Original Research Article

THE EFFECT OF PROTEIN TYROSINE PHOSPHATASE 1B INHIBITOR ON GLUCONEOGENESIS *INVITRO*

Abstract

Obesity is considered to have association with an increased risk of metabolic syndrome such as type 2 diabetes, insulin resistance, dyslipidaemia and non-alcoholic fatty liver disease. Obesity occurs as a result of imbalance between food intake and energy expenditure leading to excessive accumulation of adipose tissue**.** NAFLD is the most common liver condition and related to resistance of insulin. Insulin resistance is associated with increase influx of lipid into the liver promoting accumulation of hepatic triglyceride. The aim of this study is to develop an experimental model of hepatic steatosis with lipid over accumulation and to test the effect of PTP1B inhibitor on gluconeogenesis in the designed model. HepG2 cells were cultured for 24 hours in free fatty acid media (1:2 palmitic acid and oleic acid respectively). Intracellular lipid content and lipotoxicity were determined by oil red O staining followed by colorimetric detection. This experiment was accomplished by defining the experimental conditions of lipid exposure that leads to significant intracellular fat accumulation in the absence of lipotoxicity with 1 mM of free fatty acid media .

Key words: tyrosine phosphatase 1B, flow cytometry, gluconeogenesis, hepatocellular carcinoma (HepG2) cells, insulin resistance, Oil Red O, Nile Red, lipotoxicity, obesity

1. **INTRODUCTION**

Increased incidence in obesity is reaching epidemic proportions because of lifestyle modification especially change in eating habits. Obesity complications including type 2 diabetes, cardiovascular disease, metabolic syndrome, non-alcoholic fatty liver and cancers [1] have raised a significant health concern in the world especially in developed countries [2,3]. Therefore, many studies of molecules regulating the development of obesity and its associated pathologies is ongoing to treat and prevent obesity [4]. An important link between obesity, hypertension and sympathetic nerve activity (SNA) is leptin [5]. Leptin is a 16 KDa protein produced by adipocytes and controls body weight by regulating appetite and energy expenditure [6,7]. Absence of functional leptin receptors or deficiency in leptin is related with distinct hyperphagia and reduced energy expenditure [8], whereas viral vectors increase leptin by overexpression of leptin gene or infusion of leptin, this lead to decrease food intake and increases expenditure of energy [9,10]. Leptin (adipocyte hormone) increases with increase proportion to adipose tissue mass and decrease with weight loss [11] and plays a key role in glucose homeostasis facilitated by its direct action on the central nervous system [12]. Glucose is an important nutrient and source of energy whose homeostasis is important to maintain proper cell functions, since the physiology of the body can be weakened by either hypoglycaemia or hyperglycaemia leading to cell death. The inability of the cells to use or take up glucose as an energy source upon stimulation by insulin is defined as insulin resistance (IR) [13]. The intrinsic protein tyrosine kinase activity is induced by binding of insulin to its receptor; the tyrosine residues are phosphorylated in the insulin receptor as a result of activating intrinsic protein tyrosine kinase activity this is the first step. "*Subsequent steps involved the activation of the heterodimeric p85/p110-PI3K complex including generation of the lipid second messenger PIP3 (Phosphatidylinositol (3, 4, 5)-trisphosphate) which activates PDPK1 (phosphoinositide-dependent protein kinase-1), PKB/Akt (Protein Kinase B) and a typical PKC isoform and recruitment of adapter molecule's insulin receptor substrates (IRS), IRS1 and IRS2"* [14,15].

The most common form of chronic liver disease is non-alcoholic fatty liver disease (NAFLD), its prevalence increase with increase incidence of obesity [1,16]. NAFLD is defined as excess accumulation of fat in the liver that is not a result of alcohol consumption, genetic disorders or drug use [17]. The accumulation of lipids in micro and macro vesicles in more than 5 % hepatocytes, mostly in the perivenular hepatocytes is known as Non-alcoholic steatohepatitis (NASH). NASH associated with steatosis and necro inflamation is a more severe form of NAFLD and may lead to hepatic fibrosis and cirrhosis [18]. Progession of simple steatosis to NASH is contributed by increased delivery of fatty acids to the liver. Fatty acids such as palmitic and oleic acid are found in triacyl-sn-glycerols of seed oils and animal depot fats in our daily diet. Palmitic acid is a saturated fatty acid that induces apoptosis in hepatocytes [19]. In the presence of oleic acid, the palmitate-induced apoptosis is reduced [19]. It is known that lipid accumulation in NASH is induced by free fatty acids (FFA) [20]. To study NAFLD, HepG2 cells have been widely used. HepG2 cells are derived from tumor cells, thus they behave differently from normal cells [21]. Injury and death to cells caused by FFAs and their metabolites is described as lipotoxicity [22]. Triglycerides is the major lipid stored in NAFLD recent data suggest that triglyceride accumulation may be hepatoprotective in the liver. A diacylglycerolacyltransferase 1 or 2 catalyze the synthesis of hepatic triglyceride [23]. Accumulation of FFA is likely to have toxic effects in hepatocytes in contrast to triglyceride. Li et al. (2009) demonstrates the main determinant of hepatocellular damaged in NAFLD is the ratio of monounsaturated FAs (MUFA) to saturated FAs (SFA) [24].

