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Effects of GSK101 Exposure on Astrocyte Mechanical Properties, Collagen Production, and Viability

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Abstract:

Transient receptor potential vanilloid 4 (TRPV4) is a cation channel protein which is thought to facilitate extracellular matrix (ECM) modification and glial scar formation following traumatic brain injury (TBI). Activation of TRPV4 has been shown to correlate with an increase in intracellular Ca^{2+} concentration. TRPV4 is subject to both chemical and mechanical activation. This study involved chemical activation of TRPV4 in cultured mouse astrocytes using GSK101, the primary agonist of TRPV4, and subsequent evaluation of mechanical and chemical changes to the ECM. Prior to GSK101 exposure, the cells were seeded into Matrigel Matrix, a gelatinous protein mixture intended to simulate a natural 3D environment for cell growth. Cells assigned to the experimental treatment group were regularly exposed to a known concentration of GSK101, while cells assigned to the control treatment group were exposed to an equivalent volumetric concentration of dimethyl sulfoxide (DMSO) accordingly. Following treatment, rheological testing was used to assess changes in the mechanical properties of each sample through application of shear stress. Following this, additional Matrigel samples were produced and treated. Each of these samples was lyophilized and hydrolyzed. The hydroxyproline assay was then used to quantify the presence of collagen, a major component of ECM, in each sample. Although the hydroxyproline assay revealed no statistically significant difference in collagen content between experimental and control samples, rheological testing revealed experimental samples to exhibit a greater storage modulus than control samples. The effects of mouse astrocyte exposure to DMSO and varying concentrations of GSK101 on cell viability were also assessed. While both exposure to high concentrations of pure DMSO and exposure to high concentrations of GSK101 suspended in DMSO were shown to reduce cell viability, there is no evidence to suggest that GSK101 alone affects cell viability.

1. Introduction

1.1 Traumatic Brain Injury

Traumatic brain injury (TBI) represents a major source of mortality and disability, causing approximately 52,000 deaths and 80,000 severe neurological disabilities each year.[1] It is the leading cause of death and severe disability in people under the age of 45 years in Western industrialized countries.[2] Children aged 0 to 4 years and adolescents aged 15 to 19 years are more likely to attain a TBI than individuals in other age groups, while the most common causes of TBI include falls and motor vehicle collisions.[3] Other common causes of TBI are shown in Figure 1. Long-term complications following TBI often include visual difficulties, headaches, and fatigue.[4] Cognitive, behavioral, and emotional changes are common as well.[4] The prevalence and chronicity of the physical and psychological complications resulting from TBI warrant further exploration of the physiological processes which take place in nervous tissue following TBI. Advanced knowledge of these processes will facilitate the development of effective treatment options focused on the restoration of healthy neural structure and function.

1.2 Astrocyte Function Following TBI

Astrocytes are the most numerous cell type in the human central nervous system and serve various important maintenance and regulatory functions, many of which are not well understood.[5] A few important functions performed by astrocytes include neurotransmitter regulation, ion homeostasis, blood brain barrier maintenance, and the production of extracellular matrix (ECM) molecules.[6] Brain tissue contains relatively few fibroblasts, leaving astrocytes to also assume the role of forming barriers known as glial scars to isolate lesion areas following TBI.[7] The formation of glial scar tissue interferes with axon regeneration through both direct

physical obstruction and the synthesis of collagens and sulfate proteoglycans which impede axon regeneration.[7] However, the formation of glial scar tissue following TBI is necessary to facilitate wound stabilization and healing, as cross links between collagen molecules and sulfate proteoglycans form and strengthen the tissue, providing a scaffold for wound contraction.[8] The general structure of a glial scar is illustrated in Figure 2. While the role of astrocytes in glial scar formation is known, the mechanisms by which these phenomena occur are not well understood.

1.3 Transient Receptor Potential Vanilloid 4

Transient receptor potential vanilloid 4 (TRPV4) is a widely expressed cation channel which has been shown to increase intracellular Ca^{2+} levels upon activation.[9] The presence of TRPV4 channels in astrocytes has been confirmed,[10] and it is possible that TRPV4 plays a role in the mechanism by which reactive astrocytes induce ECM remodeling. Studies have shown mechanical activation of TRPV4 in astrocytes to result in an increase in glial scar size and increased expression of glial fibrillary acidic protein, a glial-specific intermediate filament protein.[11] It is thought that the increase in intracellular Ca^{2+} concentration resulting from TRPV4 activation serves as an early injury signal which triggers signal transduction cascades for astrogliosis.[11] TRPV4 has been shown to exhibit osmotic sensitivity in chondrocytes, suggesting that TRPV4 also plays a role in chondrocyte volume regulation during periods of osmotic stress.[12] In addition to these methods of activation, TRPV4 can be chemically activated using GSK101, the TRPV4 agonist, and chemically inhibited using GSK205, the TRPV4 antagonist.[13] In this study, GSK101 was used to activate TRPV4 channels on mouse astrocyte cells. The effects of TRPV4 activation on collagen production and mechanical properties of the ECM were then evaluated using the hydroxyproline assay and rheological testing, respectively. The effect of GSK101 exposure on cell viability was also assessed.

