Development of a Collagen Assay and testing pro- and antifibrotic compounds on fibroblasts in vitro

MASTER PROJECT

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Abstract

Collagen is the most abundant protein found in mammals, making up to 30% of the total protein content. Disruption of collagen homeostasis can lead to diseases like liver fibrosis and cirrhosis. Due to the unique molecular structure of collagen, various assays have been developed to measure this protein. In this study, three assays will be compared to each other to determine the optimal assay for measuring collagen in NIH-3T3 fibroblasts. The three assays; Sirius Red assay, hydroxyproline assay and DPHAA assay each use a different characteristic to produce a signal from collagen. For these assays the positive control will be TGFβ. Additionally, the effect of IFNγ (a cytokine) and two natural compounds harmine and honokiol will be tested on the gene expression of fibrosis markers COL1A1, Fibronectin 1 (FN1) and α smooth muscle actin (αSMA). After which the IFNγ will be tested in combination with the three assays.

The findings suggest that the Sirius Red assay is not suitable for collagen measurement in cell culture due to the fact that the dye also binds to the serum proteins. The hydroxyproline assay is not suitable because it needs large quantities of collagen to accurately measure the collagen content, while concentration of collagen in cell culture is significantly lower. The DPHAA shows promising results on measuring collagen in cell culture. Further optimization is needed however to increase the reliability of the assay.

It was found that IFNγ reduces the gene expression of COL1A1 and FN1 but not αSMA in NIH-3T3 cells. Harmine and Honokiol showed a reduction in COL1A1 but further studies have to be made to support this observation.

Introduction

Collagen & Liver fibrosis

Collagen is a protein that can be found in numerous places throughout the body. It plays a crucial role in cell structure and is a fundamental component of the extracellular matrix. In mammals, up to 30% of the protein content in the body consists of collagen[1]. Disruption in the production and deposition of collagen can lead to various diseases like liver fibrosis and cirrhosis. These diseases cause the liver to lose function and can result in serious health problems over time.[2]

Collagen has a unique molecular structure which is composed of three alpha chains that are in a triple helix configuration. Each chain has a repeating amino acid sequence, the most common are glycine (Gly), proline (Pro) and hydroxyproline (HPro).[3]

Measuring collagen could be a way of monitoring or diagnosing liver fibrosis. Therefore a reliable collagen assay is needed to measure collagen in vitro. Currently, no reproducible easy-to-use collagen assay is available at the current lab for measuring collagen in vitro.

Methods and Assays for collagen measurement

There have been various methods developed to measure collagen in cell culture and liver tissue. The most used one is the Sirius Red assay, which relies on the principle of the binding of a dye exclusively to the fibers in collagen molecules. Making it useful for analyzing structure and distribution in cell culture and tissues.[4] In theory it is also possible to quantify collagen in cell culture using the Sirius Red assay, but modifications have to be made to the standard protocol.[5] Performing the Sirius Red assay is relatively cheap and time efficient.

A different method of quantifying collagen is through the hydroxyproline assay. In principle, this assay works by using the fact that hydroxyproline is quite unique to the collagen molecule and thus would make for a specific marker of collagen. By first hydrolyzing the collagen molecule, the individual amino acids are available for reaction. The hydroxyproline is then oxidized and later reacted to a chromophore (D in Figure 1) which can be measured with a plate reader (Figure 1).[6]

In more recent years, a method has been developed where a fluorophore can be formed from collagen molecules. This method uses the N-terminal Gly-containing peptides to form fluorophores through a chemical reaction. Since a single collagen molecule contains numerous Gly-containing peptides in its alpha helix, the fluorescent signal has the ability to greatly increase[7]. For an overview of the assay see Figure 2.

Because collagen can be closely related to fibrotic diseases, a selective, easy-to-run, high throughput assay is essential for testing future therapies and compounds on their effects.

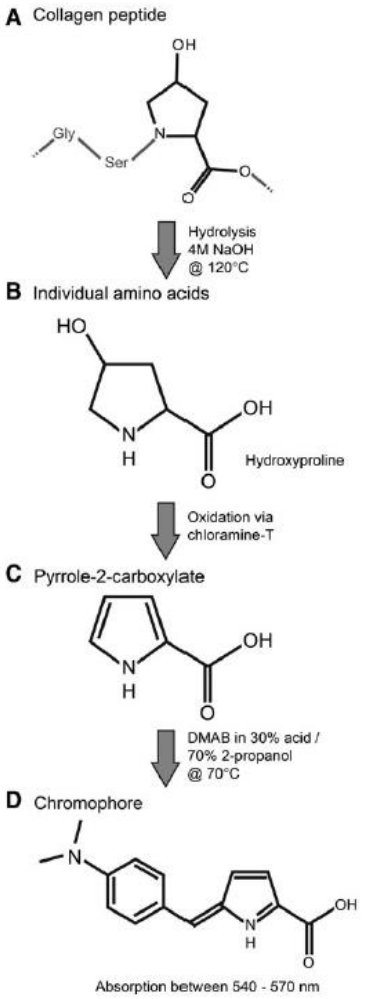

Figure 1. This figure shows the principle of the hydroxyproline assay.[6]

In this study, the collagen assays will be tested with TGFβ as a positive control. Literature suggests that the cytokines IFNγ and IL-10 have antifibrotic properties[8]. In this study, Interferon-γ (IFNγ) is used to possibly reduce collagen production in cell culture. Literature also states

that the compounds harmine and honokiol possibly contain antifibrotic properties. Therefore these compounds will also be tested with the different collagen assays.

Properties of Interferon-γ

Interferon-γ (IFNγ) is known to have opposing effects on cells in the liver. By stimulating macrophages to produce TNFα and interleukin-12 immune cells are activated, protecting the liver from infection. Stimulated macrophages can also kill phagocytosed microbes and cancer cells. Furthermore, IFNγ causes hepatic macrophages to be in an inflammatory activation state, which causes the production of proinflammatory factors. These factors can activate the hepatic stellate cell (HSC) to become profibrotic fibroblasts.[9] Contrarily, IFNγ can exert a direct antifibrotic effect on activated HSCs. Increased levels of IFNγ is supported by increased induction of apoptosis and cell cycle arrest, through the TNF-related apoptosis inducing ligand (TRAIL) expression in Natural Killer cells (NK cells). Additionally, IFNγ has been shown to reduce α smooth muscle actin (α SMA), collagen levels and HSC proliferation in fibroblasts[8–13].

Properties of Harmine and Honokiol

Harmine is a β-carbonyl alkaloid that occurs in a variety of plants, like the Syrian rue and the *Banisteriopsis caapi*. Due to its strong monoamine oxidase A inhibition (MOA-A), Harmine has been a suggested antidepressant and possibly also an effective antipsychotic.[14,15] It has recently been discovered that Harmine behaves as a DYRK1A-specific inhibitor. Dual-specificity tyrosine-regulated kinases (DYRKs) like DYRK1A, DYRK1B and DYRK2 have recently been shown to have involvement with the development of liver cancer.[16] Specifically DYRK1A has been reported to be involved with fibrosis[17]

Honokiol can be found in the bark, leaves and cones from trees belonging to the *Magnolia* genus. It has been reported that Honokiol induces apoptosis of HSCs in rats.[18] Research suggests that Honokiol is also an inhibitor of the TGFβ/SMAD pathway. TGFβ can induce liver fibrosis through binding and phosphorylation of the TGFβ type 1 receptor. This induces the phosphorylation of SMAD proteins, particularly SMAD3. The presence of SMAD3 during hepatic stellate cells (HSCs) activation causes the promotion of collagen type I and III gene expression. The extracellular matrix consists primarily of collagen type I and fibronectin.[19]

Retinol and platelet-derived growth factor (PDGF)

In this study, it was attempted to grow NIH-3T3 cells in 0% serum conditions to avoid background signal for the Sirius Red assay. Literature suggests that the only survival factors for NIH-3T3 cells are retinol (vitamin A) and PDGF[22].