Protein tyrosine phosphatases (PTPs) have been involved in the modulation of glucose homeostasis *in vivo,* including prototypic PTP1B [25]. The IR PTK in liver and muscle is dephosphorylated by PTP1B to regulate glucose homeostasis. Increasing the expression of PTP1B leads to insulin resistance in people and rodents and knockout of PTP1B is associated with leanness and insulin sensitivity in rodents, this suggests that PTP1B is an important molecular target for the treatment of diabetes and obesity. Due to the role of PTP1B in IR and leptin signalling it can be used as a target for the development of therapeutics for treatment of obesity and type 2 diabetes. Antisense oligonucleotides that target PTP1B are in clinical trials whereas drugs inhibiting the activity of PTP1B are in preclinical development [26]. Decreasing the level of PTP1B in peripheral tissues is associated with improved insulin sensitivity, regulates hyperglycaemia and reduced obesity in these mice [27]. This suggests that PTP1B plays an important role in regulation of body mass in one or more peripheral tissues independent on effects in the brain [27]. A common model to study hepatic steatosis is FFA induced lipid accumulation in hepatocytes [28]. The aim of this study is to develop an experimental model of hepatic steatosis using HepG2 cell line and test the effect of PTP1B inhibitor on gluconeogenesis in the designed model. To do obtain the aim, the study used Oil red O and Nile red staining to measure lipid levels in HepG2 cell lines.

2. MATERIALS AND METHODS

2.1. Materials

Human hepatocellular carcinoma (HepG2) cells were supplied by Sheffield Hallam University. Cells were grown in high glucose (4.5 g/L) Dulbecco's modified Eagle's medium with ultra-glutamine (DMEM) (Lonza, UK), 10 % Fecal Calf Serum (FCS) (Gibco, UK) and 1 % penicillin/streptomycin (Lonza, UK).

Basic culture media was prepared with 200 ml of DMEM (Lonza, UK), 2000 µl 1 % penicillin/streptomycin (Lonza, UK) and 2 g of 1 % fatty acid free Bovine Serum Albumin (BSA) (Thermo-Fisher).

Oleic acid (Sigma-Aldrich) and palmitic acid (Sigma-Aldrich) dissolved in dimethyl sulfoxide were diluted in basic media to obtain 30 mM of free fatty acid (FFA) media (stock solution). This stock solution was further diluted with basic culture media to obtain 1 mM FFA media.

2.2. Oil red O colorimetric assay

Stock solution of Oil red O (ORO) was prepared by dissolving 0.7 g of ORO in 200 ml of Isopropyl alcohol. Bakers' formalin was prepared by adding 10 ml of 37 % formaldehyde and 10 ml of 10 % (w/v) calcium chloride solution to 80 ml of water and stored at 4°C. To prepare glycerol gelatine, 5 g gelatine was gently mixed with 50 ml glycerol in 50 ml water at 50ºC, and stored at 4ºC, then heating at 55-60ºC before use.

Cells were seeded in 12-well plate for 24 hours before treatment with 1 mM fatty acid at a density of 50000 cells per well. A half of the plate (6 wells) was used as control samples and the other half was treated with 1 mM of FFA and incubated for 24 hours at 37ºC and 5 % CO_2, in SANYO incubator before staining with Oil red O.

A 60 % ORO solution was prepared by diluting stock ORO with water at 3:2 ratio. Harris haematoxylin (Sigma Aldrich, UK) and 60 % ORO were filtered before use. Treatment and basic media were removed from each well and cells were washed 2 times in Hanks Balanced Salt solution (Sigma Aldrich, UK), the excess was poured off and blotted with a dry tissue. Each well was fixed with 780 µl of bakers' formalin at 4ºC for 30 minutes, the excess was poured off and blotted with a dry tissue. 780 µl of 60 % ORO was added to each well and left to incubate for 10 minutes at room temperature. Cells were rinsed with water for 5 minutes after staining. Excessive water was blotted with dry tissue before staining cells with 780 µl of

Harris haematoxylin and incubated for 2 minutes at room temperature. Cells were rinsed with water for 5 minutes. Excessive water was blotted with dry tissue and 2 drops of glycerol gelatine were added to each well. Stained cells were observed under x400 magnification of the microscope and quantified using Image J program. Image J was opened and microscopic picture were loaded. Threshold was set at 163 and 213 for minimum and maximum, respectively.