Materials and Methods:

2.1 Cell Culture

A vial of approximately 1,000,000 C8-D1A mouse astrocyte clones was retrieved from storage in liquid nitrogen and thawed in a 37 $^{\circ}$C water bath. The thawed cell suspension was then centrifuged at 300 RPM for 10 minutes, and the freezing media was aspirated. The cells were resuspended in 1 mL of cell culture media consisting of Dulbecco's modified eagle's medium supplemented with 10% fetal bovine serum, 1% L-glutamine, and 1% penicillin streptomycin. This suspension was added to 6 mL of media in a T75 cell culture flask, and the flask was stored in a CO_2 incubator at 37 $^{\circ}$C. The media within the flask was aspirated and replaced with fresh media 3 times per week until a confluency of approximately 90% was reached.

Once the desired level of confluency was reached, the cells were detached and passaged into 2 T175 cell culture flasks. First, the media was aspirated, and 7 mL of trypsin supplemented with 0.25% ethylenediaminetetraacetic acid was added to the flask to facilitate detachment of the cells. After approximately 8 minutes, the suspension within the flask was neutralized with 7 mL of media and collected. The suspension was centrifuged at 300 RPM for 10 minutes, and the supernatant was aspirated. The cells were then resuspended in 2 mL of media, and 1 mL of the suspension was transferred to each of 2 T175 cell culture flasks. Media was added such to increase the total volume of suspension in each flask to 17 mL. The flasks were stored in the CO_2 incubator at 37 $^{\circ}$C, and the media was aspirated and replaced 3 times per week until a confluency of approximately 90% was attained in both flasks. The cells were then detached, centrifuged, and resuspended using the aforementioned method.

2.2 Matrigel Seeding

The cell density of the suspension was determined through the use of a hemocytometer. First, a small sample of the cell suspension was diluted with trypan blue using a dilution factor of 2. This ensured that living cells were able to be distinguished from dead cells. Approximately 10 µL of this sample was placed into the hemocytometer counting chamber using a micropipette, and cells within certain regions of the chamber were counted. The metric dimensions of these regions, the dilution factor, and the total number of counted cells were then used to calculate the cell density of the suspension. Once the cell density was determined, media was added to the suspension such to adjust the cell density to 5,000,000 $\frac{cells}{mL}$.

Next, 100 µL of the cell suspension was added to each of 12 Eppendorf tubes containing 400 µL of thawed Matrigel Matrix (Corning). Chilled micropipette tips were used to prevent gelation of the Matrigel. The contents of each tube were mixed thoroughly. Each 500 µL sample was then transferred to a rubber mold sealed against a coverslip. The 12 molds were contained in 2 6-well plates. These plates are pictured in Figure 3. The plates were placed in the CO_2 incubator at 37 $^{\circ}$C for 30 minutes, allowing gelation to occur. 5 mL of media was then added to each well. The plates were stored in the CO_2 incubator at 37 °C for 72 hours before sample treatment began.

2.3 Matrigel Sample Treatment

Solid GSK101 (Sigma-Aldrich) was weighed and diluted with dimethyl sulfoxide (DMSO) such to produce a stock solution of GSK101 at a concentration of 10 $\frac{mg}{mL}$. Prior to each treatment, 10 µL of this solution was added to 753 µL of sterile 1X phosphate buffered saline (PBS). Following this, 150 µL of this diluted solution was added to 30 mL of media, creating a media solution which contained 1,000 nM GSK101. This media was applied to the samples in

the experimental treatment group during exposure. This procedure was repeated using 10 µL of pure DMSO in place of the stock GSK101 solution. This media was applied to the samples in the control treatment group during exposure. Both of these solutions were made just prior to each treatment.

One 6-well plate was assigned to the experimental condition, while the other was assigned to the control condition. Three days following Matrigel seeding, the media in each well was aspirated and replaced with the appropriate treatment media. The plates were then placed in the CO_2 incubator at 37 $^{\circ}$C. After 30 minutes of exposure, the treatment media was aspirated from each well and replaced with regular media. This treatment was performed 3 times per week for 3 weeks.

2.4 Matrigel Sample Rheological Testing

Following treatment, the mechanical properties of the Matrigel samples were assessed using a Discovery HR-2 rheometer with 10 mm parallel plate attachments. Prior to testing, the Matrigel samples were removed from the molds using a 15 mm biopsy punch. The rheometer friction, inertia, and rotational mapping were then calibrated. The bottom rheometer plate was warmed to 37 °C, and a plate gap of 1,500 µm was consistently used. Each sample was placed on the plate to be tested individually. Drops of 1X PBS were then placed around each sample to prevent dehydration during testing. A strain level of 10% was applied to each sample at a frequency of 1 Hz. Storage modulus and loss modulus values were collected for each sample. The plates were cleaned with 70% ethanol and allowed to dry between tests.

