



Platelet Signalling Dynamics: A Clinico-Relevant Derived Cell Marker in Fibrotic Liver Disease Determination

Amarachi Favour C¹, Igere Bright E^{2,3*} and James Pritchett¹

¹Division of Life Science, Department of Clinical Biochemistry, Biomedical Sciences, Manchester Metropolitan University, Manchester, United Kingdom

²Biotechnology Unit, Department of Microbiology, Delta State University, Abraka, Delta State, Nigeria

³Biotechnology and Emerging Environmental Infectious Pathogens Research Group (BEEIPREG), Biotechnology Unit, Department of Microbiology, Delta State University, Abraka, Delta State, Nigeria



Abstract

Liver Fibrosis has been reported with severity in recent cases as 11th most frequent cause of morbidity and mortality worldwide as evidences associates it with diverse biomarkers and dearth of understanding on the interplay activities. This study investigates how platelets from various donors (with normal BMIs (Body-Mass-Index) and healthy-lifestyles) produce differing influences on fibrotic cells. Blood samples were collected from consented donors, for platelet extraction and used to challenge/treat both immortalised human-hepatic-stellate cell line (LX-2 cells which is a fibrogenic cell line), and mouse Hepatic stellate cells. QuA quantitative approach was used which involved relative quantification method of collected data using SYBR-Green and some known fibrotic target genes in qPCR assay to measure gene expression. Recovered data were analyzed using a one-way ANOVA performed with the appropriate post hoc. Our results revealed increase in gene expression of target specific fibrotic genes when platelets are added to fibrotic cells suggesting platelets momentous role in liver fibrosis in association with HSCs activation. Such highly expressed genes include COL1 (Collagen-1), ACTA2 (Actin alpha-2), CTGF (Connective Tissues Growth Factor), YAP1 (Yes-Associated Protein 1) compared with the reference gene GUSB (Beta-Glucuronidase) used, in both human and mouse hepatic stellate cells. Although recent knowledge has indicated that platelets have beneficial roles in liver fibrosis, it has also been proven that platelets influence hepatic stellate cell activation in liver fibrosis development. Our findings are limited only to apparently healthy non-obese individuals suggesting the need for future studies to include obese, poor health and poor lifestyle individuals.

Keywords: Liver Fibrosis; Hepatic Stellate Cells (HSCs); Platelets; Gene Expression; quantitative Polymerase Chain Reaction (qPCR)

Background

Liver diseases have a significant worldwide annual fatality rate of approximately 2 million. It has been documented that people with chronic liver disease might present with cirrhosis and hepatocellular carcinoma symptoms [1]. These types of liver cancer are often initiated due to liver injury leading to inflammation that is progressive to fibrosis, and ultimately causing cirrhosis which leads to death [2].

Currently, severe liver fibrosis is the 11th most common cause of mortality worldwide. The most common type of chronic liver illnesses is characterized by liver fibrosis, which is the excessive buildup of extracellular matrix proteins, including collagen. Cirrhosis, liver failure, and portal hypertension are side effects of advanced liver fibrosis, which usually warrants liver transplantation [3].

The cell that results hepatic fibrosis appears to be the myofibroblast which are activated during the process. The activated myofibroblast-like phenotype originates from bone marrow precursors and quiescent Hepatic Stellate Cells (HSCs). Oxidative stress, an increase in transforming growth factor beta, hepatocyte mortality, and chronic inflammation is closely linked to hepatic fibrosis. Fibrosis may be reversed if the active myofibroblast is removed.

The progressive accumulation of fibrillar Extra-Cellular Matrix (ECM), is associated with the result of continued liver tissue damage which may be caused by viral infections (hepatitis B and C viruses), toxins/drugs (alcohol), metabolism, and autoimmune cases and finally, the long-term

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*Correspondence:

Igere Bright E, Biotechnology Unit,
Department of Microbiology, Delta State
University, Abraka, Delta State, Nigeria,
E-mail: ibe22002@yahoo.com

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triggering of wound-healing reaction of the liver to chronic diseases. Hepatic failure and clinically obvious liver cirrhosis are the outcomes of the process [4].

Recent literature suggests that a heterogeneous population of cells is linked to an accumulation of ECM after a chronic liver injury which is in contrast to the mechanism of ECM proteins release in liver suffering from fibrosis as previously reported [5]. Myofibroblasts, or liver fibrogenic cells, are now recognized to play a significant role during liver fibrosis. Numerous sources of Myofibroblasts (MFs) have been found *via* significant research into their origin [5]. Hepatic Stellate Cells (HSCs) are now thought to be the main source of MFs since they are the primary ECM-producing cells in any wounded liver. Additionally, bone marrow-derived mesenchymal cells and portal fibroblasts may be the sources of hepatic MFs. The transition of endothelium to mesenchymal and the transition of epithelial to mesenchymal are two additional, if modest, sources of fibrogenic cells [6].