2.3. Proliferation Assay

100x stock solution of alamar blue (Sigma-Aldrich, UK) was prepared by dissolving 1 mg alamar blue in 1 ml of 1x PBS. Working concentration was achieved by diluting 30 µl of 100x stock solution in 30 ml of basic media. To measure cell proliferation, negative control was achieved by adding 1 ml of 1 % Triton X-100 solution to each control and treated sample, then incubated for 1 hours before staining with Alamar Blue. After incubation of the negative control, all cell media was removed and 2 ml of alamar blue solution was added followed by a 4-hour and 20-hour incubations at 37ºC and 5 % CO_2, in SANYO incubator. Absorbance was measured at 570 nm using GENESYS 10S UV-VIS (Thermo-Fisher) after 4 and 20 incubation hours.

2.4. Protein quantification

Cells were seeded in a 6-well plate for 24 hours before treatment with 1 mM fatty acid at a seeding density of 50000 cells per well. Cells were incubated at 37 ºC and 5 % CO_2, in SANYO incubator. Half (3) of the plate was used as control wells and the other half was treated with 1 mM concentration for 24 hr at 37ºC and 5 % CO_2, in SANYO incubator. Bradford reagent was prepared by dissolving 50 mg Coomassie brilliant blue in 24 ml 99 % ethanol; 50 ml of 85 % (w/v) phosphoric acid was added and diluted to a final volume of 500 ml with water, this solution was filtered before use. Protein standard was made by dissolving 0.05 g BSA in 50 ml warm water at a concentration of 1000 µg/ml. A serial dilution was prepared in the following concentrations of 1000, 500, 250, 125, 63, 31 and 0 µg/ml.

After treatment, basic media was removed from each well and cells were washed with 1x PBS. 20 µl of cell lytic M reagent was added to each well and incubated at room temperature for 15 minutes on a shaker. Lysed cells were collected by scraping. The lysed cells were centrifuged for 15 minutes at 12000 x g at room temperature. The supernatants were removed and the pellets were re-suspended in 100 µl of water. Each pellet and supernatant sample were diluted 1:10 with water, 10 µl was pipetted in triplicate into a flat bottomed 96-well plate. Standards were also added to the plate in duplicate. 200 µl of Bradford reagent was added into each well and absorbance was measured by MULTISCAN FC; Thermo-Fisher, UK at 595 nm.

2.5. Nile Red fluorescence assay

Cells were seeded in a 6-well plate for 24 hours before treatment with 1 mM fatty acid at a seeding density of 50000 cells per well. Cells were incubated at 37ºC and 5 % CO_2, in SANYO incubator. The half (3 wells) of each plate was used as control wells and the other half was treated with 1 mM concentration for 24 hours at 37ºC and 5 % CO_2, in SANYO incubator. Working solution of Nile Red (1 µg/ml) was achieved by diluting 1 mg in 1 ml methanol before diluting 5 µl with 5 ml 1x PBS.

Cells were washed with 500 µl of PBS (Lonza, UK). 250 µL of trypsin was added to each well and placed in an incubator for 5 min for the cells to dislodge. 250 µl of media was added to each well and pipetted into different tubes. Cells were centrifuged at 500 x g for 5 minutes. Supernatants were discarded and pellets were re-suspended in 1.5 ml 1xPBS and centrifuged at 500 x g for 5 minutes. 500 µl of 1x Nile Red in PBS was added and incubated for 5 minutes on ice. Cells were washed in 1.5 ml PBS, supernatant was discarded and pellet was re-suspended in 500 µl of PBS. Nile red fluorescence was determined by flow cytometry with Becton Dickinson FACS Calibur System, FL2 emission channel, at excitation wavelength of 488 nm and emission wavelength of 550 nm. CELLQUEST software from BD Biosciences was used for data analysis.

3. RESULTS

3.1. Oil red O colorimetric assay

After 24-hour incubation with 1 mM of FFA media in a 12-well plate, cells were stained with Oil red O to observe the accumulation of lipid (palmitic acid and oleic acid) at x400 magnification of the microscope. Compared with the control samples, the appearance of red spots in Figure 1B shows lipid accumulation in FFA-treated HepG2 cells. The quantification of stained HepG2 cells using Image J is expressed as % average areas of 6 wells. In control cells, the percentage area was approximately 12% while that was nearly 16% in treated cells.