2.5 Hydroxyproline Assay

The entire protocol detailed in sections 2.1 – 2.3 was repeated, and the collagen content of each new sample was determined using the hydroxyproline assay. Hydroxyproline is a non-coded amino acid which is exclusively present in collagen.[14] This principle renders the quantification of hydroxyproline useful in the direct measurement of collagen content.[15] Following hydrolysis of collagen, chloramine-T is able to oxidize free hydroxyproline molecules to form pyrrole molecules.[15] The subsequent addition of 4-dimethylaminobenzaldehyde results in the formation of chromophores which can be quantified using spectrophotometry.[15]

First, the samples were removed from the molds with a biopsy punch, as detailed in section 2.4, and placed in Eppendorf tubes. The openings of the tubes were sealed with Kimwipes, and the samples were lyophilized for 24 hours. Following lyophilization, the samples were weighed and placed in screw cap tubes. Each sample was diluted to a concentration of 40 $\frac{mg}{mL}$ using 6M HCl, and the samples were placed in an oven at 110 °C to hydrolyze for 18 hours. The samples were then stored at 4 °C overnight.

Following hydrolysis of the samples, the different buffers necessary for the hydroxyproline assay were produced. The hydroxyproline buffer consisted of 600 mL of dH_2O, 25 g of citric acid monohydrate, 6 mL of glacial acetic acid, 36 g of anhydrous sodium acetate, 17 g of sodium hydroxide, 150 mL of 1-propanol, and 750 µL of toluene. This buffer was primarily used as a dilution buffer in performing serial dilutions to generate a standard curve. Buffer A consisted of 3.5 mL of dH_2O, 122.5 mg of chloramine-T trihydrate, 3.5 mL of 1-propanol, and 28 mL of hydroxyproline buffer. This buffer was used to oxidize the free hydroxyproline present in the hydrolyzed samples. Buffer B consisted of 1.05 g of 4-dimethylaminobenzaldehyde, 5 mL of 1-propanol, and 1.82 mL of 70% $HClO_4$. This buffer was used to facilitate chromophore formation following hydroxyproline oxidation by buffer A.

Once the buffers were produced, serial dilution of a hydroxyproline standard solution was performed. This enabled the generation of a standard curve during data analysis. Exactly 20.2 mg of trans-4-hydroxy-L-proline was added to 200 mL of hydroxyproline buffer, forming a 0.101 $\frac{mg}{mL}$ hydroxyproline standard solution. This standard solution was serially diluted with hydroxyproline buffer 10 times using a dilution factor of 2. This serial dilution was performed in quadruplicate using library tubes.

The hydrolyzed samples were diluted with dH_2O using a dilution factor of 5. This partially neutralized the pH of each sample. Four 50 µL samples of each diluted sample were then placed in a 96-well plate, along with 50 µL of each component of the serial dilution. Four 50 µL samples of pure hydroxyproline buffer were also included as blanks.

Once all components had been added in 50 µL volumes to the 96-well plate, 100 µL of buffer A was added to each well, facilitating the oxidation of hydroxyproline. The plate was then covered and incubated at room temperature for 20 minutes. Next, 100 µL of buffer B was added to each well, increasing the total volume in each well to 250 µL and facilitating chromophore formation. The plate was then incubated at 65 °C for 25 minutes. The completed plate following the addition of both buffers and both incubation periods is pictured in Figure 4. The absorbance of 570 nm light was determined for each well using a BioTek Synergy Mx microplate reader.

2.6 Cell Viability Testing

Mouse astrocytes were cultured until two 90% confluent T175 flasks were obtained, as detailed in section 2.1, and the cell density was determined using a hemocytometer, as detailed in section 2.2. Using the surface area of a T175 flask (175 cm^2) and the surface area of one well of a 6-well plate (9.6 cm^2), the theoretical number of cells present in one well of a 6-well plate at

90% confluency was determined, and this number of cells was added of each well of 5 6-well plates. The total volume of cell suspension in each well was adjusted to 2 mL, and the plates were placed in the CO_2 incubator at 37 °C.

Two days following cell seeding, the media in each well of the 5 plates was aspirated. One plate was treated with pure media, while one plate was treated with media containing a concentration of DMSO equal to that present in the media applied to the control samples discussed in section 2.3. The other plates were treated with various concentrations of GSK101. One plate was exposed to media containing 1,000 nM GSK101, one plate was exposed to media containing 100 nM GSK101, and one plate was exposed to media containing 10 nM GSK101. Media containing 1,000 nM GSK101 was produced using the method detailed in section 2.3, while media containing 100 nM GSK101 was produced by diluting 40 µL of the PBS-diluted GSK101 solution with 360 µL of 1X PBS prior to dilution in 30 mL of media. Likewise, media containing 10 nM GSK101 was produced by further diluting 40 µL of this diluted solution in 360 µL of 1X PBS prior to dilution in 30 mL of media.

The cells were exposed to treatment media for 30 minutes in the CO_2 incubator at 37 °C before all treatment media was aspirated and replaced with regular media. At this point, the plates were placed back in the CO_2 incubator at 37 °C. One day later, the cell density in each well was determined. Working with only one plate at a time, the media was aspirated from each well and replaced with 500 µL of trypsin. After approximately 8 minutes, cell detachment was complete. An equivalent volume of media was added to each well, neutralizing the trypsin and increasing the total volume in each well to 1 mL. The cell density in each well was then determined using the hemocytometer counting method discussed in section 2.2. This process was repeated for each plate.