Although it has been shown that liver fibrosis may be reversed, the precise process remains unknown, and liver fibrosis does not currently have an effective treatment. Furthermore, gene therapy has been shown to be an effective anti-fibrosis treatment [7] however, other reports have shown that an underlying organ damage cannot be repaired by gene therapy. Gene therapy may lessen CF symptoms and prevent future damage, but it cannot reverse scarring or other irreversible damage that existed before the treatment [7]. *In vitro* research using the cultivation of primary HSCs that are activated and extracted from rat, mouse, or human liver have provided some knowledge gaps on the cell and molecular biology of hepatic fibrosis. Other ECM-producing cells including the fibroblasts, circulating "fibrocytes" and the myofibroblasts of the portal tract, probably encourages fibrosis of liver. In addition, Liver fibrosis is proposed to be affected by platelets as it may aid the regulation of HSC cells activation. This necessitates studies on the option of applying platelets as treatment strategy in liver fibrosis cases. A few strategies that have recently come to light as important ways to stop the onset and progression of fibrosis of the liver include the use of anti-inflammatory drugs and protection of liver, control of triggering of Hepatic Stellate Cells (HSCs), control of production of ECM, and accelerated breakdown of ECM. The target of this study focusses on HSCs response to platelets derived signals as a way of preventing fibrosis progression. The study hopes to bring to light the contribution of platelets to HSC activation in the fibrosis of the liver. In addition, it hopes to summarize the current knowledge of the relation of platelets with fibrosis of the liver and validate the role of platelets in hepatic fibrosis.

Materials and Methods

The LX2 HSC cells required were provided by Dr. J Pritchett from department of Clinical Biochemistry, Biomedical Sciences, Manchester Metropolitan University, Manchester, United Kingdom (MMU) Tissue Culture Room. The blood samples required for platelet extraction were collected from 3 donors with the help of a phlebotomist, Dr. Amanda Unsworth (MMU) following the previous recommendation of Xu *et al.*, [8].

Preparation of Tyrode's stock

Tyrode's solution was prepared which was frequently used to perfuse and clean tissues in the bodies of animals, and it is particularly effective in isolated constant-muscle tests to keep isolated tissues functioning normally [9]. Firstly 0.4 g glucose was added to 50 ml of

Tyrode's solution and filtered. The filter was then sterilized to prepare Stock 1. Stock 2 was prepared by diluting 500 mM of CaCl_2 in the Stock 1 at the ratio of 1:100 to provide CaCl_2 concentration.

Platelet extraction

The blood for platelet extraction was collected from 3 different donors by certified phlebotomist Dr. Amanda Unsworth (MMU). Ethical approval for the blood collection was requested by the investigators and granted by the Manchester Metropolitan University Research Ethics Committee (MMU-REC).

Platelet Rich Plasma (PRP) was prepared from human whole blood by centrifugation at 100xg for 20 mins at 24°C. Then 150 μl of anticoagulant acid citrate dextrose was added to it. PRP was counted on the SYSMEX device. Centrifuge again at 350*g for 20 mins to collect pellet. The collected pellets were resuspended in Stock 1 and Stock 2 in 2 tubes respectively. The tubes were incubated at 37°C for 30mins, centrifuged at 1400 x g for 10 mins to get the platelet release. This process was done for each assay using blood from each of the 3 donors creating biological repeats [10].

LX-2 cell culture

The LX-2 cell lines are immortalized normal human hepatic stellate cells [8], which were provided by Dr. J Pritchett (MMU), were tested for contamination using Mycoplasma test. Once the results were negative, the LX-2 cells were isolated and cultured as described [11].

The culture media consisted of the Dulbecco's Modified Eagle Medium (DMEM) which is 4.5g/l glucose, sodium pyruvate; penicillin/streptomycin; glutamine and 1% of Fetal Bovine Serum (FBS). The LX-2 cells which were kept suspended in the media and stored in the hydrogen storage. 200 μl of 1% FBS DMEM base media was added along with 2ml of LX-2 cells and was mixed properly. 8ml of FBS and 2.5ml of LX-2 cells in another T75 flask was mixed properly, both incubated overnight, and cells were monitored under the microscope [8].

In addition to this, mouse hepatic stellate cells (mHSCs) were also isolated and cultured as previously described [12]. The mHSCs were subsequently isolated using an improved density gradient centrifugation. These primary mHSCs were treated with Tyrode's buffer with no calcium and platelet releasate from donor 3, for 24 hours in 0% serum by Dr. J Pritchett.

Trypsinization of LX-2 cells and cell count

Culture media was removed from the T75 flask and 4ml trypsin was added to the adhered cells in flask, incubated for 4 minutes while cell scraper was used to lift the cells. A total of 8 ml of media was added to cells, transferred to falcon tube and centrifuged at 1000 rpm for 5 mins.

The cells in suspension were stained with trypan blue to distinguish live and dead cells. Then, the cell counts were made with the help of hemocytometer. Calculation was done to know how many ml of suspended cells per well. The cells were then seeded in 12 well plates with 4 conditions each. The cells were also seeded in 3 wells for one condition, with 50,000 cells per each well. This seeding was all done in 1:1000 dilutions of platelet releasate and serum [12].

RNA isolation

RNA Isolation is a crucial step in qPCR gene expression analysis, it was done to separate single stranded pure RNA from residual proteins

Table 1: RNA concentration and absorbance ratio by nanodrop from donor 1.

Conditions	ng/ μ l	A_{260}/A_{280}	A_{260}/A_{230}
-Ca+G+T	6.7	1.22	0.05
+Ca+G+T	5.7	2.47	0.02
P-Ca	5.7	1.10	0.13
P+Ca	3.7	1.83	0.01

Table 2: RNA concentration and absorbance ratio by nanodrop from donor 2.