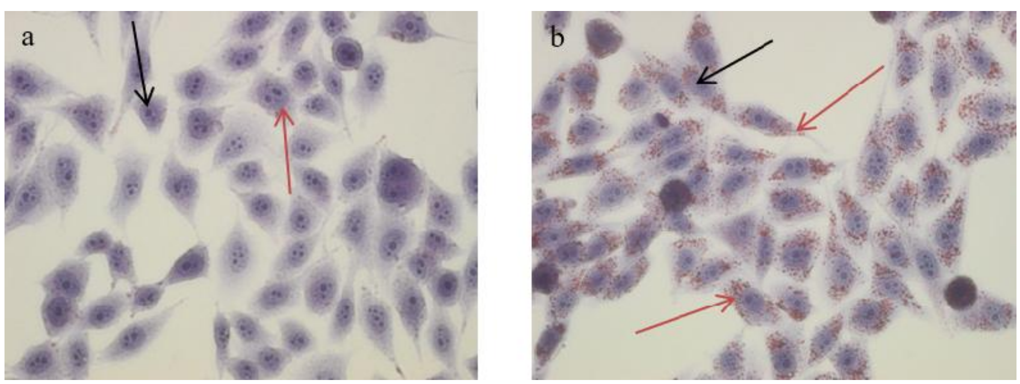


Figure 1. Oil Red O staining of HepG2 cells in
A) control samples, B) FFA-treated HepG2 cells samples
** red arrow shows lipid droplet; black arrow shows cell nucleus*

3.2 Proliferation Assay

Proliferation assay was used to monitor the response and health of HepG2 cells in culture after treatment with 1 mM FFA. Figure 2 shows the lipotoxicity of HepG2 cells. As can be seen, treatment of HepG2 cells with 1 mM of FFA media did not significantly decrease the cell viability, compared to control cells. After incubation in 4 hours, the amount of alamar blue that was reduced in both the control and treated cells is 17 % while this proportion is 52 % and 57 % respectively after 20-hour incubation.

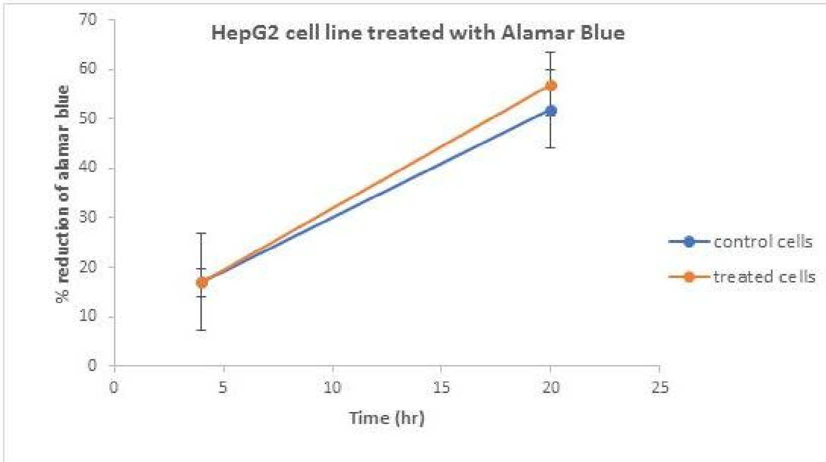


Figure 2. % reduction of Alamar blue by HepG2 cells

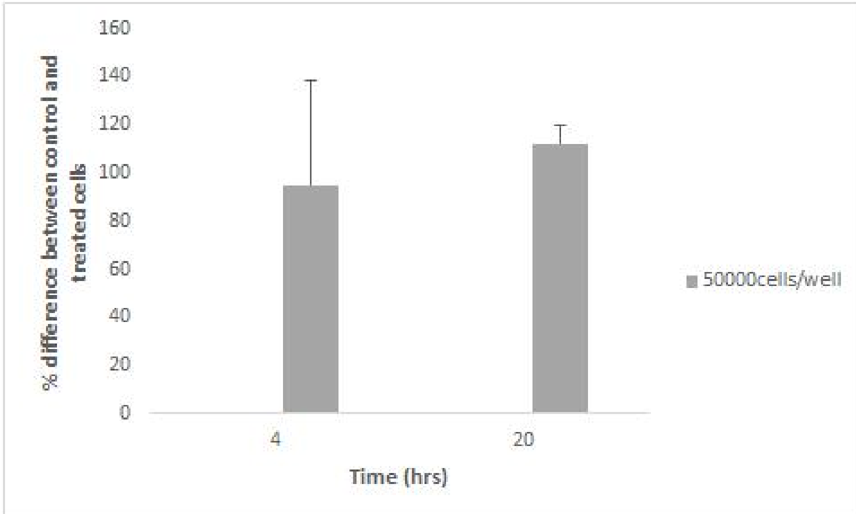


Figure 3. % difference between control and treated HepG2 cells

To determine the percentage of alamar blue that is reduced by the treated cells compared to control cells or % growth inhibition of cells in the treated cells compared to the control cells, proliferation assay was performed. After incubation in 4 hours, the amount of alamar blue that was reduced in treated cells is 95 % while this proportion is 112 % after 20-hour incubation.

