Activated Glycogen Synthase Kinase-3β's Ability to Inhibit Keloid Formation

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Abtract

Keloid scars are abnormal wound healing processes characterized by excessive collagen deposition that exceed the borders of the original wound. Inactivated glycogen synthase kinase 3-β (GSK-3β), a molecule that breaks down β-catenin which is used by the Wnt/β-catenin pathway in hypertrophic scarring, has been found to be abundant in keloid fibroblasts. The Wnt/β-catenin pathway is known to influence hypertrophic scarring, and the activation of GSK-3β has been found to inhibit other hypertrophic scarring diseases. This project focuses on determining if activating GSK-3β will inhibit keloid formation by decreasing the collagen, elastin, and fibronectin levels. Human keloid fibroblasts were cultured in vitro and treated with sodium nitroprusside to activate GSK-3β, lithium chloride to inhibit GSK-3β, and a negative control. Protein levels were then assessed through Western Blot analysis. The results of this study suggest that GSK-3β is partially inactive in keloid fibroblasts and that activation of the protein activates the Wnt signaling pathway and decreases collagen and elastin production. Fibronectin levels were found to increase with treatment, but this may be explained by its role in cell adhesion and contraction in wound healing. Further research is needed to fully identify activated GSK-3β as a therapeutic target for keloid scar patients and individuals who are prone to keloid scarring.

Activated GSK-3β's Ability to Inhibit Keloid Formation

Although the effects of scarring on quality of life may seem less than other chronic conditions, they are important to consider when assessing patient needs and have implications on clinical practice; the impact of scars on patient quality of life has been shown to be similar to other chronic skin diseases and disfiguring conditions (Brown et al., 2008). One of the main issues concerns the stigma surrounding scars, which can impact a patient's social and psychological well-being (Mundy et al., 2016). In addition to the negative psychosocial effects, there are also negative physical impacts that scars can have on quality of life. Keloid and hypertrophic scars occur in areas of high skin tension and on the chest wall, which in addition to being in regions difficult to hide, can result in restricted mobility (Bock et al., 2006). Keloid scars are also associated with small nerve fiber neuropathy, which can manifest in itch and pain (Lee et al., 2004). Some other distinguishing features of keloids further suggests the importance of their treatment and prevention when compared to other hypertrophic scars—while hypertrophic scars stay within the confines of the original wound, keloids extend beyond the original wound's borders, which can further restrict mobility or cause discomfort (Chaudet et al., 2020). Hypertrophic scars may also ultimately regress and have a low rate of recurrence after excision, but keloids persist nearly indefinitely and almost always recur, suggesting the need for outside intervention. Although the effects of scarring on quality of life may seem less than other chronic conditions, they are important to consider in clinical practice. Not only can patient satisfaction be negatively affected if scarring occurs as a result of a medical procedure, instances

of clinicians being dismissive towards scars and delaying treatment have resulted in increased patient anxiety (Brown et al., 2008).

Keloids are fibroproliferative lesions often associated with hypertrophic scars, both of which are characterized by the excessive deposition of collagen during wound healing (Chaudet et al., 2020). Although the exact pathogenesis is unknown, the Wnt/β-catenin pathway has been implied to be involved in both hypertrophic scarring and keloid formation. This pathway is activated by wounding and results in β-catenin entering the nucleus to activate transcription, playing an important role in activating fibroblasts during the proliferative phase of healing (Houschyar et al., 2019). In this phase, the wound surface is covered with granulation tissue, while extracellular matrix proteins are produced by fibroblasts to lay the foundation for the new connective tissue matrix and restoration of mechanical strength. β-catenin acts as a regulator of fibroblast activity during this process, evidenced by the increased β-catenin levels and transcriptional activity during this phase which then returns to normal in the following remodeling phase.

In hypertrophic scars and keloids, the presence of elevated β-catenin levels suggest that abnormal Wnt/β-catenin signaling, or prolonged β-catenin activity during the proliferative phase of wound healing in particular, is involved in excessive fibrosis and scar formation (Houschyar et al., 2019). When Wnt signaling is not present, β-catenin is phosphorylated by a complex consisting of casein kinase and glycogen synthase kinase-3 (GSK3), which marks it for ubiquitination and degradation (Chaudet et al., 2020). Previous studies have shown that when the

Wnt/β-catenin pathway was inhibited, the collagen, fibronectin, and elastin in keloid samples decreased, suggesting the Wnt/β-catenin pathway to be a possible therapeutic target.