2.7 Statistical Analysis

Following all experiments, treatment groups were compared using a one-way ANOVA test and Tukey-Kramer HSD test. A significance level of 0.05 was used. All bar graphs compare mean values, and all error bars correspond to standard deviation within specific treatment groups.

Results

3.1 Matrigel Sample Testing

Following treatment, 4',6-diamidino-2-phenylindole (DAPI) staining was used to confirm the presence of cells in samples assigned to both the control and experimental treatment groups. As illustrated in Figure 5, cell density was roughly consistent across the two treatment groups.

At a strain level of 10% and frequency of 1 Hz, the mean storage modulus values of samples in the control and experimental treatment groups were 147.503 Pa and 184.468 Pa, respectively. The mean storage modulus of the experimental samples was significantly higher than that of the control samples ($p = 0.0422$). At a strain level of 10% and frequency of 1 Hz, the mean loss modulus values of samples in the control and experimental treatment groups were 17.686 Pa and 22.685 Pa, respectively. The difference in mean loss modulus between the two treatment groups is not statistically significant. This data is depicted in Figure 6.

The mean 570 nm absorbance values of samples in the control and experimental treatment groups were 0.10265 and 0.10475, respectively. After subtracting the mean blank absorbance value from all sample and standard curve absorbance values and using the standard curve to convert absorbance to hydroxyproline concentration, the mean concentration of hydroxyproline in samples assigned to the control and experimental treatment groups were

determined to be 0.61207 $\frac{\mu g}{mL}$ and 0.65765 $\frac{\mu g}{mL}$, respectively. Considering this information, a 7.4468% increase in collagen content was observed in the samples assigned to the experimental treatment group relative to the samples assigned to the control treatment group. However, this difference is not statistically significant (p = 0.3605). This data is depicted in Figure 7.

3.2 Cell Viability Testing

Mean cell densities of 1,127,500 $\frac{cells}{mL}$, 855,000 $\frac{cells}{mL}$, 936,667 $\frac{cells}{mL}$, 906,667 $\frac{cells}{mL}$, and 823,333 $\frac{cells}{mL}$ were recorded for cells exposed to pure media, media containing DMSO, media containing 10 nM GSK101, media containing 100 nM GSK101, and media containing 1,000 nM GSK101, respectively. Cell density following exposure to media containing DMSO was found to be significantly lower than cell density following exposure to pure media (p = 0.0239). Cell density following exposure to media containing 1,000 nM GSK101 was also found to be significantly lower than cell density following exposure to pure media (p = 0.0098). This data is depicted in Figure 8.

Discussion

The primary purpose of this study was the assessment of the effects of TRPV4 activation on ECM remodeling by mouse astrocytes. The rheological testing results indicate that chemical activation of TRPV4 causes an increase in ECM elasticity, as the mean storage modulus of the samples in the experimental treatment group was significantly higher than that of the samples in the control treatment group, and TRPV4 had no significant effect on the loss modulus. It is possible that this change in elasticity is due to increased collagen content, as the triple helix structure of collagen is known to contribute to elasticity in collagen fibers.[16]

Although a slight increase in collagen content was observed in the experimental samples relative to the control samples using the hydroxyproline assay, this increase is not statistically

significant. It is possible that the acidity of the hydrolyzed samples interfered with the reactions which took place during the application of buffer A and buffer B. One potential protocol modification involves neutralization of the samples with base in place of the dH_2O dilution detailed in section 2.5.

The statistically significant difference in cell density between samples exposed to pure media and both samples exposed to media containing DMSO and samples exposed to media containing 1,000 nM GSK101 suggests that DMSO negatively affects cell viability. There is no evidence to suggest that GSK101 affects cell viability, as there was no statistically significant difference in cell density between samples exposed to media containing DMSO and any of the treatment groups exposed to media containing GSK101. The protocol used for this test could possibly be improved through the exposure of additional treatment groups to media containing various concentrations of DMSO corresponding to the concentrations of GSK101 used. In addition to this, use of a longer exposure period or multiple exposure periods could possibly augment potential differences in cell density between treatment groups. Direct dissolution of solid GSK101 in media could also be useful in isolating GSK101 as an independent variable.

Overall, the results of this study confirm that TRPV4 plays a role in ECM remodeling following chemical activation using GSK101. Although increased collagen content following TRPV4 activation was not directly observed using the hydroxyproline assay, the increased elasticity of the ECM following TRPV4 activation and past studies focused on the function of TRPV4 suggest that increased collagen production may have taken place. There is no evidence to suggest that GSK101 affects cell viability. Through the implementation of the aforementioned protocol modifications, more conclusive findings could possibly be derived in future studies using methods similar to those employed in this study.