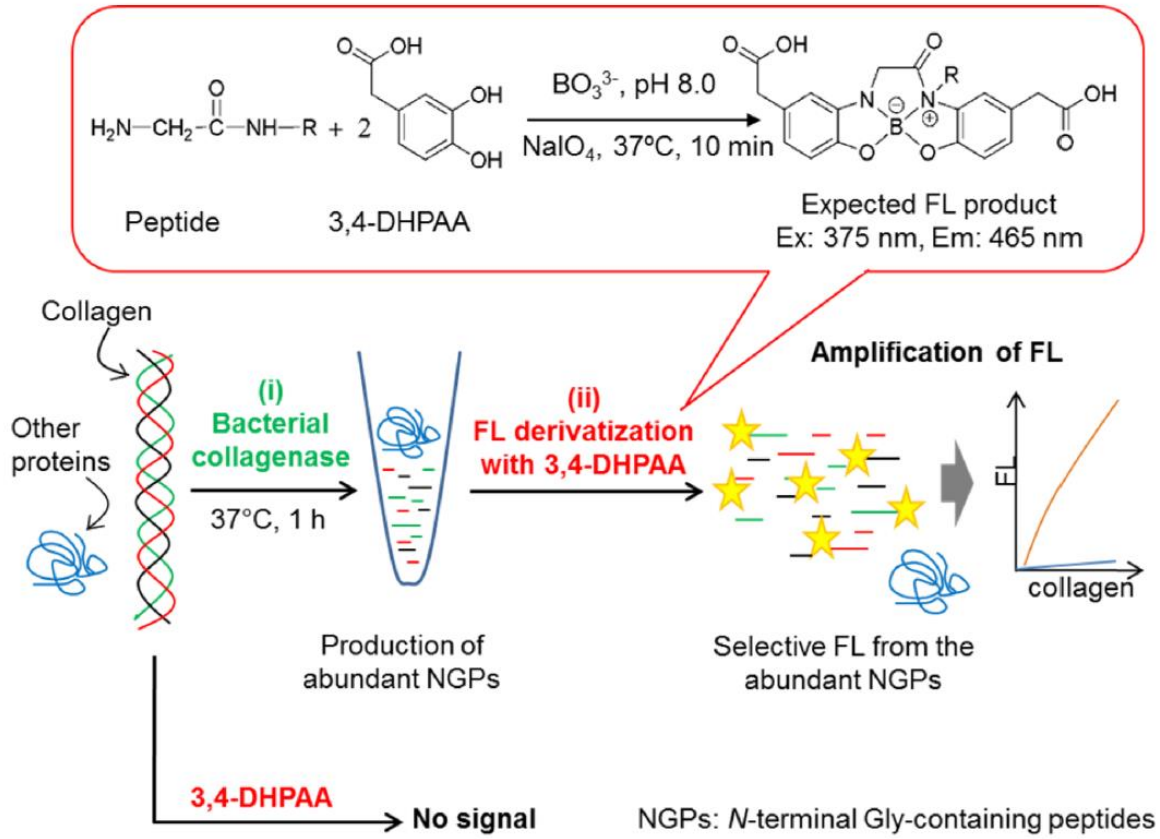

Figure 2. This figure shows the principle and chemical reactions of the 3,4-dihydroxyphenylacetic acid (3,4 DPHAA) assay. FL: Fluorescence[7].

Aims of the study

This study has the key aim of developing a collagen assay with TGFβ as a positive control. The tested assays are Sirius Red assay, Hydroxyproline assay and 3,4-DPHAA assay.

Subsequently, the antifibrotic properties of IFNγ, harmine and honokiol will be tested on these assays by measuring a possible decrease in collagen production.

Additionally, the antifibrotic effect of IFNγ is examined further by seeing if the effect is caused by a reduction in collagen production.

Materials & Methods

Cell culture

NIH-3T3 fibroblasts that were obtained from the American Type Culture Collection were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 10% Fetal Bovine Serum and penicillin/streptomycin. The cells were incubated (starting at $5*10^3$ cells/cm^2) in a T-75 flask and grown till 80-90% confluence before detaching them using trypsin. The cells were incubated at 37°C with 5% CO_2.

Collagen estimation in NIH-3T3 fibroblasts

Sirius Red Assay

First 3T3 cells were seeded in a 24-wells plate with a density of 15000 cells/well. These cells were incubated for 24h. Then the medium was removed and replaced with medium containing either stimulants or normal medium without stimulants.

In later experiments, after 24h incubation in normal media, de media is changed to DMEM without serum but with the addition of retinol (vitamin A, 2µM) and PDGF (50ng/ml).

After the replacement of media, the cells were incubated for 24h, 48h or 72h.

For extracellular collagen estimation, 500µL supernatant was collected. 200µL 1% Sirius Red was added to the supernatant. This was then incubated for 1-2h at room temperature. The solution was then centrifuged for 15 min at 15000 rpm. The supernatant was discarded and the pellet was washed with 0.1M HCL till the liquid was no longer pink/red. Now dissolve the pellet in 500µL 0.5M NaOH. Of this solution, 200µL (in duplicate) was transferred to a 96-wells plate. Absorbance was measured at 540nm in a plate reader.

For intracellular collagen estimation, all media was removed from the wells. Then the cells were washed twice with 1X PBS. 500µL of Kahle fixate was added to each well (30 mL demi water, 14 mL 96% methanol, 1 mL glacial acetic acid, and 5 mL 37% formaldehyde) to fixate. After 30min incubation at room temperature, the Kahle fixate was removed and 200µL 1% Sirius Red was added to each well. This was incubated for 1-2h at room temperature. The wells were washed with 0.1M HCL till no more pink/red color could be observed from the liquid (normally washing 3 times is enough). 500 µL 0.5M NaOH was added to each well to dissolve the collagen. In order to homogenize the samples, the solution was pipetted up and down in the well and incubated for 30min at room temperature. 200µL (in duplicate) was transferred to a 96-wells plate and the absorbance was measured at 540nm in a plate reader. The concentration of collagen was estimated using the calibration curve which was obtained using the scheme in Table 1

Table 1. This table shows the pipetting scheme for making a calibration curve for the Sirius Red assay to measure collagen.

Concentration collagen (µg/ml)	Volume collagen (stock) (µL)	Volume medium (µL)	Total volume (µL)
1000 (stock)	500	.	500
800	400	100	500
600	300	200	500
400	200	300	500
200	100	400	500
100	50	450	500
50	25	475	500

Hydroxyproline assay

Roughly $3*10^6$ NIH-3T3 cells were collected in a centrifuge tube and spun down for 5 min at 300g. The supernatant was removed and the cells were washed with PBS, after which the cells were spun down again. The cell pellet was then dissolved in 100 µL of 6M HCL.

The dissolved cells were then transferred to a small glass ampule. This ampule was then sealed using high heat with a Bunsen burner. Hydrolyzation of the collagen in the cell suspension was achieved by incubation for roughly 16 hours at 115°C.

After hydrolyzation 5 µL of each sample was transferred to a tube and 50 µL citric acetate buffer (5% citric acid, 7.24% sodium acetate, 3.4% NaOH, 1.2% glacial acetic acid) and 100 µL Chloramine T solution (32 mL Citric Acetate buffer, 4 mL water, 4 mL n-propanol, 564mg Chloramine T) was added. The samples were then incubated at room temperature.

After the incubation 100 µL of Ehrlich's reagent (18.6 mL n-propanol, 7.8 mL 30% HCL, 4.5 g 4-dimethylaminobenzaldehyde) was added to the samples and 200 µL was transferred to a 96-wells plate. The absorption was measured at 550nm in a plate reader. After the measurement, the plate was covered and incubated for 25min at 65°C. After incubation, the absorption was measured again at 550nm in a plate reader. The collagen concentration was calculated using the calibration line made using Table 2

Table 2 This table shows the pipetting scheme used for making the calibration line in the hydroxyproline assay.

Concentration collagen (µg/ml)	Collagen Stock (400ug/ml) (µL)	Water (µL)
0	-	1000
10	25	975
20	50	950
40	100	900
60	150	850
80	200	800
100	250	750
200	500	500

DPHAA assay

First NIH-3T3 cells were seeded onto a 6-wells plate with 250.000, 125.000 and 75.000 cells per well (for 24, 48, 72h incubation time respectively) and incubated for 24h at 37°C. After 24h the media was removed and new media with possible treatment factors was introduced to the cells. The cells were washed with 1XPBS and harvested after 24-72h (until 85-95% confluence) using trypsin and transferred to tubes.

This assay is also suitable for measuring collagen from liver tissue. When using liver tissue: 400 µL of Milli Q water was added to the liver tissue in a tube. Small glass beads were added to this tube. Then the sample was homogenized with a mini bead beater for 2X45sec. These samples were stored on ice until the assay was executed.

The cells were centrifuged for 5min at 300g to form a cell pellet. The supernatant was removed and the cell pellet is stored at -80°C until further use.

The cell pellet was lysed in 350 µL of water. Per sample 200 µL (also for liver tissue samples) was taken to perform the assay. To each sample 25 µL 0.5M sodium borate buffer (20mM $CaCl_2$, pH:7.5), 5 µL water and 20 µL of 0.1 mg/ml (1.0mM) bacterial collagenase was added. The solution was mixed but

not vortexed to ensure enzyme function. The samples were incubated overnight (roughly 20 hours) at 37°C.