Current Research

The pathogenesis of keloids is still debated, but it is known to be involved in inflammatory, proliferative, humoral, and/or genetic reasons (Nangole & Agak, 2019). The inhibition of glycogen synthase kinase-3β (GSK-3β) in particular appears to be involved in keloid formation; it was discovered that the phosphorylated form was abundant in keloid fibroblasts treated with Wnt5a (Chaudet et al., 2020). GSK-3β has been studied in conjunction with other hypertrophic scarring diseases, such as diabetic kidney disease in mice (Mariappan et al., 2014). Diabetic kidney disease can result in renal tissue fibrosis due to an accumulation of matrix proteins through TGFβ signaling, a cytokine also involved in regulating matrix protein production during the proliferative stage of epidermal wound healing (Houschyar et al., 2019; Mariappan et al., 2014). GSK-3β, when activated by sodium nitroprusside (SNP), was found to inhibit kidney hypertrophy following diabetic scarring on the macroscopic level (Mariappan et al., 2014). On the molecular level, phosphorylation of GSK-3β induced by diabetes was reversed with the addition of SNP, suggesting that GSK-3β was reactivated. Under normal circumstances, GSK-3β inhibits mTORC1 activation, which regulates kidney matrix protein production. As a result, GSK-3β has been proposed as a therapeutic target in diabetic kidney disease due to its ability to inhibit the overproduction of scar tissue. However, the ability of GSK3, especially the isoform GSK-3β, when activated by SNP to inhibit the Wnt signaling pathway in keloid fibroblasts has not yet been studied, but the similarity between the overproduction of matrix

proteins and evidence that phosphorylated GSK-3β is also present in keloid fibroblasts suggests it can also be a therapeutic target for keloid scarring.

Aims and Objectives

Introduction

If more ways are identified to prevent keloid scarring, especially as a result of trauma induced during necessary medical procedures, patient satisfaction and quality of life can be increased. This project will investigate glycogen synthase kinase-3β (GSK-3β) as a possible inhibitor of keloid formation. Keloid fibroblasts will be grown in the lab and treated with sodium nitroprusside (SNP), which has been found to activate GSK-3β and ameliorate other hypertrophic scarring diseases. The production of extracellular matrix proteins in the SNP treated cells will be compared to the protein production of cells grown in a negative control and lithium solution, which is already known to inhibit GSK-3β (Kurgan et al., 2019).

Aims

1. To investigate if the activation of GSK-3β has any effect on the collagen, elastin, and fibronectin production of keloid fibroblasts using Western blot analysis.

2. To investigate sodium nitroprusside's ability to activate GSK-3β in response to keloid formation in comparison to lithium's ability to inhibit GSK-3β.

3. To identify if sodium nitroprusside can activate GSK-3β in keloid fibroblasts without affecting the cells' viability.

Objectives

1. The overproduction and abnormal formation of collagen, elastin, and fibronectin is one of the characteristics of keloid scarring. If the activation of GSK-3β inhibits this overproduction, it can be used as a therapeutic target for keloid scar formation.

2. Sodium nitroprusside is relatively inexpensive and commonly used as a medication to lower blood pressure. If it is found to sufficiently be able to activate GSK-3β and inhibit keloid scar formation, it will be safe and convenient for patient use and can further be investigated as a preventive measure against keloid scarring prior to surgery. This ability will be evaluated by comparing the activity of keloid fibroblasts treated with SNP to a positive control and lithium, which is known to inhibit GSK-3β activity.

3. The estimated amount of sodium nitroprusside directly applied to the fibroblasts can be related to the amount that should be in the body to prevent keloid scarring. If the estimated amount is not toxic to the body, it reinforces the possibility of using sodium nitroprusside as a preventive measure against keloid scarring and can give insight into the best way to integrate it into the body.