Acknowledgements:

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Appendix

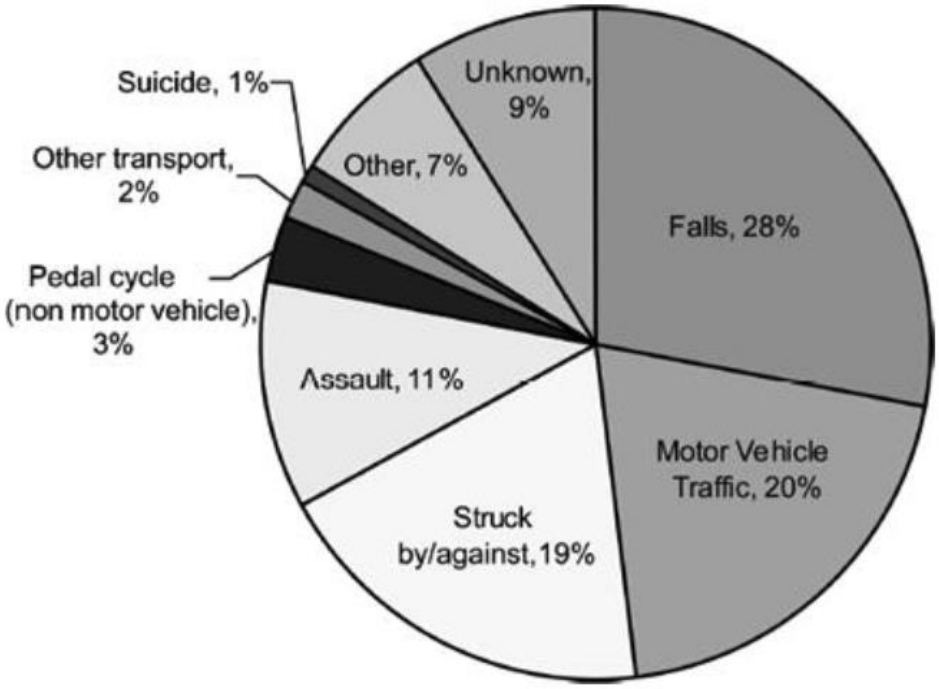

Figure 1: The chart above reveals different causes of TBI and the relative prevalence of each cause.[3]

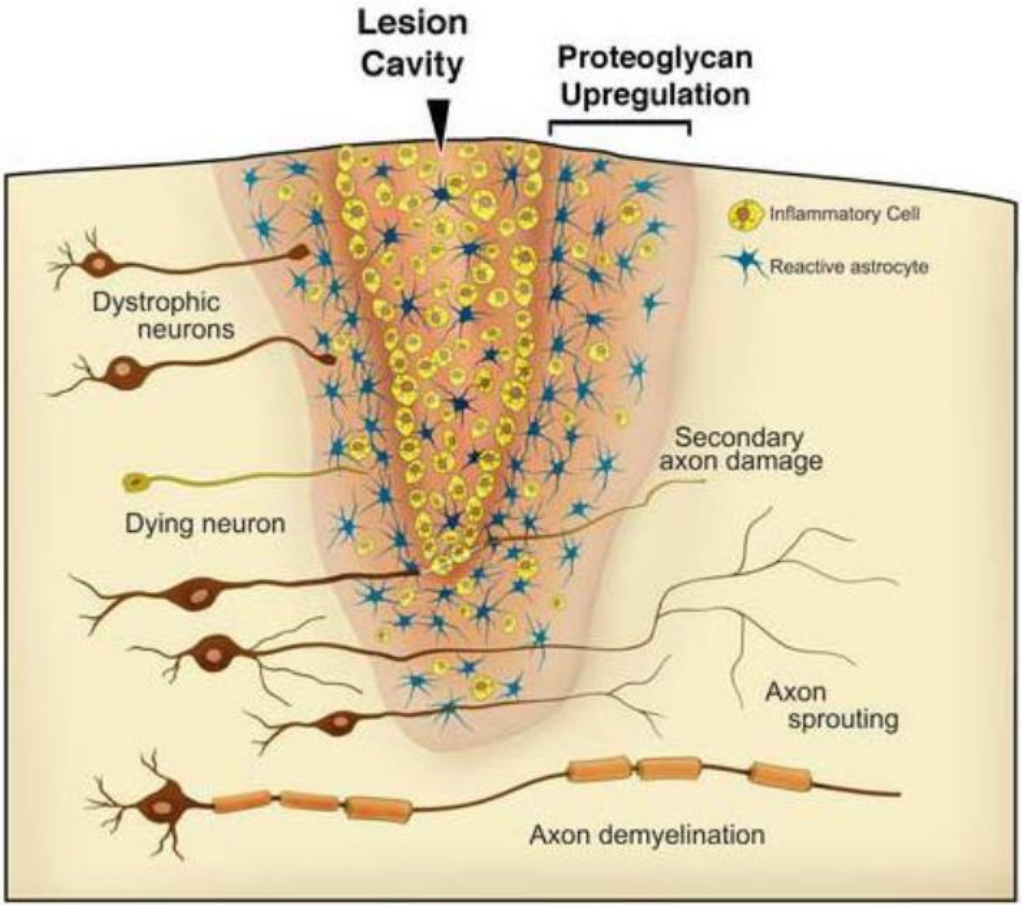

Figure 2: The image above illustrates the structure of a glial scar.[6]