3.2. Protein quantification

The amount of protein has been successfully measured in HepG2 cells. In the standard curve, the absorbance increased when increasing the concentration of protein from 0 to 31, 63, 125, 250, 500 and 1000 µg/ml. The regression equation was A = 0.0012C + 0.2698 with the (R^2 = 0.9962).

Table 1. Concentration of protein calculated from the standard curve

Samples		Protein concentration (µg/ml) in triplicate		
Control cells	pellet	0.54	0.51	0.45
	suspension	2.01	2.09	1.83
Treated cells	pellet	0.62	0.81	0.59
	suspension	1.69	1.87	1.09

Table 1 shows the difference in concentration of proteins extracted from pellet and suspension of the samples, and difference between untreated and treated cells. High protein concentrations were seen in the suspension of both the control and treated cells. The pellet of cells had low protein concentration.

3.3. Nile red fluorescence assay

The content of intracellular lipid droplets was determined by Nile red staining and the cellular FFA uptake was quantified by flow cytometric assay. Unstained control cells in figure 4A were used to adjust the settings of the flow cytometer. Cells exposed to 1 mM FFA for 24 hours induced fat accumulation. Compared to stained control cells, only 1000 intensity, the fluorescence signal of lipid in the stained treated cells was evidently higher, up to roundly 4500 intensity (Figure 5C & 5D). The difference in fluorescence intensity shows an increase in intracellular lipid accumulation in cells treated with FFA media.

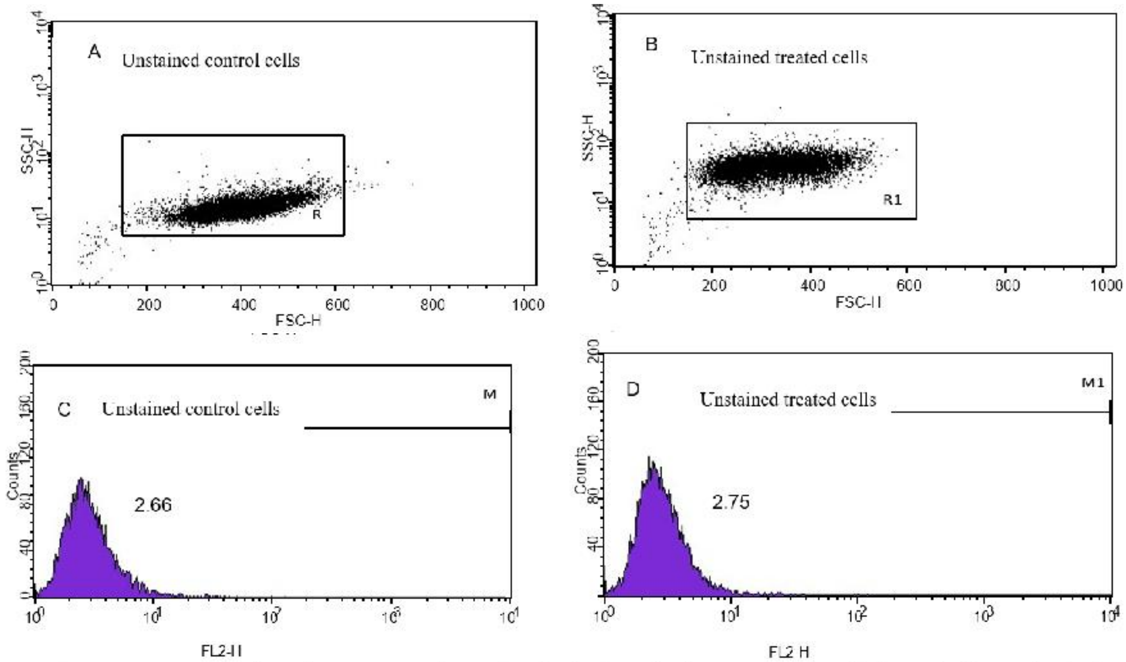
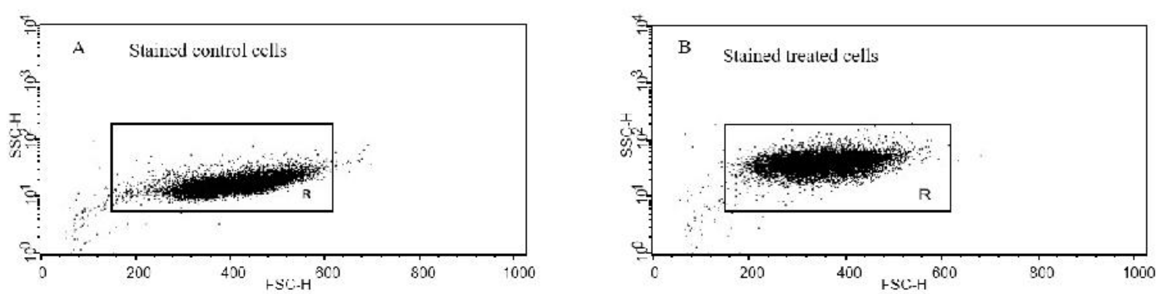