Conditions	ng/ μ l	A_{260}/A_{280}	A_{260}/A_{230}
-Ca+G+T	8.3	1.76	0.09
+Ca+G+T	11.4	1.54	0.07
P-Ca	10.7	1.59	0.10
P+Ca	9.5	1.69	0.07

in aqueous solution that might interfere with absorbance reading [13]. For RNA isolation, the cell-culture medium was completely removed from the cell-culture plate. The remaining cells were then rinsed with PBS twice. Then 350 μ l of buffer RLT was added to the cell-culture dish. Buffer RLT is a lysis buffer and used to break down cells for RNA isolation. The remains called the lysate were collected with the help of a cell scraper and transferred into a microcentrifuge. The lysate was mixed by gentle pipetting, while 350 μ l of 70% ethanol solution was added to the homogenized lysate and centrifuged at 8000 x g for 15seconds. The spin column membrane was washed twice using 700 μ l of Buffer RW1, and centrifuged for 15 seconds at 8000 x g. Then the RNeasy spin column membrane was further washed twice with 500 μ l of Buffer RPE and centrifuged for 15 seconds at 8000 x g. RNeasy spin column was placed in a new 2ml collection tube and centrifuged at full speed for 1 minutes to eliminate any possible carry-over of the Buffer RPE (www.Qiagen.com). Then 30 μ l RNase-free water was added to the RNeasy spin column membrane, centrifuged for 1 minute at 8000 x g to elute RNA samples and kept on ice.

DNase treatment

When extracting proteins from prokaryotic organisms, Deoxyribonuclease (DNase) is frequently employed to get rid of DNA contaminants. Cell wall breakdown is frequently involved in protein extraction. It frequently happens that the frail and damaged cell wall is unintentionally lysed, releasing the desirable proteins and unwanted DNA [14]. Specific volume of 1.75 μ l DNase I, and 3.5 μ l of 10X buffer were also added to each sample of RNA extract, incubated for 15mins in room temperature, then 3.5 μ l of stop solution was added and incubated at 70°C pre-heated water bath for 10mins. RNA extract concentration was quantified for purity using a nanodrop (Table 1 and 2).

Table 3: Calculated cDNA preparation table.

Components	-Ca+G+T		+Ca+G+T		P-Ca		P+Ca		Master mix	
Reaction mixers	+RT	-RT	+RT	-RT	+RT	-RT	+RT	-RT	+RT	-RT
2 x rt buffer mix	10 μ l	10 μ l	10 μ l	10 μ l	10 μ l	10 μ l	10 μ l	10 μ l	44 μ l	44
20 x rt enzyme mix	1 μ l	1 μ l	1 μ l	1 μ l	1 μ l	1 μ l	1 μ l	1 μ l	4.4 μ l	4.4H ₂ O
RNA sample	9 μ l	9 μ l	6.5 μ l	6.5 μ l	5.3	5.3 μ l	7.3 μ l	7.3 μ l	0	-
Nuclease free H ₂ O	0	0	2.5	2.5 μ l	3.7 μ l	3.7 μ l	1.7 μ l	1.7 μ l	0	-
Total vol. per reaction	20 μ l	20 μ l	20 μ l	20 μ l	20 μ l	20 μ l	20 μ l	20 μ l	48.4	48.4 μ l

RT: Reverse-Transcriptase enzyme

Making of complementary DNA (cDNA)

cDNA synthesis process is very vital in gene expression experiment using qPCR approach. Reverse Transcriptase (RT) is the enzyme-catalyst which synthesizes the conversion of single RNA template to a double stranded complementary DNA, required for qPCR analysis (Table 3).

The components comprise of reagents used for cDNA making and Master mix of +RT and -RT made for reverse transcriptase and the table includes buffer mix, enzyme mix, the calculated RNA samples for each condition and nuclease free water added were necessary to have equal sample solution per reaction. Referring to the Table 1 to 3 above, four conditions were adopted which includes 'Free calcium plus glucose plus Tyrode's', 'Calcium plus glucose plus Tyrode's', 'Platelet releasate' and 'Platelet releasate plus Calcium'. Calculated volume of reaction of each component and total per reaction were equal -RT and +RT. Master Mix (MM) were made for each condition having a total of 4.4 μ l for -RT and 44 for +RT. 11 μ l of +RT of MM was added to each of the 4 conditions +RT and same for -RT. RNA and dH₂O was add in each sample as seen in Table 1 to 3, to have equal total volume per reaction (20 μ l).

Measurement of the fibrotic gene expression by qPCR

One of the fluorescent dyes most frequently used in qPCR is SYBR Green. It intercalates between the DNA bases and binds double-stranded DNA molecules. SYBR Green loses mobility after being intercalated to DNA, releasing its energy as fluorescence. In amplification procedures, primers and DNA-binding dyes can be used to track the appearance of double-stranded DNA (dsDNA). However, for increased specificity in a qPCR assay, fluorescently labeled probes can be employed. For each of the donor samples, the whole process of the qPCR was done after making cDNA. Firstly, the qPCR experiment planning pad was used for primer design of the qPCR experiment. Diluted cDNA, which were prepared in the previous step, were taken. Then the Reverse Transcriptase master mixes prepared in the previous step were also taken as Master Mix 1. After these diluted primers of the RNA samples were prepared. The diluted primers prepared were both the forward and reverse primers. To prepare diluted primers, 390 μ l of sterile distilled water was added to 10 μ l of the sample to produce 1:40 diluted primer. Then, the target mix was prepared as Master Mix 2. To prepare target mix the SYBR GREEN dye was added to each of the diluted primers according to the calculations done and 1 tube of master mix 2 per target was prepared.