Materials and Methods

Cell Culture

Keloid fibroblasts were obtained from Gibco™ and grown to 80% confluence while kept at 37°C with 5% carbon dioxide. Trials were conducted with 4 replicate wells for each treatment and all trials were completed simultaneously at the same passage number to minimize uncontrollable variables between conditions.

Experimental Reagents

Once the cells reached 80% confluence, they were treated with the experimental reagents. Four wells were treated with sodium nitroprusside (SNP), another four wells were treated with lithium chloride (LiCl), and the final four wells were treated with fresh medium as the negative control. Each well was exposed to 1 mL of its respective treatment.

Sodium Nitroprusside Treatment

Cells were exposed to 50 μM of sodium nitroprusside for 30 minutes.

Lithium Chloride Treatment

Cells were exposed to 0.5 mM of lithium chloride for 30 minutes.

Negative Control Treatment

Cells were exposed to DMEM with 10% FBS for 30 minutes.

Western Blot

After the cells were treated for 30 minutes with their respective treatments and incubated for another 24 hours, cell protein was harvested in radioimmunoprecipitation assay lysis buffer (RIPA) and frozen at -80°C for protein analysis. The BCA assay was then used to determine the protein concentration of each unknown sample. The standard curve produced from the BCA assay is provided in the appendix. After protein quantification, equi-protein aliquots were combined with loading buffer and run on 8% Tris-HCl SDS-PAGE gels. The gels were then transferred to nitrocellulose for blotting.

The protocols for protein gel electrophoresis and western blot analysis were adapted from *Protein Methods* (Bollag et al., 1996). Blots were blocked with Tris-buffered saline (TBS), 0.05% Tween 20, and 2% bovine serum albumin (BSA) and probed with the primary antibodies mouse anti-collagen type-I, mouse anti-collagen type-III, mouse anti-elastin, and mouse anti-fibronectin. The dilutions for each antibody are provided in the appendix. The secondary antibody used was goat anti-rabbit HRP conjugate. Enhanced chemiluminescent (ECL) detection reagent was applied and the blots were imaged on the Chemi-Doc system with Kodak imaging software.

Analysis

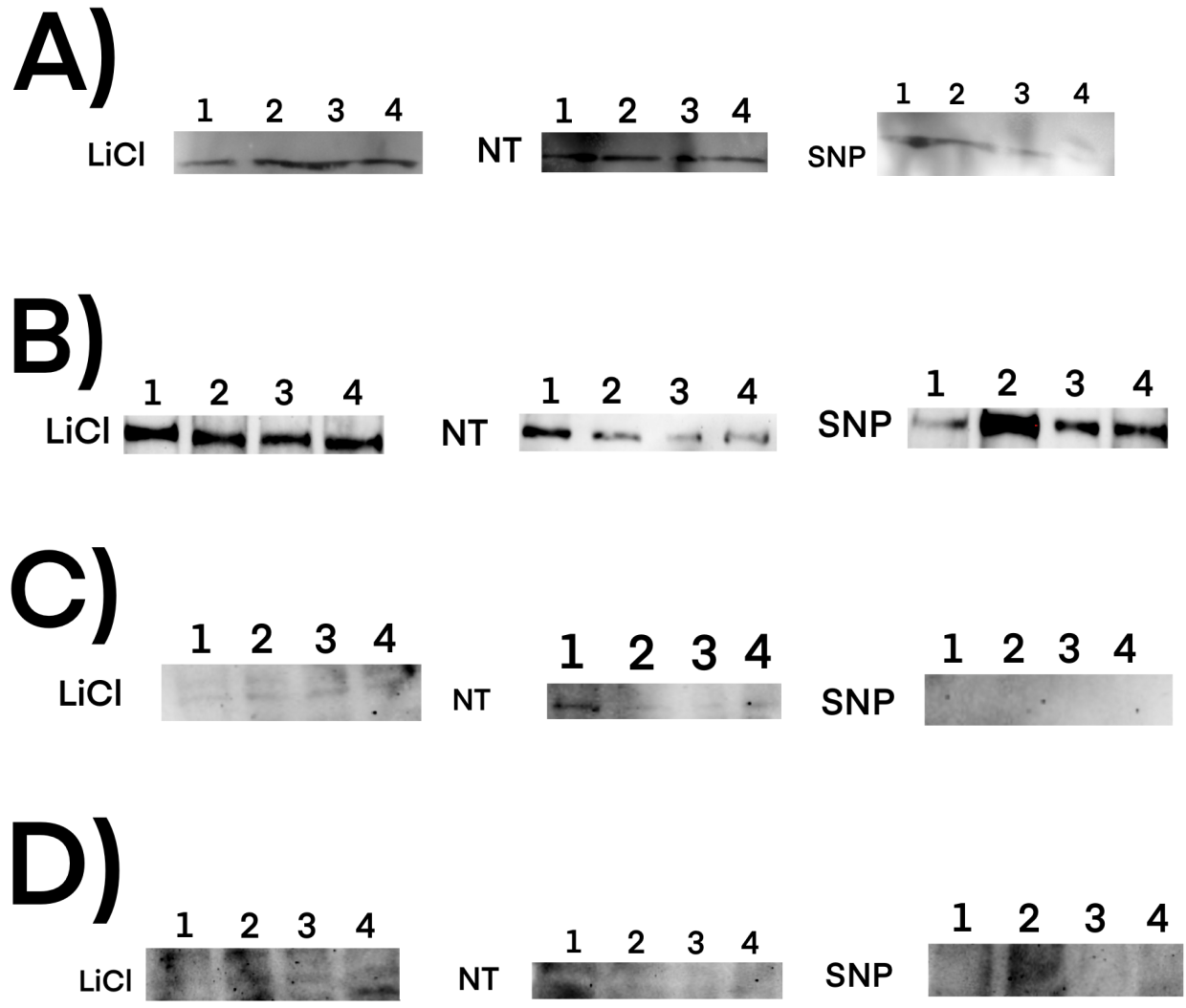
The blots were analyzed using ImageJ. ImageJ is an inexpensive image analysis software designed at the National Institutes of Health that can be used to statistically compare densitometry on Western blot images (Gallo-Oller et al., 2018). Each Western blot image was

