neocollagen fluorescent labeling technique to image the collagen production by TCCT fibroblasts for the whole culture duration. This method allowed for a better determination of the fibrosis response, in addition to proliferation assessments. These findings offer a useful insight for further study into the wound-healing response triggered by aqueous fluid outflow after glaucoma surgery.

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