Following the plan designed for the Primer Design at the start of qPCR experimentation, 6 μ l of the master mix 1 was pipetted into each well using 1 pipette filter tip per well. Then 14 μ l of the master mix 2 was pipetted into each well as planned in the Primer Design using 1 pipette filter tip per well as with master mix 1. This resulted in wells with a total volume of 20 μ l each per reaction. The well

Table 4: cDNA RT sample mix.

	No of wells	Total volume	cDNA	dH ₂ O
+RT	18	118.8	16.5	99
-RT	6	39.6	6.6	33

Table 5: Target mix for SYBR green and primers.

No. of wells/targets	SYBR Green	Forward primer	Reverse primer
16	176	35.2	35.2

Table 6: Number of platelets counts per donor.

Donor	Platelet count/L
1	4.01×10^8
2	3.18×10^8
3	5.21×10^8

plates were sealed and covered properly and then centrifuged. After centrifugation, the qPCR prepared mix were run on each of the well plates.

qPCR setup (Table 4 and 5)

Calculation of delta delta CT values and statistical analysis

The delta-delta CT ($\Delta\Delta CT$) method (see steps below) was used to calculate the fold change in the gene expression of the target gene.

1. Find the average Ct value for all technical replicates
2. Calculate the delta Ct for each sample by using the average Ct values
3. $\Delta Ct = \text{Average Ct (gene of interest)} - \text{Average Ct (housekeeping gene)}$
4. Calculate the delta delta Ct for each sample
5. $\Delta\Delta Ct = \Delta Ct \text{ of target gene (gene of interest)} - \Delta Ct \text{ of reference gene (housekeeping gene)}$
6. Calculate the fold gene expression values
7. Fold gene expression = $2^{-\Delta\Delta Ct}$

A Two-way ANOVA Dunnett's multiple comparison test was performed to statistically analyse the fold changes in the gene expression.

ANOVA - Shapiro-wilk Normality Test for each plate.

Results

As a first step of this study, it was first needed to acquire platelets from the donor participants. Below is the quantization or quantity of platelets recovered from donor.

Platelet counts

The Table 6 below illustrates the results from the platelets counts acquired from the donors of this study. A variation in the number of platelets was observed between the three donors. Donor 3 was recorded with the highest number of platelets whereas Donor 2 with the lowest.

Mycoplasma test result

Prior to the platelet counts, mycoplasma test was performed to check for and ascertain LX2 cell purity as well as any microbial or mycoplasma contamination. This was because the cells were

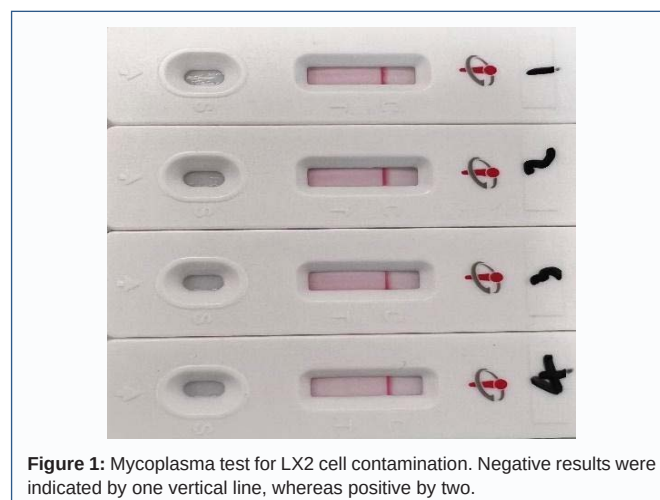


Figure 1: Mycoplasma test for LX2 cell contamination. Negative results were indicated by one vertical line, whereas positive by two.

stored in the hydrogen tank and some individual cells in the same hydrogen tank were found contaminated. Supervisor requested we run mycoplasma test for purity of cell.

The mycoplasma test illustrated in Figure 1, indicates that the LX-2 cells were not contaminated. Hence experiments were continued or carried out.

qPCR results from donor 1

Following the quality tests described previously, genetic analysis was carried out on the samples of the three donors to investigate the gene expression of ACTA2, COL1, CTGF and YAP1.

Figure 2A represented a schematic similarity in the two different peaks of COL1 and RPLO (reference gene) with the same level of $-d(RFU)/d$, which indicated a single discrete species of the amplified double-stranded DNA of both. Figure 2B compared COL1 single target gene ($n=3$) and RPLO in two conditions of P-Ca and P+Ca to record their relative fluorescent levels of melting temperature. This comparison indicates that COL1 had the same melting temperature of the technical replicate and close different from the reference gene RPLO. Figure 2C showed increased expression of COL1 and YAP1 with statistical significance in asterisk. Slightly higher expression of platelet releasate + Calcium was observed compared with platelet releasate. While ACTA2 increased in expression with both platelet releasate and platelet releasate + Calcium, no statistical significance was seen. CTGF showed insignificant gene expression. Conclusively COL1 and YAP1 showed significant expression in the first donor sample compared to ACTA2 and CTGF. Viewing error bars, standard error of mean of COL1 and YAP1 indicates that samples are closely distributed around the population mean, implying that the reported measurement value might be close to the true value, while CTGF and ACTA2 standard error are higher, signifying uncertainty of accurate measurement. Figure 2D showed the Volcano statistical analysis of YAP1, COL1, ACTA2 and CTGF. Each point schematically represents a target gene. P-Value levels of relative normalized expression of releasate, YAP1 and COL1 above passed the statistical significance cut off line indicating they are statistically significant and high expression of gene. ACTA2 in red shows expression of gene but not statistically significant and CTGF had no statistical significance and expression.