transformed into an 8-bit image and inverted prior to analysis. Bands were individually selected and circumscribed with the rectangular region of interest selection. Data was obtained as arbitrary area and mean values, which were multiplied together to find the quantification of the band.

Results

Figure 1

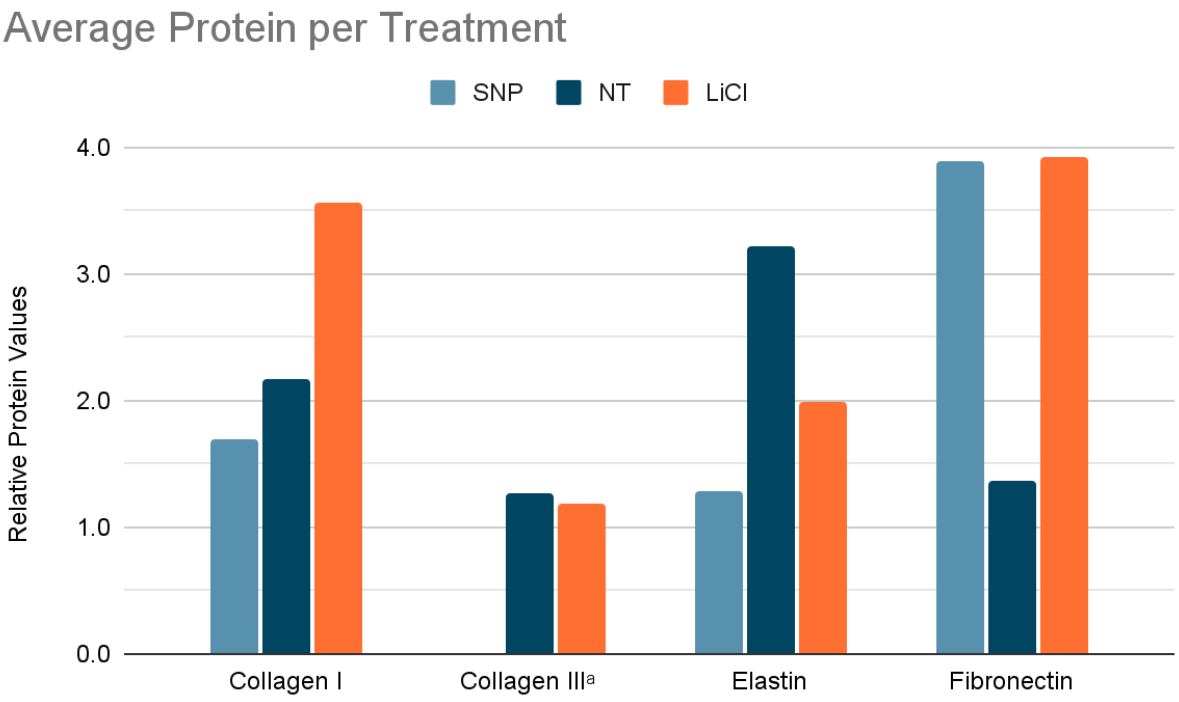
Western Blot Analysis of the Effect of Lithium Chloride (LiCl), No Treatment (NT), and Sodium Nitroprusside (SNP) on the Expression of Extracellular Matrix in Cultured Keloid Fibroblasts