To the enzymatic solution, 250 µL of 0.75mM 3,4-DPHAA, 250 µL sodium borate buffer (5mM CaCl2, pH:8.0) and 250 µL 1.25mM $NaIO_4$ was added. The formation of the fluorophore was carried out at 37°C for 10 mins. After the reaction the samples were transferred to a fluorophotometer appropriate 96-wells plate and the fluorescence was measured at 375nm excitation and 465nm emission. The collagen concentration was calculated with a calibration line which consisted of the following points: (Table 3) See *Appendix B* for the full protocol.

Table 3 This table shows the pipetting scheme for the calibration line used in the DPHAA assay for collagen measurement.

Concentration collagen (µg/ml)	Collagen Stock (400ug/ml) (µL)	Water (µL)
0	-	2000
0.5	25	1975
1	50	1950
1.5	75	1925
2	100	1900
2.5	125	1875
3	150	1850
4	200	1800
8	400	1600
16	800	1200

Performing a quantitative polymerase chain reaction: preparation of samples

RNA isolation of cultured NIH-3T3 cells in a 6-wells plate

3T3 cells were seeded in a 6-wells plate at a density of 250.000 cells/well. After 24h incubation at 37°C the cells were stimulated IFN-γ (50 ng/ml) and TGFβ (10 ng/ml) and were then incubated again for 24h at 37°C. After incubation, the wells containing the cells were put on ice and were first washed twice with 1XPBS.

Then 200 µL of Homogenization Solution (HB)(from Maxwell 16 LEV simplyRNA Cells Kit) containing 20µL per 1 mL HB was added to each well. Each well was homogenized by scraping the backside of a 1mL pipet over the bottom till a sludge-like substance was formed. After homogenization, the samples were transferred to a 1.5mL tube (also on ice).

To each tube, 200 µL (pre-chilled) lysis buffer was added and immediately vortexed for 15 sec. After being vortexed the samples were transferred to the Maxwell RNA cartridge. The Maxwell 16 machine was used to purify the samples with the simply RNA© program. After purification, the quantity of the RNA was measured using a Nanodrop spectrophotometer (Thermo Fischer) after which the samples were stored at -80°C.

RNA conversion to cDNA

Before the cDNA conversion, samples were made which contained 0.5ug RNA in 5 µL H2O. Depending on the number of samples, 6 samples were made in duplicate for creating a standard curve. The samples were stored on ice during the preparation of the samples and master mix. To each sample 5 µL of the master mix was added. The quantity of the components in the master mix can be found in Table 4.

Table 4 This table shows the quantity's of the components which make up the master mix for cDNA conversion from RNA.

Component	Amount per sample (µL)
RT Buffer	2
dNTP's mix (40 mM)	0,25
RNasin (20– 40 units/µL)	0,25
Random hexamers (500 µg/mL)	0,5
M-MLV reverse transcriptase (10,000 U/mL)	0,5
RNase-free water	1,5
Total volume	5 µL

After addition of the master mix, the samples were converted using an Eppendorf Master cycler gradient thermocycler using the parameters listed in Table 5.

Table 5 This table shows the time and temperature cycles used to convert RNA to cDNA.

Temperature (°C)	Time (min)
20	10
42	30
20	10
99	5
20	5

Real-time quantitative polymerase chain reaction (qPCR)

After the cDNA conversion, the samples were diluted 10x using RNA-free water. Except for the samples dedicated to the standard curve: these were pooled and a standard curve is made according to Table 6.

The primer (see Table 9) solutions were prepared by adding 20 µL of both the forward (50µM) and reverse (50µM) primer to 60 µL RNA-free H2O. Then the master mix for the PCR was made following the amounts in Table 7.

Then the 384-wells plate was filled with first 2 µL of the samples in duplo. After which 8 µL of Sybr Green master mix was added to each well. The plate was spun down to promote homogenized samples and the PCR reaction was run using a QuantStudio Flex 7 (Thermo Fischer). The PCR protocol can be found in Table 8.

Table 6 This table shows the pipetting scheme to prepare the standard curve for the qPCR.

STD nr	Volume (µL)	Water (µL)
STD 4	All pooled cDNA samples (60 µL)	90
STD 2	60 µL of STD4	60
STD 1	60 µL of STD2	60
STD 0.5	60 µL of STD1	60
STD 0.25	60 µL of STD0.5	60

Table 7 This table shows the components and quantities of the PCR master mix.

Component	Volume per well (µL)
primermix 10 µM	0,3
RNA-free water	2,7
Sybr Green Mix	5

Table 8 This table shows the heat cycles used during the PCR protocol.

Stage	Time	Temperature °C
Activation of Taqman	10 min	95
Amplification (40 cycles)	15 sec	95
	30 sec	60
Melt curve	15 sec	95
	1 min	60
	60°C -> 95°C in 0.05°C/sec	

Table 9 This table shows the gene sequence of the used primers.

Gene	Forward Primer	Reverse Primer
β actin	ATCGTGGCGTGACATCAAAGA	ATGCCACAGGATTCCATACC
COL 1A1	TGACTGGAAGAGCGGAGAGT	ATCCATCGGTCATGCTCTCT
FN1 (fibronectin)	GCGACTCTGACTGGCCTTAC	CCGTGTAAGGGTCAAAGCAT
A SMA	ACTACTGCCGAGCGTGAGAT	CCAATGAAAGATGGCTGGAA

Pierce BCA protein assay

To measure protein content in the liver tissue samples, the Pierce BCA protein assay was used. In 96-wells plate, 5 µL of each standard concentration was pipetted in triplo (see Table 10 for the pipetting scheme). 5 µL of each sample was also pipetted into the plate in triplo. 200 µL of WorkReagens (20 µL of reagent B per 1 mL of reagent A) was added to each well. The plate was covered and incubated for 30min at 37°C. Then the plate was cooled to room temperature and the absorption was measured at 562nm in a plate reader.

Table 10 This table shows the pipetting scheme used for making a calibration line for the BCA protein assay.

Standard (µg/µL) (Stock: 20ug/ml)	BSA (Stock: 20ug/µL)	Water (µL)
10	20 µL of stock	20
8	16 µL of stock	24
6	12 µL of stock	28
4	8 µL of stock	32
3	6 µL of stock	34
2	4 µL of stock	36
1	4 µL of stock	76
0.5	40 µL of 1 µg/µL	40
0.25	40 µL of 0.5 µg/µL	40
0.125	40 µL of 0.25 µg/µL	40

Statistical analysis

The statistical analysis performed in this study has been calculated using GraphPad Prism 9, using the unpaired t-test.

Results & Discussion

Collagen estimation with Sirius Red assay

The first assay to measure collagen with was the Sirius Red assay. For this experiment, the cells were treated with different compounds and cytokines for 24h and 48h to see if collagen concentrations would rise over time. TGFβ acts as a positive control in this experiment. The concentrations of the treatments can be found in the description of Figure 5.

In Figure 5 it can be seen that for the extracellular collagen, most of the treatments have little to no effect on collagen production by the 3T3 cells. IL-10 shows a marginal increase in measured collagen. TNFα shows an increased collagen level of around 20 µg compared to the 10 µg seen with the control, with a significance of $p < 0.0001$. It needs to be mentioned, however, that during the treatment with TNFα, the cells became detached from the bottom of the well. This correlates with the TNFα treatment bar in the intracellular graph, where this bar is greatly reduced compared to control and the other treatments.

The graph for intracellular collagen shows no real changes compared to control apart from the significant decrease with the TNFα group.