qPCR results from donor 2

In Figure 3A, it can be observed different levels of $-d(RFU)/d$

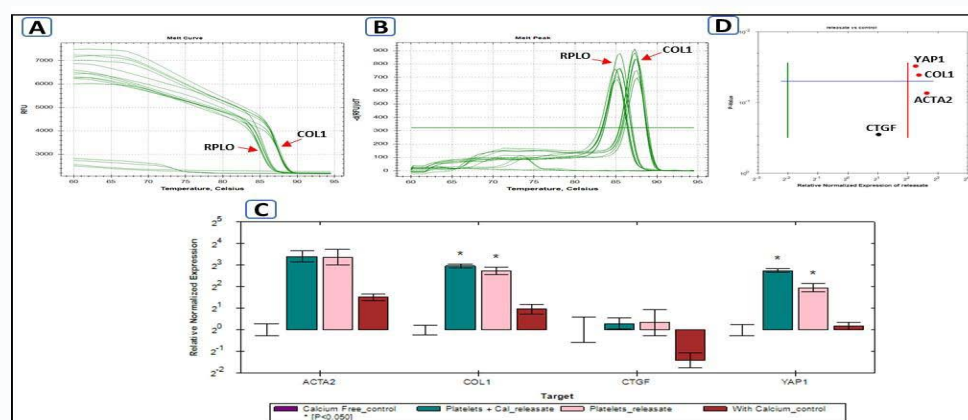


Figure 2: Gene analysis of donor 1. (A) Relative fluorescence unit level of temperature comparison of COL1 target gene and the reference gene RPLO of P-Ca and P+Ca; (B) Negative derivative rate of fluorescence variation level of melting temperature comparison of COL1 and the reference gene RPLO of P-Ca and P+Ca; (C) Gene expression analysis of ACTA2, COL1, CTGF and YAP1; (D) Scatter plot Statistical significance (p value) level of Magnitude of change in normalized relative expression releasate versus control.

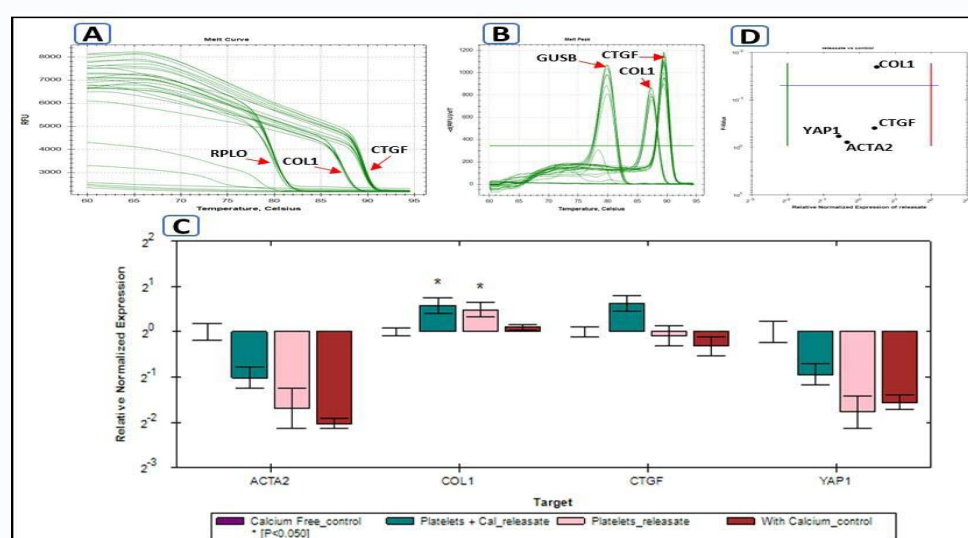


Figure 3: Gene analysis of donor 2. (A) Relative fluorescence unit level of temperature comparison of COL1, CTGF and the Reference gene GUSB for P-Ca and P+Ca; (B) Negative derivative rate of fluorescence variation level of time of melting comparison of the reference GUSB with COL1 and CTGF for P-Ca and P+Ca; (C) Gene expression analysis of ACTA2, COL1, CTGF and YAP1; (D) Volcano plot Statistical significance (p value) level of Magnitude of change in normalized relative expression releasate versus control.

of CTGF and COL1 compared to the reference gene (GUSB). a schematic similarity in the two different peaks of COL1 and RPLO (reference gene). That indicated a single discrete species of the amplified double-stranded DNA between the target genes but different of the reference gene. Figure 3B compared the reference gene (GUSB) with the target genes (COL1, CTGF). This comparison indicated that the target genes had the same melting temperature of the technical replicate and close different from the reference gene GUSB. In Figure 3C, it was overall observed that addition of calcium has shown increase in expression of the four genes (ACTA2, COL1, CTGF and YAP). However, statistically significant increase in the gene expression upon calcium was only observed on COL1 platelets releasate + Ca when compared to the control. Interestingly, addition of calcium on the control sample of COL1 has shown slight increase in the gene expression whereas reduction in the gene expression was observed in ACTA2, CTGF and YAP1 genes. Figure 3D showed the Volcano statistical analysis of YAP1, COL1, ACTA2 and CTGF. Each

point schematically represents a target gene. P -Value levels of relative normalized expression of releasate, COL1 above passed the statistical significance cut off point indicating its statistically significance and high expression of gene. On the other hand, ACTA2, YAP1 and CTGF were recorded below the cutoff point illustrating no statistical difference.