Figure 4. Fluorescent intensity of unstained control and treated HepG2 cells
A and C) Unstained control cells; B and D) Unstained treated cells with 1 mM FFA media



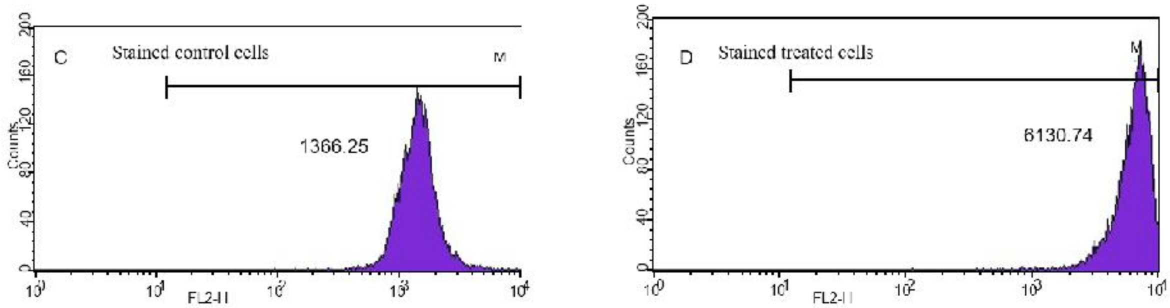


Figure 5. Fluorescent intensity of stained control and treated HepG2 cells
A and C) Stained control cells; B and D) stained treated cells with 1 mM FFA media

DISCUSSION

NAFLD causes chronic liver disease and prevalence increase with growing epidemic of obesity worldwide. The prevalence and impact of NAFLD on the growing epidemic of obesity make NAFLD become the most common cause of liver disease [29]. NAFLD is associated with several non-hepatic related complications and has the potential to develop to hepatic fibrosis and end-stage liver disease [30]. There is a general understanding that patients with NASH have increased lipolysis and subsequently a high circulating FFA level, accumulation of lipid in liver cells is contributed by increase in FFA inflow and de novo lipid synthesis [31]. A model for investigating NASH that is usually used is incubating hepatocytes with FFA [28]. The structure of fatty acids affects their activity biologically and they are classified as saturated and unsaturated fatty acids. The saturated FAs induce lipotoxicity, insulin resistance and apoptosis. A saturated fatty acid that induces apoptosis in liver cells is palmitic acid. Oleic acid is discovered to be more steatogenic but less apoptotic than palmitic acid [19,32]. Co-incubation of liver cells with both palmitic and oleic acid results in higher amount of fat accumulation than incubation with only palmitic acid. This co-incubation also lowers palmitate-mediated apoptosis, indicating protective feature of oleic acid [19,32]. In this study, HepG2 cells were incubated with a mixture of PA and OA (1:2) respectively, to develop a model to test the effect of PTP1B inhibitor on gluconeogenesis *in vitro*. To determine if HepG2 cells accumulate lipid, Oil red O was performed, flow cytometry based on Nile red staining was performed to determine the intracellular lipid content.

With this experiment, the quantification with ImageJ shows that HepG2 cell line can accumulate FFA, which was stained by ORO and viewed under microscope x 400 magnification. The cells were cultured in high glucose media, some lipids were seen in control but fewer in number compared to the treated cells. This result is similar with the study of Chavez-Tapia et al. (2011) [33], reaching the conclusion that hepatic cell line (HuH7) and no tumoral immortalized human hepatocytes (IHH) used could accumulate FFA, and the increase in fat content was not related to critical deterioration of the cell integrity, in accordance with clinical and *in vivo* experimental information.

Yao, *et al*., 2011 demonstrate that 1 mM concentration of FFAs caused fat accumulation but not lipotoxicity [34]; however, group of HepG2 cells treated with 2 and 3 mM FFAs significantly increased the lipotoxicity in cells. The proliferation assay was used to determine how proliferative HepG2 cells are and to confirm if the concentration of FFA causes lipotoxicity in this study. In this study, after incubation in 4 hours, there was 17% reduction of alamar blue in both the control and treated cells, while 20-hour incubation gave 52% and 57% reduction of alamar blue in control and treated wells, respectively. This means that the cells are dividing rapidly in 20 hours than 4 hours because of long period of incubation. 1 mM FFA concentration showed no significant difference in the proliferation of the treated HepG2 cells compared to the control cells; this indicates that 1 mM FFAs cause's lipid accumulation but not cell toxicity. This is similar to previous study [34] on lipotoxicity in HepG2 cells that support the findings in this practical. In 4-hour incubation, treated cells are differentiating at 95% of the rate of control cells, this indicate that the treated cells have reduced alamar blue by 95% of what the control had done or growth in treated is inhibited by 5% compared to control. However, in 20-hour incubation, treated cells are differentiating at 112% of the rate of control cells, this indicates that there is 0% growth inhibition in the treated cells compared to control cells.