Note. A) Denatured Collagen I (30 KD) B) Fibronectin (250 KD) C) Pro- and Pre-Collagen III (130 KD) D) Elastin (70 KD)

Figure 2

Average Collagen I, Pro- and Pre-Collagen III, Elastin, and Fibronectin levels in cultured keloid fibroblasts treated with Lithium Chloride (LiCl), No Treatment (NT), and Sodium Nitroprusside (SNP) quantified with ImageJ



[a]As the Collagen III levels in the SNP treated samples were faint and difficult to quantify, the average Collagen III level for the SNP treated samples was omitted.

As shown in Figure 1 A, C, and D, Western blot analysis revealed that sodium nitroprusside (SNP) treatment of cultured keloid fibroblasts resulted in decreased levels of collagen type I, collagen type III, and elastin. Collagen I levels in the SNP treated cells decreased around 22% over the negative control and 52% over the lithium chloride (LiCl) treated samples.

Collagen III levels were faint and difficult to quantify, but noticeably less than the negative control and LiCl samples. Elastin levels decreased 60% over the negative control and 35% over the LiCl samples. Fibronectin levels in the SNP treated cells increased 185% over the negative control and decreased 0.7% over the LiCl samples. Tables of the protein quantification are provided in the appendix. These results support the hypothesis that SNP reverses the phosphorylation of glycogen synthase kinase-3β (GSK-3β), activating the kinase and allowing it to phorphorylate β-catenin for degradation, therefore inhibitng the Wnt signaling pathway and decreasing extracellular matrix deposition in comparison to lithium treated and non-treated keloid fibroblasts. In contrast to the original hypothesis, fibronectin protein levels were found to increase with SNP when compared to protein levels from non-treated keloid fibroblasts. This could be explained by fibronectin's role in cell adhesion and contraction in wound healing, which was also seen in lung fibroblasts exposed to SNP (Liu et al., 2000). Further research is needed to fully identify activated GSK-3β as a therapeutic target for keloid scar patients and individuals who are prone to keloid scarring.

Discussion

This project focuses on determining if activating glycogen synthase kinaes-3β (GSK-3β) will inhibit keloid formation by decreasing the collagen, elastin, and fibronectin levels through treatment with sodium nitroprusside (SNP). The results of this study suggest that GSK-3β is partially inactive in keloid fibroblasts and its activation promotes the Wnt signaling pathway and decreases collagen and elastin production when compared to lithium treated and non-treated keloid fibroblasts. As the treatment of SNP effectively decreased collagen and elastin levels

without affecting the viability of the cells, it has demonstrated potential to be a safe and efficient means to prevent keloid formation.