A second replicate of the donor 2 was performed and shown in Figure 4. Figure 4A represented a schematic difference with two different peaks of CTGF and GUSB (reference gene) indicating different species of the amplified double-stranded DNA. Figure 4B compared GUSB and target gene CTGF in two conditions of P-Ca and P+Ca to record their relative fluorescent levels of melting temperature. This comparison indicates that CTGF had different melting from the reference gene GUSB. Figure 4C showed the genetic profile of genes expressed in the presence and absence of calcium. Noteworthy, is the different gene response observed between the first

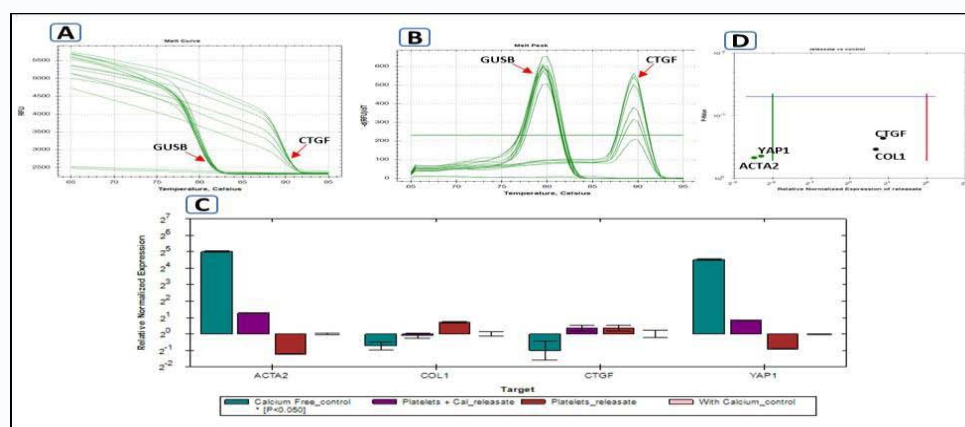


Figure 4: Gene analysis of donor 2 (2nd replicate). (A) Relative fluorescence unit level of temperature comparison of CTGF target gene and the Reference gene GUSB for P-Ca and P+Ca; (B) Negative derivative rate of fluorescence variation level of time of melting comparison of the reference GUSB and CTGF gene for P-Ca and P+Ca; (C) Gene expression analysis of ACTA2, COL1, CTGF and YAP; (D) Volcano plot Statistical significance (*p* value) level of Magnitude of change in normalized relative expression releasate versus control.

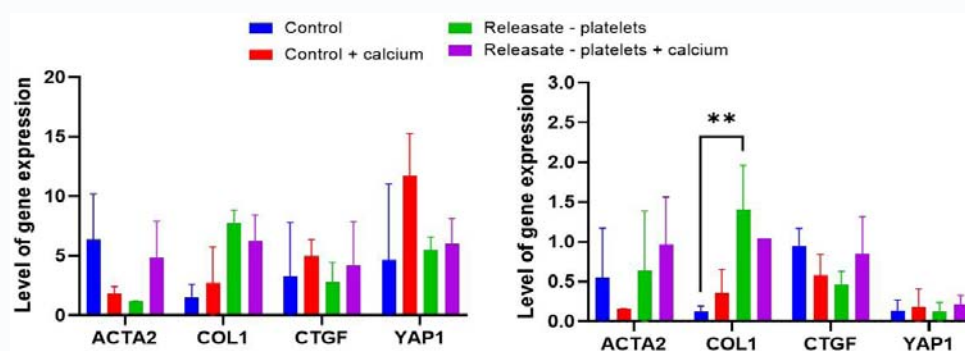


Figure 5: Average level of gene expression of donor 1 and 2 in the presence of releasate-platelets and/or calcium. Fold change was normalized with two reference genes: RPLO (left graph) and GUSB (right graph). Statistical analysis was performed by two-way ANOVA Dunnett's multiple comparison test.

and second replicate of donor 2. In the second replicate presented in Figure 4C, presence of calcium caused increased gene expression on ACTA2 and YAP1 control and platelet-releasate. However, decrease in the gene expression upon calcium was observed on COL1 and CTGF genes. Moreover, Volcano statistical analysis in Figure 4D showed that all the genes, YAP1, COL1, ACTA2 and CTGF were below the cut off line indicating no statistically significant expression of the genes.

qPCR average results from donor 1 and 2

The fold change in gene expression presented in Figures 4 and 5 was averaged and presented in Figure 6. The two reference genes were used to normalize the gene expression.

The data below clearly illustrate statistically significant increase in the fold change by 1x of COL1 gene upon the addition of PR when normalized with RPLO and GUSB. However, a different expression pattern was observed with the other target genes. Noteworthy is the gene YAP1 and ACTA2 showed increased in gene expression when treated with PR and calcium combined. Whereas CTGF target gene was observed with variable expression when normalized with RPLO and GUSB.

Reduction in the gene expression of ACTA2 was observed when compared the control and control with calcium. However, higher reduction was mainly observed on the ACTA2 normalized with

RPLO where 5 units reductions was seen. Similar phenomenon was detected on CTGF gene when normalized with GUSB reference gene.

In general, higher levels of gene expression were observed on genes normalized with RPLO compared to GUSB. Regardless of the magnitude level, similar expression pattern upon addition of PR, calcium or PR + calcium was observed on COL1 and CTGF genes.

qPCR average results from donor 3 (mHSC)

Platelets of the donor 3 were used against primary mHSCs to observe any differences between LX-2 (Figure 4) and mHSCs (Figure 5) cell response.