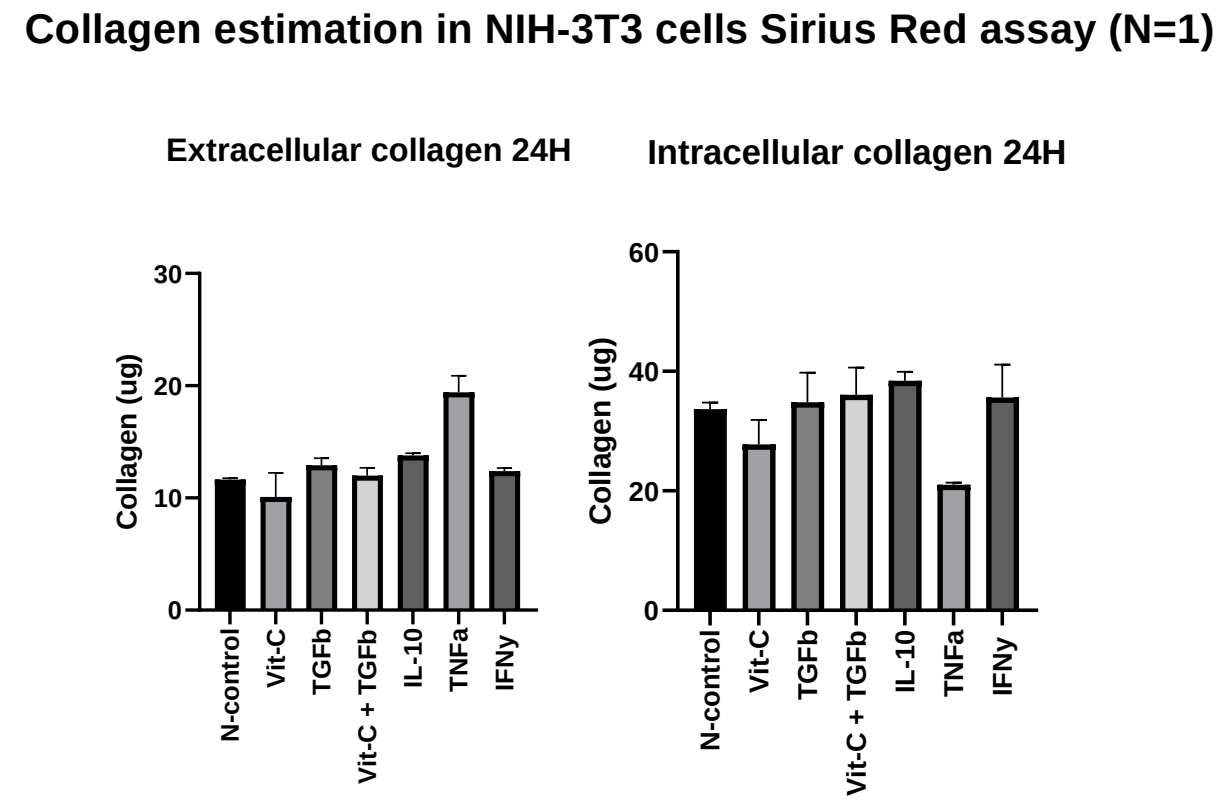

Figure 3. This figure shows 2 graphs showing the measured collagen with the Sirius Red assay. The cells were treated with the following: Vit-C; 0.16mmol, TGFβ; 10 ng/ml, IL-10; 10ng/ml, TNFα; 5ng/ml, IFNγ; 50ng/ml. Note that the experiment has only been performed once. The SD shown is the SD between duplicates.

Figure 6 shows the measured collagen with the different treatments for 48h. Note the overall increase in collagen production for all treatments compared to the 24h mark. The extracellular collagen is only increased for the TNFα and IFNγ groups, both having an increase of around 5ug of collagen, showing a significance of $p < 0.01$. Note that the cells also became detached in this well.

For the intracellular collagen TNFα ($p<0.01$) and IFNγ ($p<0.05$) show a reduction of collagen going from 50 µg for the control to 42-44 ng for TNFα and IFNγ respectively.

Ultimately an MTT assay is necessary to correct for the quantity of living cells to be able to compare individual experiments.

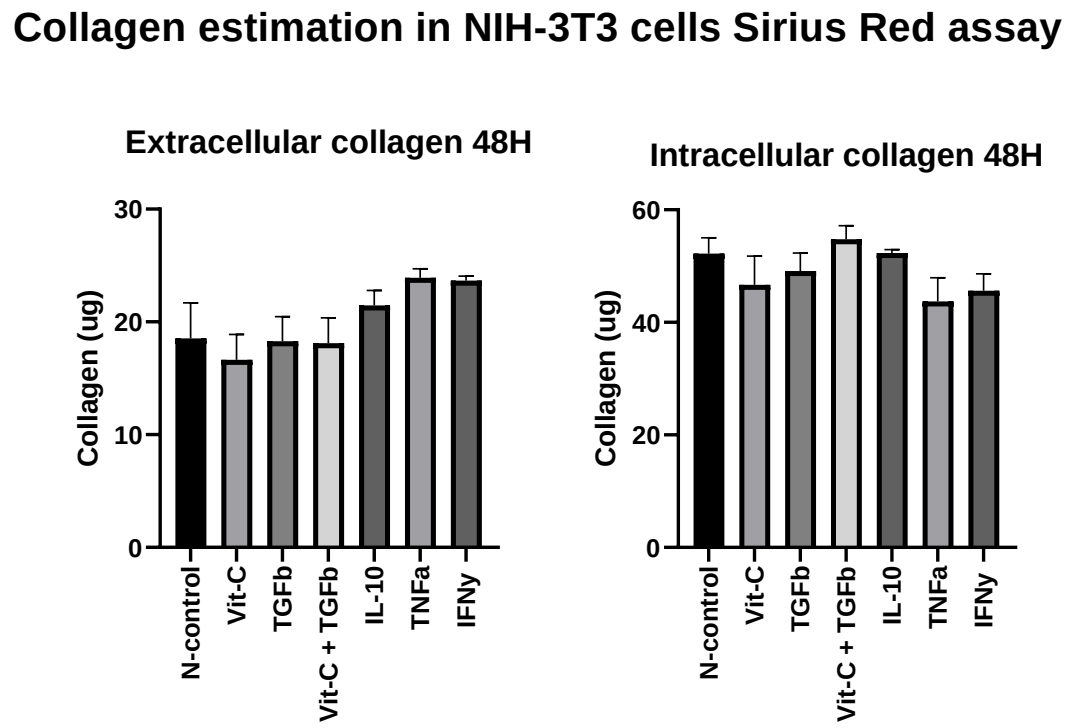

Figure 4. This figure shows 2 graphs showing the measured collagen with the Sirius Red assay. The cells were treated with the following: Vit-C; 0.16mmol, TGFβ; 10 ng/ml, IL-10; 10ng/ml, TNFα; 5ng/ml, IFNγ; 50ng/ml. Note that the experiment has only been performed once. The SD shown is the SD between duplicates.

At first glance, the Sirius Red assay is optimal for measuring collagen in cell culture, but after many experiments it became clear that this is not the case.

In the first round of experiments the used protocol produced samples that gave absorption values that were too high to be measured. It became clear that the red dye might be not collagen specific after all[5], see Figure 7.Figure 7. This figure shows the absorption of different serum concentrations and the signals for diluted samples with serum concentrations. The undiluted samples gave immeasurable absorption values.

The 1:2 dilution serum sample with 0.5% serum already gives a signal of over 1 A. This means that the assay is disturbed by the Fetal Bovine Serum (FBS) in the media.

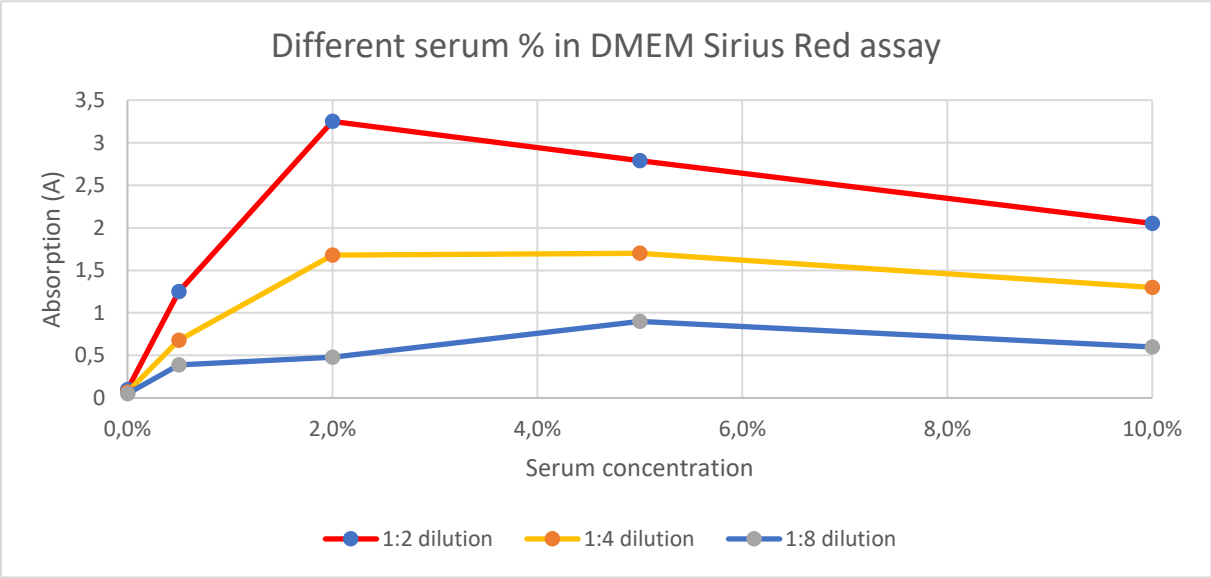

Figure 5. This figure shows the absorption of different serum concentrations and the signals for diluted samples with serum concentrations.