Contrary to the hypothesis, fibronectin levels were found to increase with treatment. This may partly be explained by its role in cell adhesion and contraction in wound healing. In a study by Liu et al. (2000), SNP was found to promote collagen gel contraction in human fetal lung fibroblasts, possibly acting through an increase in fibronectin levels and decrease in PGE_2 levels. In contrast, other nitric oxide compounds were found to have no effect on fibroblast-mediated collagen contraction, suggesting this to be a unique property of SNP that does not act through the NO-cGMP pathway on fibroblast-mediated contraction. In wound healing, fibronectin promotes cellular responses such as cellular attachment and interaction with the extracellular matrix through integrin specific binding (Chester & Brown, 2017). Wound contraction in particular is partly instituted by the tight cell adhesions formed between myofibroblasts and the fibronectin in the granulation tissue, in addition to fibroblasts attaching to collagen fibrils and migrating to exert traction forces on the collagen lattice (Grinnel, 1994; Li & Wang, 2011). Exactly how SNP augments fibroblast-mediated gel contraction is not yet fully understood and the mechanism through which fibronectin levels increase with SNP is a possible point for future research. It is likely that increased fibronectin unfolding and binding to different integrins which can stimulate other cellular processes in conjunction with prolonged fibroblast activity through the Wnt/β-catenin pathway contributes to keloid scar formation, and that overproduction of the extracellular matrix and abnormal wound contraction should be targeted to effectively prevent and treat these scars (Chester & Brown, 2017).

Future Directions

This experiment could be expanded upon by testing the effects of different treatment concentrations on the resulting unphosphorylated levels of glycogen synthase kinase-3β (GSK-3β) to determine a more precise effect of sodium nitroprusside's ability to inhibit the Wnt/β-catenin pathway through GSK. One of the major limitations of this experiment was that the ratio of unphosphorylated to phosphorylated GSK-3β was not measured before and after the treatments, so it is unclear if the treatments worked directly through the Wnt/β-catenin pathway. Repeating this experiment with the addition of testing multiple concentrations of each treatment and identifying the resulting levels of unphosphorylated and phosphorylated GSK-3β would help confirm the decrease in extracellular matrix occurred through the Wnt/β-catenin pathway, which could promote the investigation and discovery of more potential treatment targets for keloid scarring.

The mechanism by which sodium nitroprusside (SNP) increases fibronectin production could be another point of further research. As seen in this experiment, the addition of SNP alone increased fibronectin production instead of decreasing it as hypothesized. It is likely that SNP interacts with fibronectin through multiple pathways other than the Wnt/β-catenin pathway for replacing the extracellular matrix following wounding. This could also lead into further studies on the interaction between extracellular matrix production and wound contraction during wound healing which could give further insight into the mechanism behind keloid formation.

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References

Bock, O., Schmid-Ott, G., Malewski, P., & Mrowietz, U. (2006). Quality of life patients with keloid and hypertrophic scarring. *Archives of Dermatological Research, 297*(10), 433-438.

Bollag, D. M., Rozycki, M. D., & Edelstein, S. J. (1996). *Protein Methods*. Wiley.

Brown, B. C., McKenna, S. P., Siddhi, K., McGrouther, D. A., & Bayat, A. (2008). The hidden cost of skin scars: Quality of life after skin scarring. *Journal of Plastic, Reconstructive & Aesthetic Surgery, 61*(9), 1049-1058.

Chaudet, K. M., Goyal, A., Veprauskas, K. R., & Nazarian, R. M. (2020). Wnt signaling pathway in scar, hypertrophic scar, and keloid: Evidence for a continuum? *The American Journal of Dermatopathology, Publish Ahead of Print*.

Chester, D., & Brown, A. C. (2017). The role of biophysical properties of provisional matrix proteins in wound repair. *Matrix Biology, 60-61*, 124-140.

Gallo-Oller, G., Ordoñez, R., & Doctor, J. (2018). A new background subtraction method for western blot densitometry band quantification through image analysis software. *Journal of Immunological Methods, 457,* 1-5.

Grinnel, F. (1994). Fibroblasts, myofibroblasts, and wound contraction. *The Journal of Cell Biology, 124*(4), 401-404.

Houschyar, K. S., Duscher, D., Rein, S., Maan, Z. N., Chelliah, M. P., Cha, J. Y., Weissenberg, K., & Siemers, F. (2019). Wnt signaling during cutaneous wound healing. In *Regenerative Medicine and Plastic Surgery* (pp. 147–155). Springer International Publishing.

Kurgan, N., Whitley, K. C., Maddalena, L. A., Moradi, F., Stoikos, J., Hamstra, S. I., Rubie, E. A., Kumar, M., Roy, B. D., Woodgett., J. R., Stuart, J. A., & Fajardo, V. A. (2019). A low-therapeutic dose of lithium inhibits GSK3 and enhances myoblast fusion in C2C12 cells. *Cells, 8*(11), 1340.