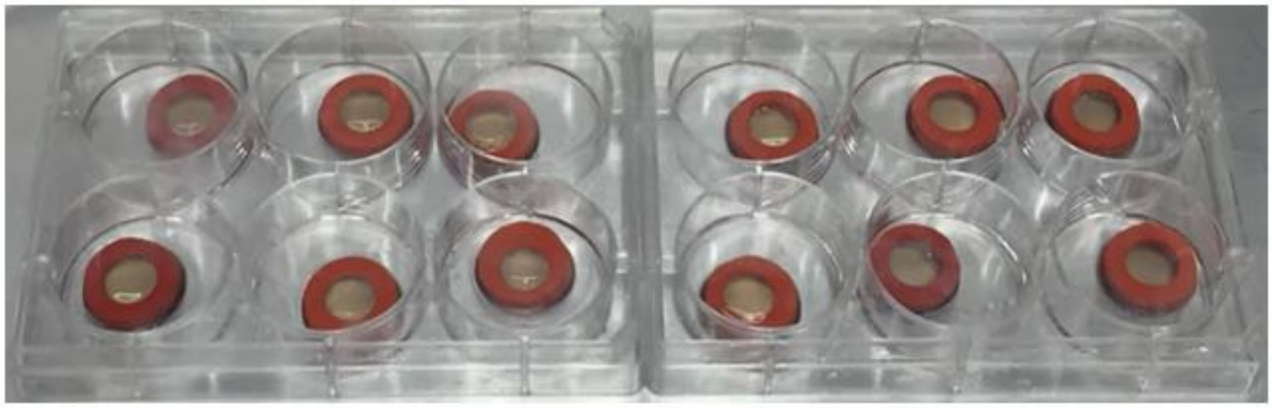

Figure 3: Matrigel samples within rubber molds are pictured above.

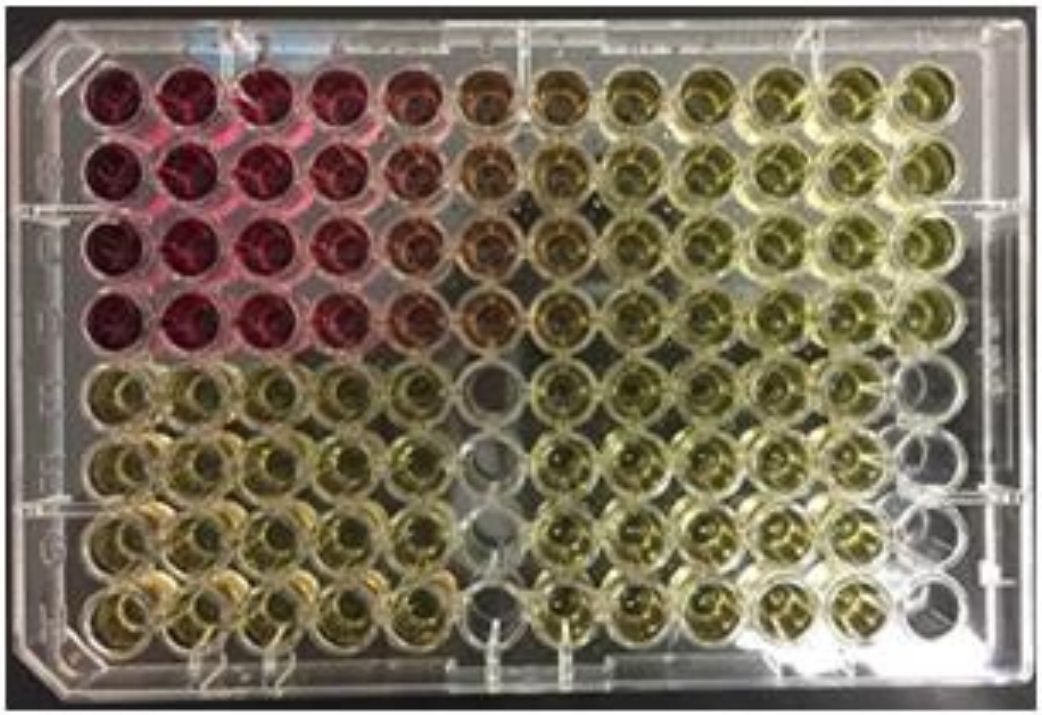

Figure 4: The plate pictured above contains the serially diluted hydroxyproline standard samples (A1 – D11), the hydroxyproline buffer samples (A12 – D12), the samples in the control treatment group (E1 – H5), and the samples in the experimental treatment group (E7 – H11). This image was captured following the addition of both buffers and both incubation periods.