Some literature states that 3T3 cells are capable of surviving in 0% media conditions with the addition of retinol and PDGF[20]. After trying to make cells survive for more than 24h with no serum, the cells looked unhealthy and therefore most likely not representative of healthy NIH-3T3 cells.

The intracellular collagen could still be measured with the Sirius Red assay, but the differences between the negative and positive control are very small (in Figure 5 the positive (TGFβ) and negative control only differ by 1-2 µg collagen). Combined with the difficulties of growing enough cells without them detaching from the well surface, it was decided that the Sirius Red assay was not properly suitable for collagen estimation in 3T3 cells.

Collagen estimation with Hydroxyproline assay.

Next to the Sirius Red-based assay, the hydroxyproline assay is another commonly used assay in literature to determine collagen in (liver) tissues.

The protocol used for the hydroxyproline assay was primarily used for large tissue samples. This experiment was set up to test if the assay could also be used for cell samples. It became clear, however, that the amount of collagen needed to measure a readable absorbance was relatively high for cell culture (10-200 mg/ml). This combined with the fact that 10mg/ml showed a signal of around 0.1A and higher quantities showed a lower signal. (Figure 8)

The reaction with Ehrlich's reagent also did not always react according to literature and came out cloudy and opaque. (See Figure 9) For the absorption values of the calibration line in this picture see *Appendix A.*

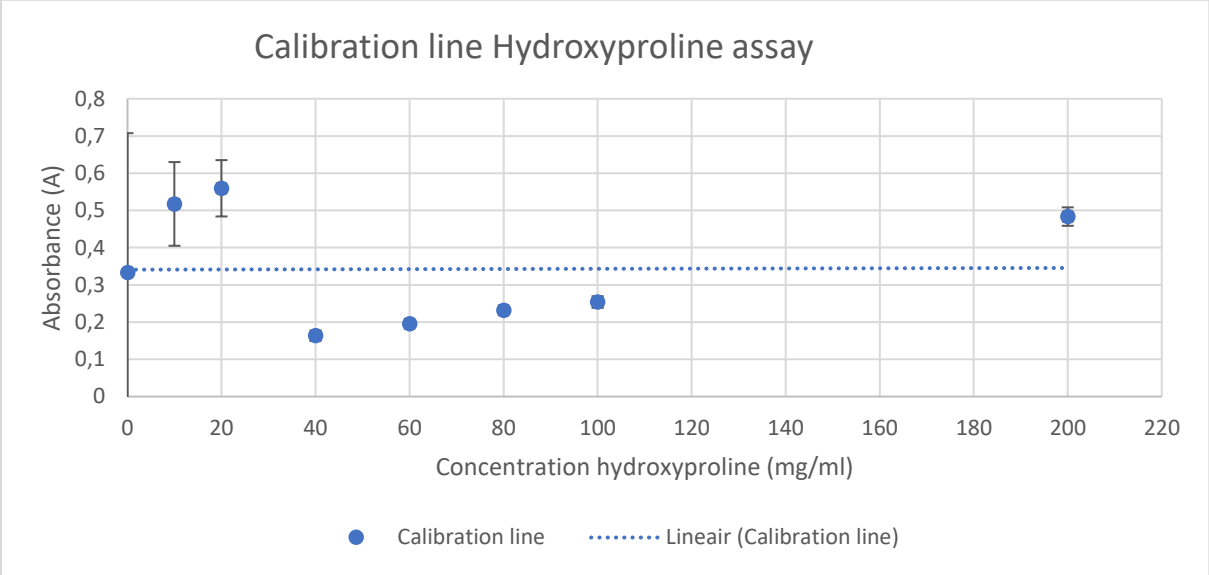

Figure 6. This figure shows the calibration curve made with the hydroxyproline assay.

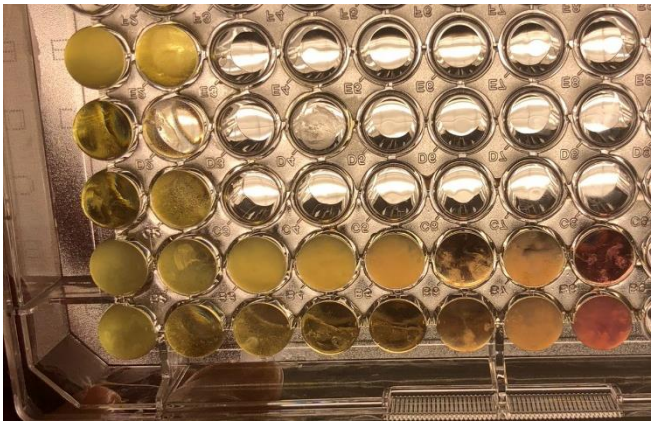

Figure 7. This picture shows the irregularities with the hydroxyproline assay. The bottom 2 rows show the calibration line, where left is 0 mg/ml and right is 200 mg/m the top 3 duplo's show cell samples.

It was decided that the hydroxyproline assay was not suitable for measuring collagen in cell culture due to the irregularities in the outcome of the reaction and the high quantity of sample needed to measure a reproducible signal. Therefore a different assay is required to measure collagen in this study.

Collagen estimation with DPHAA assay

A completely new collagen assay was recently published, this method is based on the creation of a fluorophore complex, which forms after first digestion of collagen and later the addition of a fluorophore.

In this experiment, the collagen was measured using the DPHAA assay. The 3T3 cells were harvested after 24, 48, and 72h incubation time after growing in a 6-wells plate. The number of cells per well is mentioned in the Materials and Method section. Due to some difficulties in obtaining a measurable sample, the samples were pooled. Meaning that instead of having an N=2, the samples were joined together making it N=1. After a failed first run (details of this run can be found in the discussion below the graphs) the material that was left of the 48h and 72h samples were pooled. This experiment was therefore only done once (N=1). The results of this run can be seen in Figure 10. TGFβ acts as a positive control in this experiment.

Figure 10 shows a relative trend from pooled samples with 48h and 72h incubation time. It also shows that the control group had a collagen concentration of roughly 0.6 µg/ml. IFNγ shows a slight decrease in collagen concentration but is not significant. TGFβ shows a slight increase in collagen concentration but again this is not significant. The combination of IFNγ + TGFβ shows an increase in collagen concentration of 0.9 µg/ml compared to the 0.6 µg/ml of the control.

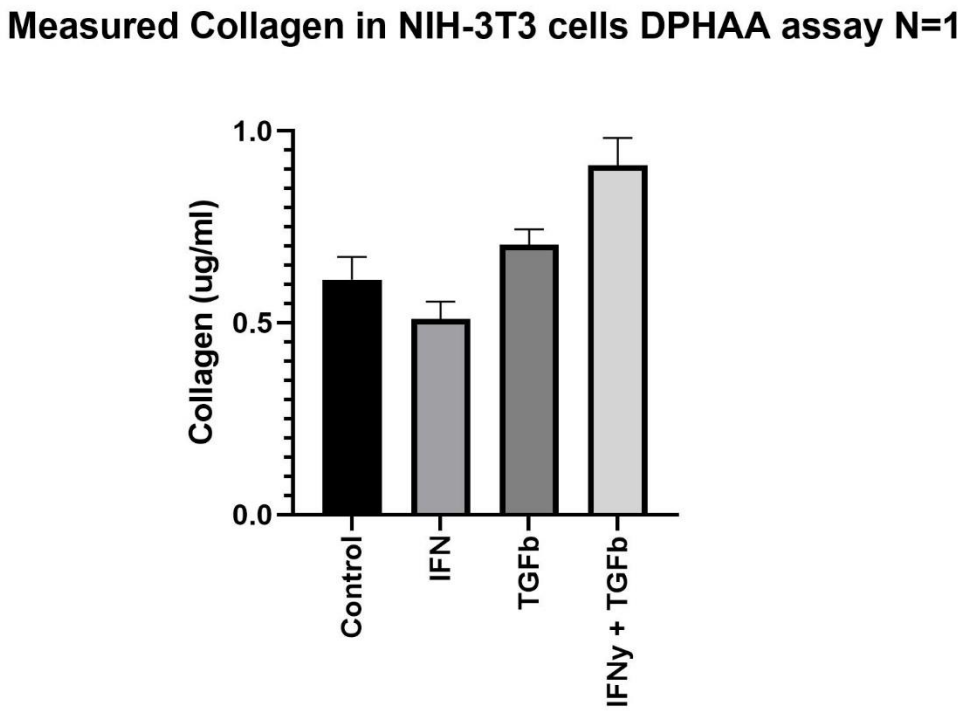

Figure 8. This graph shows the measured collagen with the DPHAA assay in 3T3 cells. The cells were treated with: IFNγ; 50ng/ml, TGFβ; 10ng/ml Note that the experiment has only been performed once. The SD shown is the SD between duplicates.