Figure 6A showed similarity in the two different peaks of COL1 and RPLO (reference gene) with similar level of -d(RFU)/d. That indicated a single discrete species of the amplified double-stranded DNA of both. Figure 6B compared COL1 single target gene and RPLO in two conditions of P-Ca and P+Ca to record their relative fluorescent levels of melting temperature. This comparison indicated that COL1 had slightly different melting temperature from the reference gene RPLO. It was observed in Figure 6C showed the effect on the gene expression of ACTA2, COL1 and CTGF genes upon the addition of Platelet Releasate (PR) to the mHSCs cells. It can be clearly seen that addition of PR significantly increases the gene expression, especially on CTGF gene. Figure 6D showed the Volcano statistical analysis of COL1, ACTA2 and CTGF. Each point schematically represents

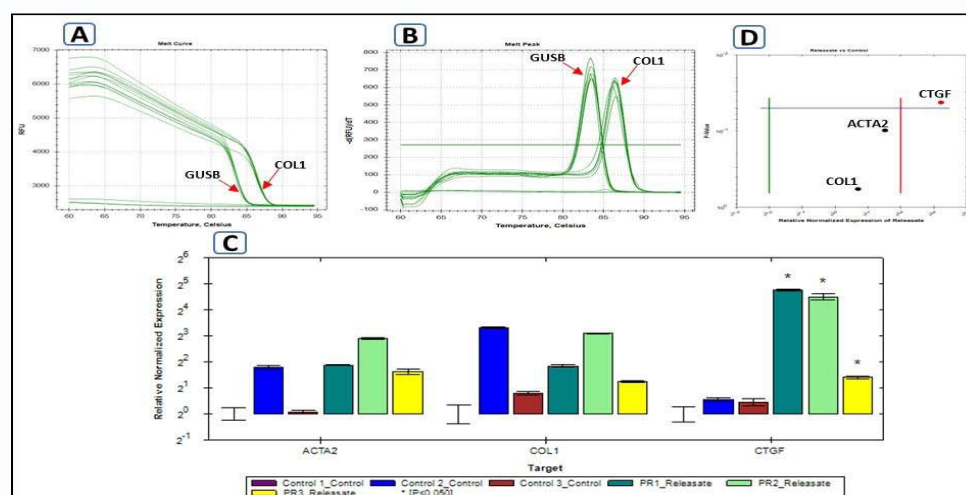


Figure 6: Gene expression of donor 3 (mHSC). (A) Relative fluorescence unit level of temperature comparison of COL1 gene and the reference gene GUSB; (B) Negative derivative rate of fluorescence variation level of melting temperature comparison of COL1 gene and the Reference gene GUSB; (C) Gene expression analysis of ACTA2, COL1 and CTGF (D) Volcano plot Statistical significance (*p* value) level of Magnitude of change in normalized relative expression releaseate versus control.

a target gene. *P*-Value levels of relative normalized expression of releaseate, CTGF passed the statistical significance cut off line indicating statistically significant high expression of gene. However, ACTA2 and COL1 detected below the cut off limit indicating no statistical significance and expression.

Discussion

The role of platelets in cases of fibrosis of the liver in recent times has welcomed diverse investigators interest as strategies towards controlling fibrogenesis continues to rise. Some investigators propose transplantation of tissues while others suggested several strategies such as anti-inflammatory agents, the use of NF- κ B inhibitors, antiplatelets etc [15, 16]. In addition, Hepatic Stellate Cells (HSCs), which become activated during liver injury promotes fibrosis and express platelet-derived growth factor receptor beta in various tissues. The inhibition of such HSCs is been suggested as potential way forward for the control/management of liver fibrosis. However, platelet which presents potential for control has continued to be emphasized by diverse investigators with dearth understanding of the activity of platelet in fibrogenesis [15]. This study investigated the expression of specific target genes including COL1, ACTA2, YAP1 and CTGF upon the addition of PR (acquired from 3 donors) and calcium on LX-2 and mHSC cells. That was to examine whether the platelets promote HSC's activation, which expresses some specific target genes such as CTGF, COL1, ACTA2, YAP1. Moreover, the impact of platelets during the period of fibrosis and its target on the respective genes was also studied. It is important to note that the aforementioned genes were targeted as they have been closely linked to liver fibrosis.

Platelet counts

The platelets count from the donors ranges from 3.18×10^8 to 5.21×10^8 per litre of blood collected. It is important to note that a normal healthy human has a platelet count ranging from 150 to $450 \times 10^9/L$. Observing such count of platelet is an indication that these donor patients were apparently healthy as previously documented by Sharma and his colleagues [17]. Such varying value of platelet count may also have a potential impact on reports.

Mycoplasma test

Confirming the sterility of LX2 cell to be free from contamination due to multiple storage of material in the same hydrogen tank was a sacrosanct procedure which revealed that LX-2 cells were not contaminated with mycoplasma.