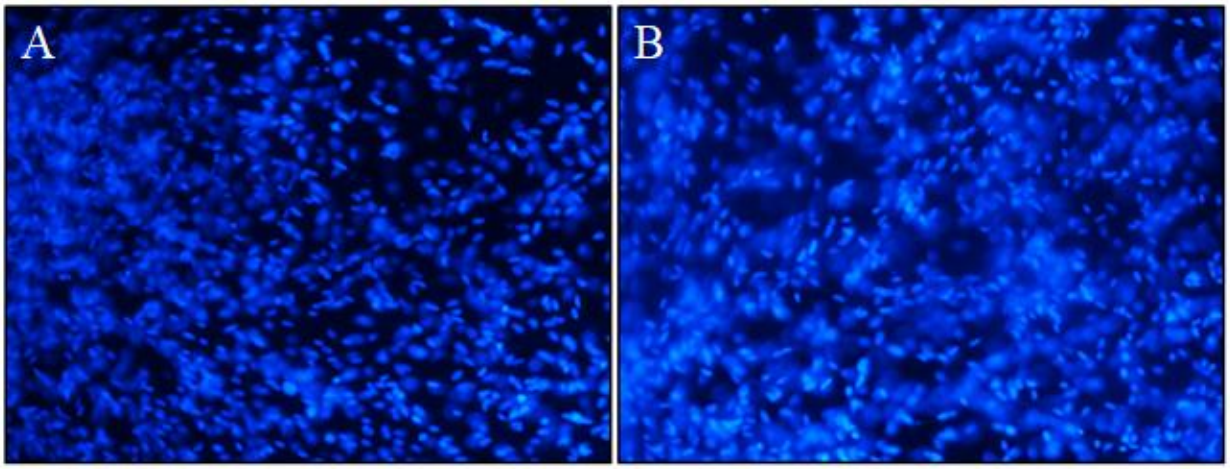

Figure 5: DAPI-stained samples assigned to the control treatment group (A) and the experimental treatment group (B) are pictured above. 20X magnification was used. Exposure times of 176 ms and 95 ms were used in capturing images A and B, respectively.

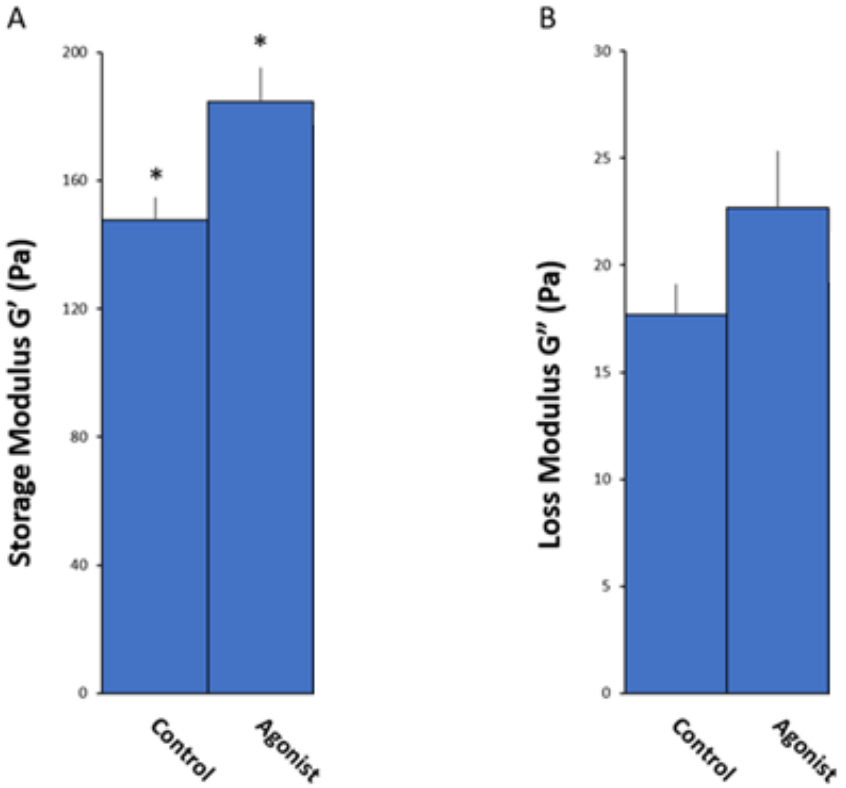

Figure 6: The mean storage modulus (A) and loss modulus (B) values of samples in both treatment conditions are pictured above. This data was obtained through the application of 10% strain at 1 Hz to each sample. A statistically significant difference in storage modulus between the two treatment groups was observed.