As stated previously, IFNγ can possibly arrest the cell cycle of 3T3 cells, which would decrease its capability to produce collagen. TGFβ can increase the amount of produced collagen by promoting the gene expression of collagen type I and III through the SMAD signaling pathway. The combination of IFNγ with TGFβ appears to significantly increase the amount of collagen produced by 3T3 cells. Reports have been made of IFNγ capability to reverse TGFβ-stimulated collagen production in subconjunctival fibroblasts[21]. If subconjunctival fibroblasts share similarities with hepatic stellate cells, the observed effect was against expectations. It has to be mentioned that since this experiment has only been performed once, the reproducibility is unknown.

For future studies, it would be interesting to repeat this experiment multiple times, possibly with the addition of Harmine and Honokiol. In future experiments, do not pool the samples from 2 individual experiments, but rather grow twice as many cells per experiment. It is also possible to increase the incubation time with the different treatments. Another essential step in achieving a comprehensive result is performing a protein assay on the cell samples, to quantify the amount of protein.

The 3,4-DPHAA assay is a new method, which needs optimizing to achieve better/readable signal from the samples and calibration lines.

The largest issue with running the DPHAA assay is obtaining enough sample for a measurable signal. Several experiments were performed to understand the time needed for the digestion of the enzyme and to obtain as high a sample concentration as possible while preserving the relatively high throughput and cost-effectiveness of the assay.

One way of increasing the amount of signal is by increasing the digestion time of the enzyme. Figure 11 shows the calibration lines made with the DPHAA assay after 1h and 20h digestion time respectively.

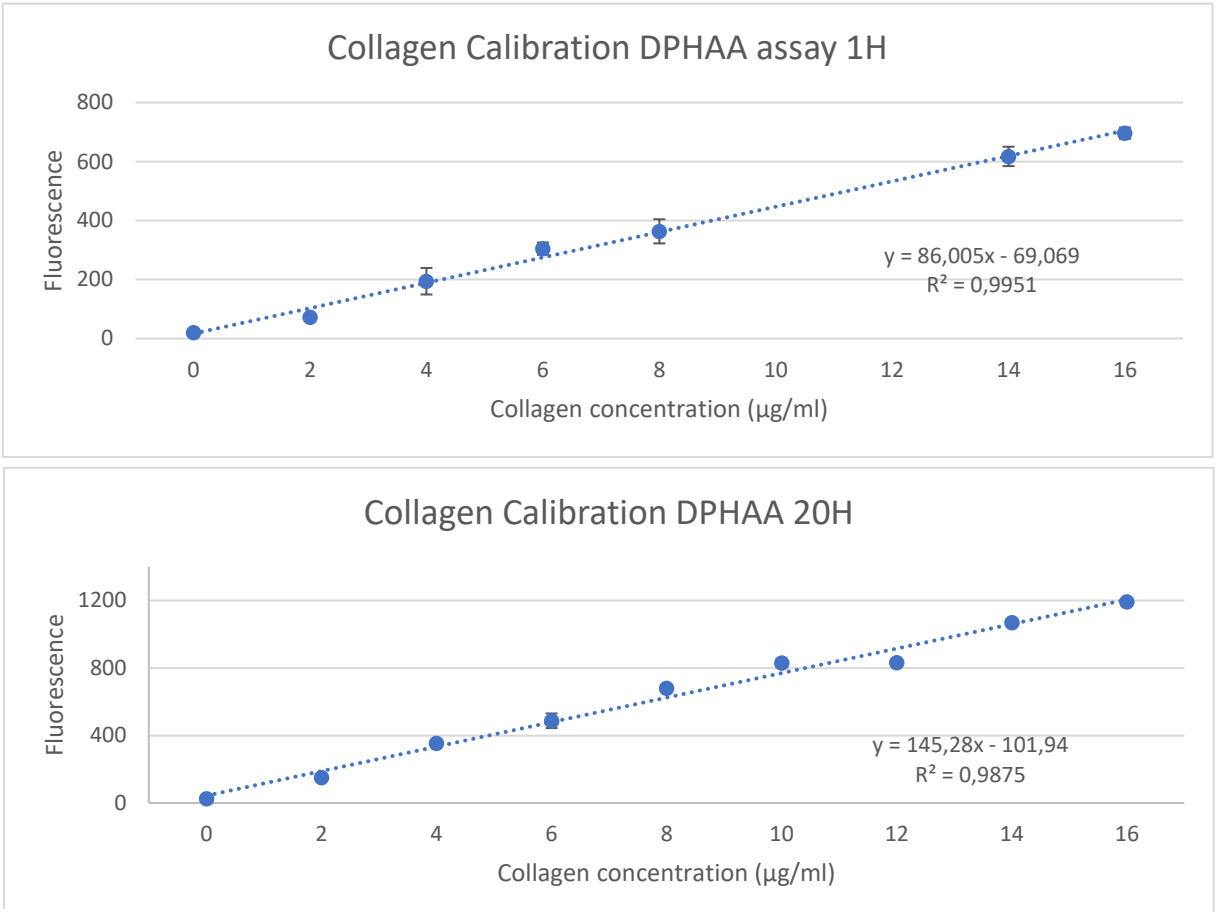

Figure 9. This figure shows the calibration line made with the DPHAA assay after 1H digestion time or 20H digestion time.

Figure 11 shows that after increasing the digestion time to 20H, the fluorescent signal increases. This means that after 1H the collagen in the sample is not completely digested into smaller N-terminal Gly-containing peptides.

It should be noted that after the concentration of the fluorescent agent 3,4-DPHAA was increased 10X (7.5 mM), the reaction was not observed anymore, (giving no readable signal, the calibration line did also not give any signal; n=1).

Since the difficulties of obtaining a measurable signal in the samples persisted, lower concentrations of collagen were introduced to the standard curve. This can be seen in Figure 12, where the points between 0.5 µg/ml and 4 µg/ml are still very well measurable.

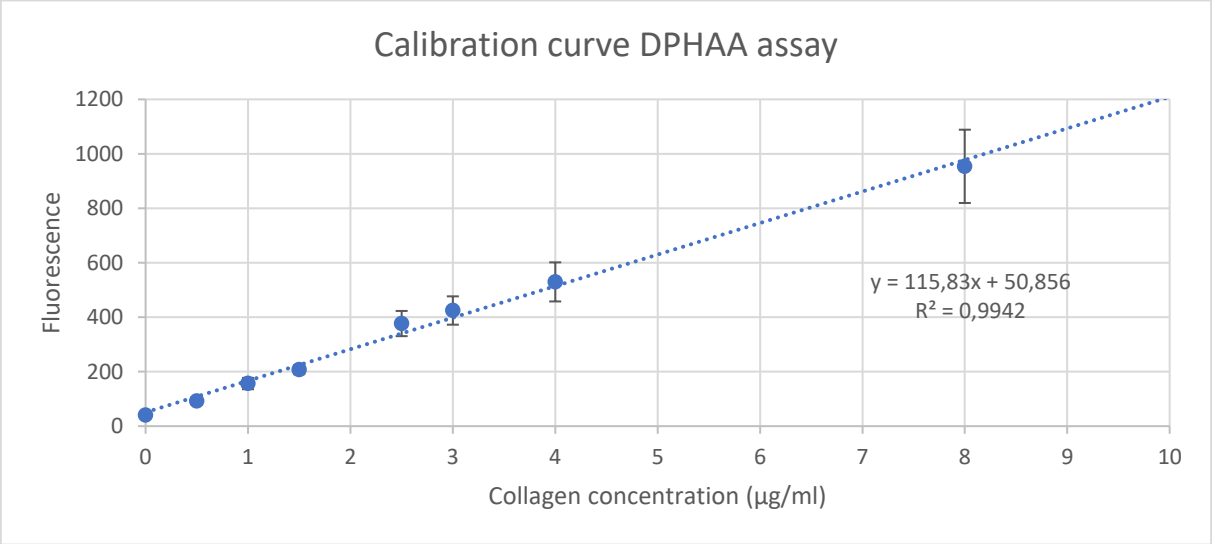

Figure 10. This figure shows the calibration line made with the DPHAA assay after 20H digestion. In this calibration line the lowest detected sample had a concentration of 0.5 µg/ml.