Li, B., & Wang, J. H. (2011). Fibroblasts and myofibroblasts in wound healing: Force generation and measurement. *Journal of Tissue Viability, 20*(4), 108-120.

Liu, X. D., Skold, C. M., Umino, T., Spurzem, J. R., Romberger D. J., & Rennard, S. I. (2000). Sodium nitroprusside augments human lung fibroblast collagen gel contraction independently of NO-cGMP pathway. *American Journal of Physiology. Lung Cellular and Molecular Physiology, 22*(5), L1032-L1038.

Mariappan, M. M., Prasad, S., D'Silva, K., Cedillo, E., Sataranatarajan, K., Barnes, J. L., Choudhury, G. G., & Kasinath, B. S. (2014). Activation of glycogen synthase kinase 3β ameliorates diabetes-induced kidney injury. *Journal of Biological Chemistry, 289*(51), 35363-35375.

Mundy, L. R., Miller, H. C., Klassen, A. F., Cano, S. J., & Pusic, A. L. (2016). Patient-reported outcome instruments for surgical and traumatic scars: A systematic review of their development, content, and psychometric validation. *Aesthetic Plastic Surgery, 40*(5), 792-800.

Nangole, F. W., & Agak, G. W. (2019). Keloid pathophysiology: fibroblast or inflammatory disorders? *JPRAS Open, 22*(44-54).

Lee, S. S., Yosipovitch, G., Chan, Y. H., & Goh, C. L. (2004). Pruritus, pain, and small nerve fiber function in keloids: A controlled study. *Journal of the American Academy of*

Dermatology, 51(6), 1002-1006.

Appendix

Figure 3

BCA Assay standard curve depicting Blank Corrected Absorbance (nm) vs Protein Concentration (µg/mL)