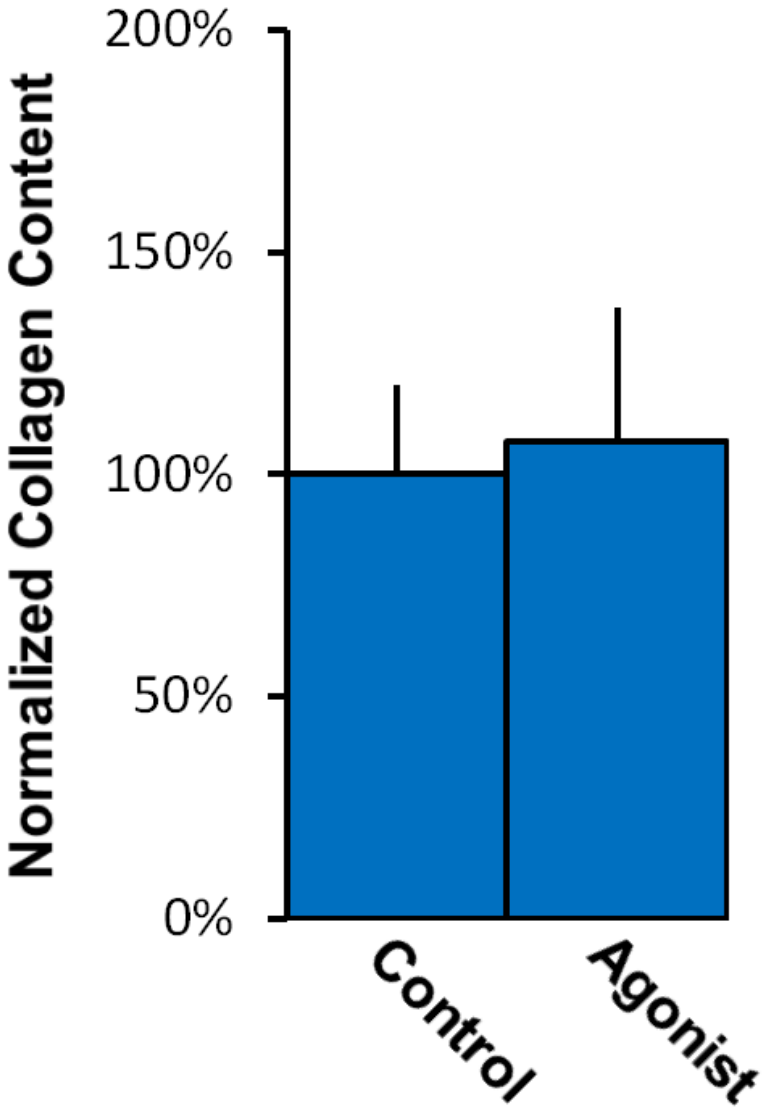

Figure 7: The mean collagen content in samples assigned to the experimental treatment group is displayed above as a percentage of the mean collagen content in samples assigned to the control treatment group. No significant difference was observed.

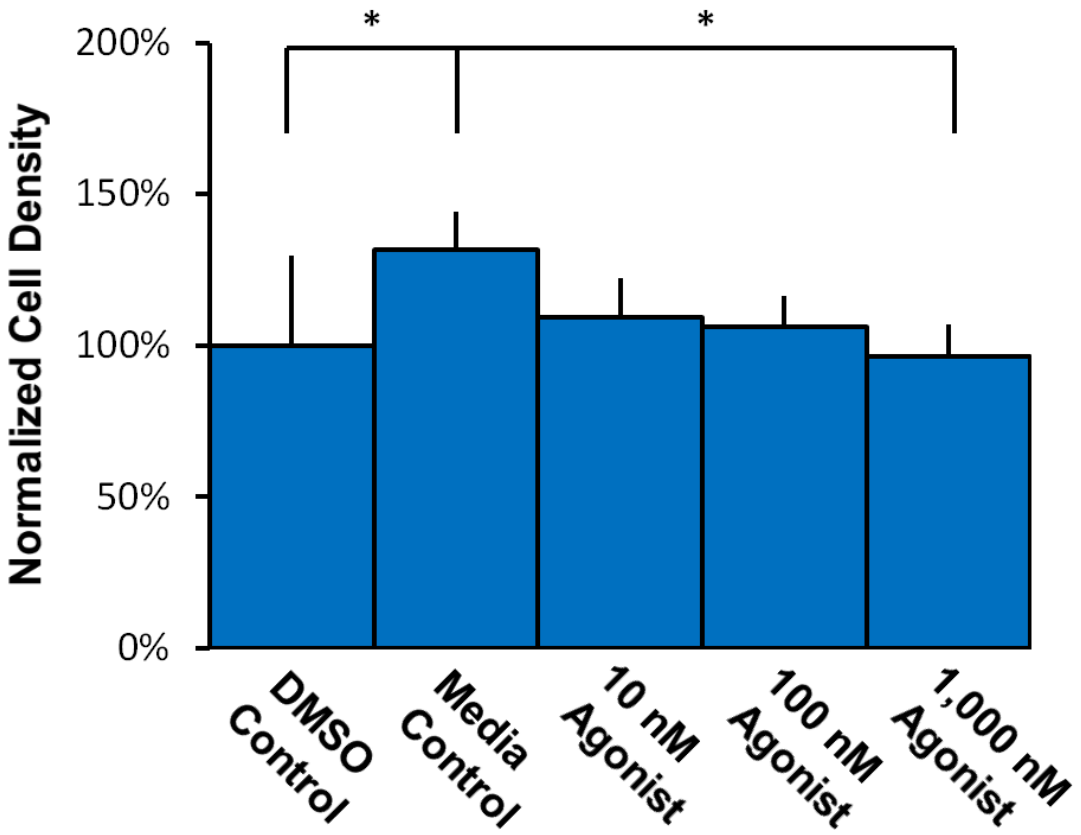

Figure 8: The mean cell density values corresponding to each treatment group are displayed above as percentages of the mean cell density of samples exposed to media containing DMSO. A significant difference in mean cell density values was observed between cells exposed only to media and both cells exposed to media containing DMSO and cells exposed to media containing 1,000 nM GSK101.