When comparing the Sirius Red assay with the DPHAA assay, it is remarkable that the amount of collagen measured is greatly different. The cells used for the Sirius Red assay are seeded in a 12-wells plate whereas the DPHAA assay requires 6-wells plates to gather sufficient sample for a signal. Despite this difference, the Sirius Red assay measures roughly 20-50 µg of collagen per sample. Where the DPHAA assay measures an end concentration of 0.9 µg/ml, which is diluted from 200 µL to 1 mL; 0.9*5 = 4.5ug/mL collagen. The total sample size is 350 µL, so 0.350*4.5 = 1.58 µg of collagen.

As previously mentioned, a protein assay would have been able to quantify the amount of protein present. This in turn would make it possible to compare the different assays completely. Measuring a total collagen content of around 20-50 µg in relatively few cells (15.000 seeded) shows that the majority of the signal is produced by the proteins present in the serum. Cells need serum to survive and even at low concentrations, it compromises the signal.

Gene expression with real-time quantitative polymerase chain reaction (qPCR)

In this experiment, IFNγ was used to examine its antifibrotic properties as an antifibrotic cytokine. The goal of this experiment is to see if IFNγ, harmine and honokiol reduce the gene expression of genes that are associated with liver fibrosis: COL1A1, FN1 and αSMA.

For this experiment, the gene expression of different genes (COL1A1, FN1 and αSMA) is measured using RT-PCR. The cells were treated with IFNγ, TGFβ, Harmine or Honokiol. The expression is tested after 24h and 48h to determine a difference in gene expression over time.

Figure 3 shows the gene expression of the 3T3 cells after 24h with different treatments. Note that both Harmine and Honokiol have only been tested once (N=1). The same applies to the entire gene expression determination of αSMA. The other samples have been repeated 3 times (N=3). TGFβ acts as a positive control in this experiment.

IFNγ, Harmine and Honokiol show a significant decrease in gene expression for COL1A1 going from around 0.8-1.0 to 1.4 for the control. This decrease has a significance of $p < 0.01$. For the FN1 gene IFNγ also shows a decreased gene expression, with a reduction of around 0.25 AU. Harmine shows an increase in gene expression for FN1 with an 0.3-0.4 increase. Both changes have a significance of $p< 0.05$. In both COL1A1 and FN1, the positive control (TGFβ) shows no difference to the control, showing a possible defect in the used TGFβ, or the 3T3 cells are already producing the maximum amount of collagen.

TGFβ is chosen as a positive control because cytokine is considered to be the most potent fibrogenic.[19] One of the ways TGFβ operates is by binding and phosphorylation of the TGFβ type 1 receptor, which induces the phosphorylation of SMAD proteins, particularly SMAD3. The presence of SMAD3 during hepatic stellate cells (HSCs) activation causes the promotion of collagen type I and III gene expression. The extracellular matrix consists primarily of collagen type I and fibronectin.[19]. An increase in expression for these genes would up-regulate the production of these proteins, creating the fibrotic effect. In this experiment, the positive control is not significantly different from the negative control (for COL1A1 and FN1).

The positive control for αSMA does show a significant increase after TGFβ treatment. Harmine shows a large increase of >1.0 compared to control, this has a significance of $p< 0.001$. Here a change in IFNγ gene expression is not seen.

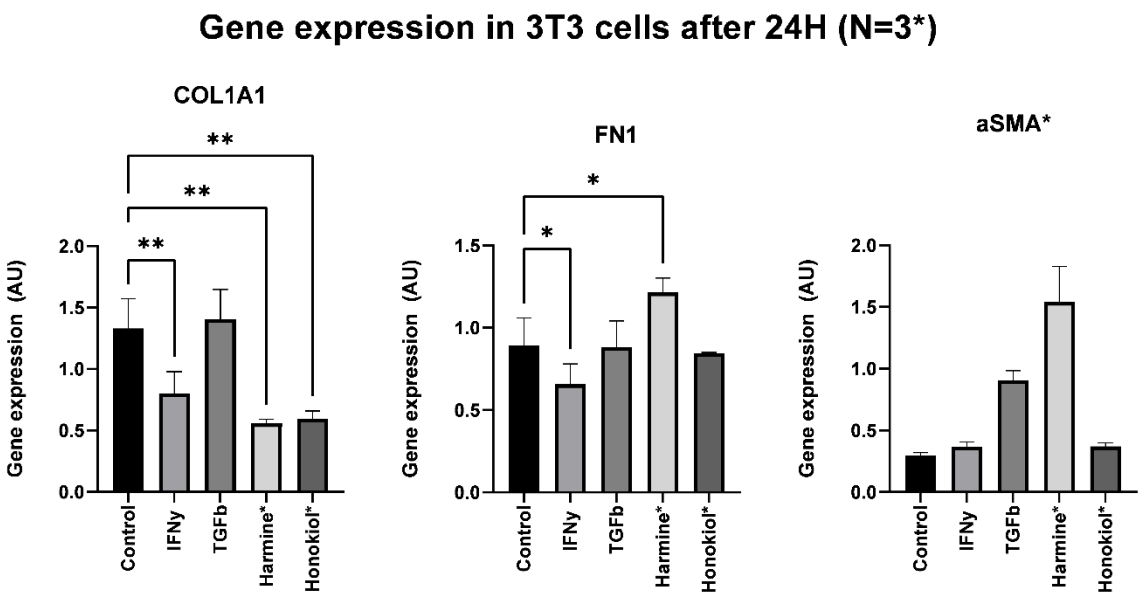

*Figure 11. Shows the relative gene expression after 24h compared to the housekeeping gene β actin, for the same well. The cells were treated with the following: IFNγ; 50ng/ml, TGFβ; 10ng/ml, Harmine; 10μM, Honokiol; 5μM. *, **, ***, **** indicate significant difference compared to control where P < 0.05, P < 0.01, P < 0.001 and P < and P < 0.0001, respectively.*

The positive control for αSMA does show a significant increase in gene expression after TGFβ stimulation. The major difference between the experiment for COL1A1-FN1 and αSMA is that for the first experiment, relatively old TGFβ was used. This means that the protein most likely has had multiple freeze-thaw cycles. This could lead to the unfolding of the protein making it inactive for the receptor. The TGFβ used in the αSMA gene expression determination was brand new, thus showing a good signal compared to the negative control.

After 3T3 cells were treated with IFNγ, it appears that the genes COL1A1 and FN1 are downregulated, COL1A1 being more reduced compared to control than FN1. Literature states that IFNγ can have opposing effects on hepatic cells.[8] When macrophages are present, IFNγ can have an activation effect on them, causing them to produce TNFα and interleukin-12 (IL-12). IFNγ can also induce an inflammatory activation state of hepatic macrophages. In this experiment, however, macrophages are not present, causing IFNγ to have a different effect on the HSCs. Literature states that IFNγ can induce apoptosis and cell cycle arrest when it interacts with HSC's.[8]. Since IFNγ can induce apoptosis and arrest the cell cycle, the viability of the cells might have been affected. For future studies, an MTT assay should be performed to examine the cell viability. The antifibrotic properties of IFNγ could explain the reduction in fibrotic markers after IFNγ treatment.

By adding harmine, the antifibrotic properties of the compound can be tested. Harmine appears to have a reducing effect on the expression of fibrotic marker COL1A1 but a promoting effect on FN1 expression. For αSMA Harmine shows a great increase in gene expression. Literature states that Harmine behaves as a DYRK1A-specific inhibitor. Dual-specificity tyrosine-regulated kinases (DYRKs) like DYRK1A, DYRK1B and DYRK2 have recently been shown to have involvement with the development of liver cancer.[16] Specifically DYRK1A has been reported to be involved with fibrosis[17]. The expectation would then be that Harmine would reduce the gene expression of all genes. This is however not the case. Pathway signaling and the overall function of the DYRK family are still poorly understood. Therefore more information needs to be available before conclusions can be drawn. The lack of information regarding DYRK pathway signaling and the fact that the experiment was only performed

once, gives the results a small significant value. The reduction in COL1A1 expression was also seen in IFNγ and honokiol.