Target genes for qPCR

Collagen 1 (COL1), Alpha-Smooth Muscle actin 2 (ACTA2), Yes-associated protein 1 (YAP1), Connective Tissue Growth Factor (CTGF)

A peak level that is similar was observed for COL1 and the reference gene RPLO with the same level of $-d(RFU)/d$, which indicates relatedness of the amplified double-stranded DNA species. The COL1 single target gene ($n=3$) and RPLO in two conditions of P-Ca and P+Ca to record their relative fluorescent levels of melting temperature. The data from donor 1 suggests that addition of calcium to LX-2 cells slightly affects the expression of COL1. A similar occurrence was observed for ACTA2 and YAP1 compared to addition of PR. However, different pattern of expression was observed on ACTA2 and YAP1 genes as there was a decrease in the expression upon addition of either calcium or PR. Such variation in the gene expression is attributable to pipette errors during the experiment, evaporation of solution and also differences in RNA concentrations (Figure 2,3 and 4).

Upon the use of Calcium alone, there was an increase in the expression of fibrotic genes, however when introduced as platelet activator, no statistical significance was observed suggesting the need for further study. On another experiment with addition of platelets from donor 1 to LX-2 cells, an increase in gene expression of COL1, ACTA2 and YAP1 were observed. Such similar observations were seen on mHSC cells where platelets from donor 3 activated the expression of ACTA1, COL1 and CTGF genes. Platelets improve expression of fibrotic genes and consequently could improve liver fibrosis and accelerate regeneration of liver [18]. Other related investigators have also reported the relevance of platelets in fibrogenesis positing that platelets promote fibrogenesis of the liver [10, 15, 19].

As observed on the various expressed genes compared to the two reference genes (RPLO and GUSB) which were used to normalize the amplification was a high expression of YAP1 and ACTA2 genes when PR and calcium were combined in the treatment process. It is important to note that such high expression of liver fibrosis associated genes implies that the treatment process influences and encourages fibrogenesis as reported by other related investigators [10, 16, 15, 19]. Although, there was a reduction in the expression of CTGF gene and ACTA2 gene when compared with the control and control with calcium when normalized with the reference, such observation may be attributable to multi-facet nature of mechanism that are at play during the process of fibrogenesis. A similar observation was reported in the study of some investigators who posited that a high expression of some fibrogenesis associated genes may be attributed to diversity of mechanisms [10, 16].

From the results obtained in this study, it is suggested that platelets play a prominent role in liver fibrosis as they heavily influence the expression of different marker genes associated with HSCs activation. It is worthy of note that a higher expression of YAP1 and ACTA2 target genes were observed in the presence of platelets. However, the exact mechanism of action in liver fibrosis remains inconclusive from the results obtained.

On another observation in this study, platelets are seen to increase the expression of fibrotic genes based on the other chemicals components present. This may be associated with physiological composition of platelets molecule carried in the bloodstream [20]. It is reported that platelets consist of heavily release molecules like PDGF, DPD, polyphosphates, ATP and TGF, which may trigger activation of HSCs resulting in increased hepatic fibrosis and other liver disease cases they interact with sinusoidal endothelial cells influencing the activation and taxis of other inflammatory cells [20].

The above-mentioned dual activity by platelets in liver fibrosis could be attributed to its derivatives and the signaling pathways (*i.e.*, PDGF, TGF, and chemokine signaling) they trigger. However, further investigation is required to shed light on the mechanism of action [21-54].

Future Studies

Based on our study and findings, our progressive future works should include to understand the differences in role of platelets in liver fibrosis in healthy and obese individuals. Such study would be conducted by focusing on apparently healthy obese individuals as blood samples collected from them will be used as donor blood for the extraction of platelets. The extracted platelets would be used to challenge/treat both immortalised HSC cell line and mouse cell line for the expression of fibrogenesis associated genes and the relevance of platelets would be determined. The report from such study would also reveal platelets relevance in other liver diseases amongst apparently healthy obese individuals. Other studies should determine the differences in the relevance of platelets in liver fibrogenesis and other liver diseases amongst poor health and poor lifestyle individuals, evaluating the role of a well-designed anti-platelet in the control and management of fibrogenesis of the liver and other related liver disease cases. It would also determine the potentials of chemotherapeutics and other ionised biomarkers in the inhibition of Hepatic Stellate Cells (HSCs) activation in liver fibrosis and cirrhosis cases as a therapeutic agent and evaluate the mechanism and the signalling pathway of platelets contribution to fibrogenesis cases from first stage to the last.

Study Limitation

During the study, specific observation may have affected the inference which may be considered in future related studies: RNA concentration was low because of splitting the LX-2 cells in 12 wells plate, making us to have lesser cells in each well/condition, which could affect the results. To improve that, we can use 6 wells plate to have more cells in each well and condition, so we can have appropriate RNA concentration after RNA isolation. In addition to that, DNase treatment should also be investigated, for proper RNA purification, which was obvious from the absorbance ratio when quantified with Nanodrop. Other related limiting concerns include; Laboratory material thawing and RNA instability may also have potential effect on delayed experimentation, Possibility of obtaining or extracting platelets comparable with normal ranges is also a limiting factor.

Conclusion

Following the study, it was observed that platelets enhanced/boosted the expression of fibrotic genes in mHSC which supports our study hypothesis. Furthermore, the addition of platelets aids the increase in expression of fibrotic genes. This is an indication and suggests that platelets play a prominent role in liver fibrosis as significant increase in the expression of different target genes associated with HSCs activation was observed. Statistically significant effect was particularly observed on COL1 and CTGF target genes. Furthermore, addition of calcium increased the expression of fibrotic genes, however no significant effect was observed when introduced as platelet activator. Data of this study also showed that platelets might reduce the expression of ACTA2 and YAP1, which are slightly enhanced with calcium as the activator, however, further studies are required as important variables such as quantity of RNA from each donor were different which could significantly alter the data.

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