To determine the intracellular lipid droplets contents, HepG2 cells were stained with Nile red. Exposure of cells to 1 mM FFA for 24 hours induced accumulation of lipid. Figure 4 (A and B) shows the forward and side scatter of the unstained control and unstained treated cells respectively. The forward scatter is used to identify the size of cells; it measures the size and shape of the cells. The side scatter is used to determine the complexity of the internal environment of the cell. Cells with a large amount of complexity in the internal environment will have a higher side scatter signal than cells with no or low complexity [35]. In figure 4 (A and B), the forward scatter shows a single cell population that are exactly the same size (HepG2 cells). Figure 4B has a higher side scatter than figure 4A, which shows figure 4B cells have more internal complexity than cells in figure 4A as a result of treating cells with 1 mM FFAs. In figure 4C and 4D, the peak was on the left side of the axis (no fluorescence) indicating the cells were not stain with Nile red. Microscopic image shows the presence of cytoplasmic lipid droplets. This data was confirmed by flow cytometry in figure 5. The highest peak was observed in figure 5D (treated stained) compared to figure 5C (control stained) due to fat accumulation in the cells. When the cells were treated with 1 mM FFA the peak shifts to the right, this corresponds to the significant increase in the geometric mean fluorescence intensity which is 6x more compared to the control stained cells. This is similar to previous study [34] that shows an increase in fluorescence intensity of lipid in treated stained cells compared to control stained cells under the microscope, they further showed the difference of fluorescence intensity in treated cells compared to control cells by flow cytometry, they observed a dose dependent increase in lipid accumulation in cells treated with FFA. Nile red attached to the lipids in the treated cells could make a more complex environment in the cells because of the lipid droplets. The more complex the internal environment of the cell was, the higher the side scatter signal was. This is why the fluorescent intensity of the treated cells was higher 5 times compared to the control stained cells. Bradford assay was used to measure the concentration of protein in a solution, which was proposed by the study of Cheng et al. (2016) [36]. To normalize the concentration of protein for western blotting, Bradford assay was performed. As a result, the highest protein concentration was seen in control suspension while the lowest concentration of protein was in control pellet.

CONCLUSION

PTP1B is an important molecular target for the treatment of diabetes and obesity. Targeting this inhibitor to reduce the complications that are associated with obesity such as hepatic steatosis and type 2 diabetes is of importance to the health care system. Some of the objectives of these study which include, identifying markers that are involved in gluconeogenesis that may affect PTP1B, measuring protein concentration using western blotting were not achieved due to time constraint. PTP1B inhibitor is supposed to be tested on the cell model that was designed but due to time constraint the inhibitor was not tested. If the inhibitor was tested on the model in all the assays performed above (Oil red O, Nile red, proliferation assay and flow cytometry), it is assumed that the cells may not have accumulate the FFA, lipotoxicity will not be seen and this will prevent non-alcoholic fatty liver disease and complications associated with it. In conclusion, the results show that HepG2 cells are induced to lipid over accumulation by mixtures of oleate and palmitate acids. 1 mM FFA did not affect the cell integrity and did not cause lipotoxicity of the cells. FFAs should be used with different concentrations of FFAs to develop further the model. Methods such as MTT assay can be used to assess the FFAs cytotoxicity to HepG2 cells. Cells treated with FFAs and stained with Annexin V/propidium iodide should be assayed for apoptosis using flow cytometry in further experiments.

REFERENCES

[1]. Starley, B. Q., Calcagno, C. J., & Harrison, S. A. Nonalcoholic fatty liver disease and hepatocellular carcinoma: a weighty connection. *Hepatology* 2010; *51*(5): 1820-1832.

[2]. Morisco, C., Lembo, G., & Trimarco, B. Insulin resistance and cardiovascular risk: New insights from molecular and cellular biology. *Trends in cardiovascular medicine* 2006; *16*(6): 183-188.

[3]. Ritchie, S. A., & Connell, J. M.. The link between abdominal obesity, metabolic syndrome and cardiovascular disease. *Nutrition, Metabolism and Cardiovascular Diseases* 2007; *17*(4): 319-326.

[4]. Delibegović, M., & Mody, N. Protein tyrosine phosphatase 1B (PTP1B) in obesity and type 2 diabetes. *Acta Medica Saliniana* 2009; *38*(1): 2-7.