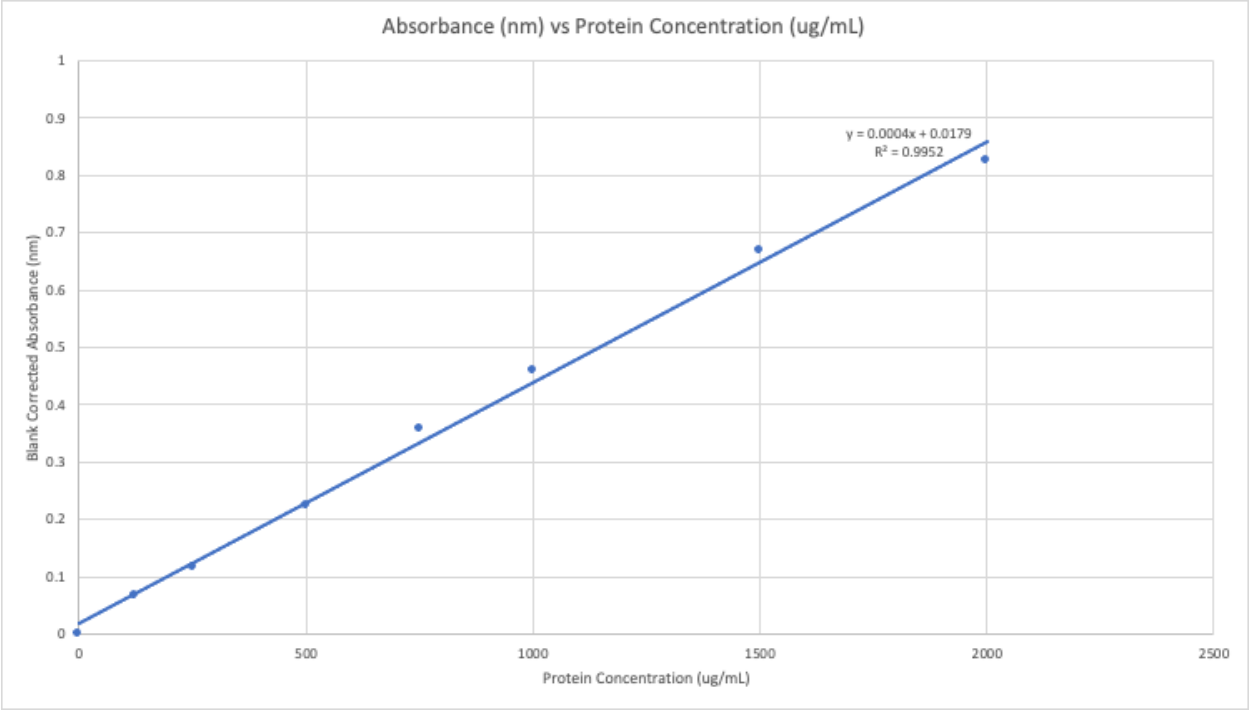

Table 1

Antibody Dilutions used during the Western Blot Analysis

Antibody	Dilution	Amount of Antibody (µL)	Amount of TBS with 2% BSA (mL)
Collagen I	1:1000	5	5
Collagen III	1:500	10	5
Elastin	1:500	10	5
Fibronectin	1:800	5	4

Table 2

Protein Quantification of the effects of No Treatment (NT), Lithium Chloride (LiCl), and Sodium Nitroprusside (SNP) on the expression of collagen I in cultured keloid fibroblasts using ImageJ

Sample	Area	Mean	Calculated Collagen I
NT 1	1455	227.152	330506.16
NT 2	1133	211.807	23934.191
NT 3/4[a]	1639	181.378	297278.542
LiCl 1	1395	132.249	184485.96
LiCl 2/3[a]	3249	177.156	575579.844
LiCl 4	1792	171.090	306593.28
SNP 1/2[a]	2831	138.545	392220.895
SNP 3	861	76.361	65746.821
SNP 4	1564	31.168	48746.752

[a] Some of the bands were difficult to distinguish from each other, so they were analyzed as a single band, i.e. NT 3/4 refers to bands NT 3 and NT 4 quantified as a single band.

Table 3

Protein Quantification of the effects of No Treatment (NT) and Lithium Chloride (LiCl) on the expression of collagen I in cultured keloid fibroblasts using ImageJ

Sample	Area	Mean	Calculated Collagen III
NT 1	1309	184.271	241210.739
NT 2	1157	105.051	121544.007
NT 3	915	60.007	54906.405
NT 4	960	92.771	89060.16

LiCl 1	966	63.818	61648.188
LiCl 2	1242	81.168	100810.656
LiCl 3	1656	86.954	143995.824
LiCl 4	1520	111.670	169738.4

Note. As the Collagen III levels in the SNP treated samples were faint and difficult to quantify, they were omitted.

Table 4

Protein Quantification of the effects of No Treatment (NT), Lithium Chloride (LiCl), and Sodium Nitroprusside (SNP) on the expression of elastin in cultured keloid fibroblasts using ImageJ

Sample	Area	Mean	Calculated Elastin
NT 1	3135	193.352	606158.52
NT 2	2929	119.361	349608.369
NT 3	1680	96.189	161597.52
NT 4	1742	98.541	171658.422
LiCl 1	1188	130.636	155195.568
LiCl 2	1324	181.615	240458.26
LiCl 3	1266	138.107	174843.462
LiCl 4	1434	155.665	223223.61
SNP 1	1530	153.968	235571.04
SNP 2	1001	154.409	154563.409
SNP 3	863	94.801	81813.263
SNP 4	375	118.669	44500.875

Table 5

Protein Quantification of the effects of No Treatment (NT), Lithium Chloride (LiCl), and Sodium Nitroprusside (SNP) on the expression of fibronectin in cultured keloid fibroblasts using ImageJ

Sample	Area	Mean	Calculated Fibronectin
NT 1	1610	164.850	265408.05
NT 2	1190	96.055	114305.45
NT 3	672	82.091	55165.152
NT 4	1323	83.896	110994.408
LiCl 1	2184	199.479	435662.136
LiCl 2	2002	199.036	398470.072
LiCl 3	1584	192.177	304408.368
LiCl 4	2241	191.606	429389.046
SNP 1	1580	102.061	161256.38
SNP 2	3784	200.110	757216.24
SNP 3	1472	172.318	253652.096
SNP 4	2156	178.847	385594.132