Honokiol shows a reduction in COL1A1 gene expression, this is in line with previous studies[22]. It shows no effect on the other genes, however. This could possibly be explained by the characteristics of Honokiol. Literature states that Honokiol is an inhibitor of the TGFβ/SMAD pathway[22,23]. As previously stated, activation of SMAD proteins promotes the expression of collagen type I and III genes. It could be possible that since the 3T3 cells are already activated, the SMAD pathway was also already activated, causing Honokiol to inhibit the expression of COL1A1. For future studies, it would be interesting to treat cells with a combination of Honokiol and TGFβ to see if the effect remains the same.

The experiments with αSMA, Harmine and Honokiol do need to be repeated at least 2 more times to show a reproducible effect.

Figure 4 shows the gene expression of COL1A1 and FN1 for 3T3 cells treated with IFNγ and TGFβ for 48h. TGFβ acts as a positive control in this experiment. As can be seen in the graphs, no significant change is measured when the cells are treated for 48h. The gene expression for FN1 is almost doubled compared to the gene expression that is seen after 24h treatment, but no differences between the treatments can be observed.

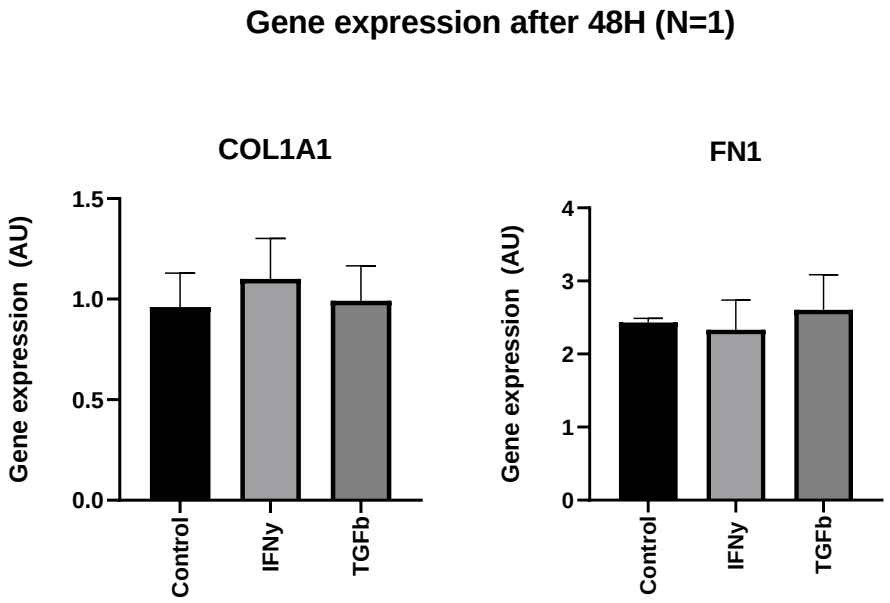

Figure 12. Shows the relative gene expression compared to the housekeeping gene β actin, for the same well. The cells were treated with the following: IFNγ; 50ng/ml, TGFβ; 10ng/ml.

After 48h most of the effect observed in the changes in gene expression is lost, compared to the measurement after 24h. It would be interesting to have multiple time points before and after the first 24h to get a visualization of the rate genes expression is altered.

Conclusion

The Sirius Red assay showed that serum can interfere with the measured signal by binding with the dye. This complies with the literature. The cells need serum to survive and conditions without serum produced unhealthy cells. The dye is not specific enough to collagen and therefore the assay was not suitable for the measurement of collagen in NIH-3T3 cells.

Because of nonlinear calibration lines and inconsistent results after the reaction with Ehrlich's reagent, the Hydroxyproline assay was not used to accurately determine collagen in NIH-3T3 cells. This assay is more suitable to measure collagen in larger pieces of tissue where the quantity of collagen is much higher.

By increasing the digestion time of the DPHAA assay, a well-readable signal is created. After initial optimization of the assay, IFNγ treatment showed a small decrease in collagen production, but not significant. The combination of IFNγ and TGFβ however did show a significant increase in collagen. These experiments need to be repeated to verify this observation. Furthermore, the DPHAA assay is a low-cost, easy-to-run, assay that shows reproducibility through straight calibration lines and readability of samples at low collagen concentrations.

Treating NIH-3T3 cells with IFNγ shows a reduction in fibrogenic gene markers COL1A1 and Fibronectin 1 after 24h. Harmine and Honokiol show a reduction in COL1A1 gene expression, but further experiments need to be conducted to confirm this observation.

IFNγ does not show a significant decrease in collagen production measured with the DPHAA assay. This observation could possibly shine light on the mechanism through which IFNγ exerts its antifibrotic effect in future studies.

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Appendix

Appendix A: hydroxyproline assay raw data

Raw data calibration line hydroxyproline assay

After reaction	mg/ml	0	10	20	40	60	80	100	200
	Absorbance in duplo	0,186	0,086	0,104	0,15	0,182	0,224	0,226	0,503
		0,139	0,133	0,128	0,048	0,046	0,06	0,053	0,047

Table 11. This table shows the raw data of the calibration line after performing a hydroxyproline assay

Appendix B: Protocol for DPHAA assay

DHPAA Collagen Assay Protocol for 3T3 cells

Preparation of reagents:

Bacterial collagenase 0.1mg/ml (1.0µM): Dissolve the bacterial collagenase in 50mM Tris buffer, pH 7.5 with 5mM CaCl2 and store at -20°C until use. Further dilution of enzyme solution is done with the same Tris buffer. Do not vortex!

Sodium Borate buffer (500 mM pH: 7.5): 25 µL per sample

For 10ml: dissolve 0.310g boric acid in 10ml demi water, add 22.2mg of CaCl2 (5mM= 5.55mg/10ml). add 12.5mg NaOH, adjust pH with HCL to 7.5.

Sodium borate (125mM pH 8.0): 250 µL per sample

For 40ml: dissolve 0.310g boric acid in 40ml demi water and add 50mg NaOH, adjust pH to 8.0

Sodium periodate (NaIO4) (1.25mM) in H20: 250 µL per sample

For 40ml: dissolve 10.7mg NaIO4 in 40ml demi water.

3,4-DHPAA (0.75mM) in H20: 250 µL per sample

For 40ml: dissolve 5.05mg DHPAA in 40ml demi water.

Preparing calibration line: Prepare the following calibration line. Store in -20°C until use.

Concentration collagen (µg/ml)	Collagen Stock (400ug/ml) (µL)	Water (µL)
0	-	2000
0.5	25	1975
1	50	1950
1.5	75	1925
2	100	1900
2.5	125	1875
3	150	1850
4	200	1800
8	400	1600
16	800	1200

Preparing the cell samples:

In a 6-wells plate grow 3T3 cells till 80-90% confluence. For each treatment use 2 wells, which will be pooled after incubation (so 1 treatment is 2x2 wells with duplo). See table for cell density for seeding:

Incubation time	Number of cells seeded.
24H	250.000
48H	125.000
72H	75.000

Wash the cells 2x with PBS, add 400 uL trypsin to each well and detach the cells. To each well add 1.6mL media and homogenize the cells. Transfer each well to 2ml tubes. Centrifuge the cells for 5min at 300g to form a pellet and remove supernatant (this pellet can be stored at -80°C).

When performing the assay: pull calibration line and samples from freezer and let it come to room temperature. Lyse the cell samples with 225 uL H2O by pipetting up and down. Once lysed pool the samples (so 2 samples become 1, with duplo so now 1x2 samples).

Enzymatic degradation:

Use 200 µL of the supernatant/homogenized solution per sample. To this sample add:
20 µL of 0.1 mg/ml (1.0 mM) bacterial collagenase,
25 µL 500mM sodium borate buffer (**pH:7.5**) with 20mM CaCl2 and
5 µL H2O.
Total volume = 250uL
Mix solution, do not vortex!
Incubate the samples overnight at 37°C.

Fluorescence detection:

After digestion spin down the samples for 5min at 300g. Collect 200 uL of the supernatant and transfer to a new tube.

To the enzymatic solution (200 µL) add:
200 µL of 0.75mM 3,4-DHPAA
200 µL of 125mM sodium borate (**pH:8.0**)
200 µL of 1.25mM NaIO4.
Vortex solution
Reaction is immediately carried out for 15 min at 37°C.
Fill black fluorescence 96-wells plate and place the pink adapter in the plate reader. The 96-wells plate goes on top of the adapter.
Measure the fluorescence intensity: excitation: 375 nm, emission: 465 nm.

NOTES:
Digestion time and reaction with DPHAA time are not optimized. Digestion might need longer to than 20H. DPHAA might need longer than 15mins.