[5]. Hall, J. E., Silva, A. A., Carmo, J. M., Dubinion, J., Hamza, S., Munusamy, S., Stec, D. E.. Obesity-induced hypertension: role of sympathetic nervous system, leptin, and melanocortins. *Journal of Biological Chemistry* 2010; *285*(23): 17271-17276.

[6]. Harris, R. B. Leptin—much more than a satiety signal. *Annual review of nutrition* 2000; *20*(1): 45-75.

[7]. Spiegelman, B. M., & Flier, J. S. (2001). Obesity and the regulation of energy balance. *Cell* 2001; *104*(4): 531-543.

[8]. Chua Jr, S. C.-P., Zhang, Y., Liu, S. M., Tartaglia, L., & Leibel, R. L. (1996). Phenotypes of mouse diabetes and rat fatty due to mutations in the OB (leptin) receptor. *Science New York Then Washington* 1996*; 271*(5251): 994-996.

[9]. Scarpace, P. J., Matheny, M., Zhang, Y., Tümer, N., Frase, C. D., Shek, E. W., Zolotukhin, S. Central leptin gene delivery evokes persistent leptin signal transduction in young and aged-obese rats but physiological responses become attenuated over time in aged-obese rats. *Neuropharmacology* 2002; 42(4): 548-561.

[10]. Tallam, L. S., da Silva, A. A., & Hall, J. E. Melanocortin-4 receptor mediates chronic cardiovascular and metabolic actions of leptin. *Hypertension* 2006; *48*(1): 58-64.

[11]. Considine, R., Sinha, M., Heiman, M., Kriauciunas, A., Stephens, T., Nyce, M., Caro, J. Serum immunoreactive-leptin concentrations in normal-weight and obese humans. *New England Journal of Medicine* 1996; 334(5): 292-295.

[12]. do Carmo, J. M., Hall, J. E., & da Silva, A. A. Chronic central leptin infusion restores cardiac sympathetic-vagal balance and baroreflex sensitivity in diabetic rats. *American Journal of Physiology-Heart and Circulatory Physiology* 2008; *295*(5): H1974-H1981.

[13]. García-Ruiz, C., Baulies, A., Mari, M., García-Rovés, P. M., & Fernandez-Checa, J. C. (2013). Mitochondrial dysfunction in non-alcoholic fatty liver disease and insulin resistance: Cause or consequence? *Free Radical Research* 2013; *47*(11): 854-868.

[14]. Cheng, Z., Tseng, Y., & White, M. F.. Insulin signaling meets mitochondria in metabolism. *Trends in Endocrinology & Metabolism* 2010; *21*(10): 589-598.

[15]. Rowland, A. F., Fazakerley, D. J., & James, D. E. Mapping insulin/GLUT4 circuitry. *Traffic* 2011; *12*(6): 672-681.

[16]. López-Velázquez, J. A., Silva-Vidal, K. V., Ponciano-Rodríguez, G., Chávez-Tapia, N. C., Arrese, M., Uribe, M., & Méndez-Sánchez, N. The prevalence of nonalcoholic fatty liver disease in the Americas. *Annals of Hepatology: Official Journal of the Mexican Association of Hepatology* 2014); *13*(2): 166-178.

[17]. Chalasani, N., Younossi, Z., Lavine, J., Diehl, A., Brunt, E., Cusi, K., Sanyal, A. (2012). The diagnosis and management of non- alcoholic fatty liver disease: Practice Guideline by the American Association for the Study of Liver Diseases, American.

[18]. AlKhater, S. A. Paediatric non- alcoholic fatty liver disease: an overview. *Obesity reviews* 2015; 16(5): 393-405.

[19]. Gentile, C. L., & Pagliassotti, M. J. The role of fatty acids in the development and progression of nonalcoholic fatty liver disease. *The Journal of nutritional biochemistry;* 2008 19(9): 567-576.

[20]. Kwan, H. Y., Fong, W. F., Yang, Z., Yu, Z. L., & Hsiao, W. L. Inhibition of DNA-dependent protein kinase reduced palmitate and oleate-induced lipid accumulation in HepG2 cells. *European journal of nutrition* 2013; *52*(6): 1621-1630.

[21]. Jiang, P., Huang, Z., Zhao, H., & Wei, T. Hydrogen peroxide impairs autophagic flux in a cell model of nonalcoholic fatty liver disease. *Biochemical and biophysical research communications* 2013; *433*(4): 408-414.

[22]. Neuschwander Tetri, B. A. Hepatic lipotoxicity and the pathogenesis of nonalcoholic steatohepatitis: the central role of nontriglyceride fatty acid metabolites. *Hepatology* 2010; *52*(2): 774-788.

[23]. Koliwad, S. K., Streeper, R. S., Monetti, M., Cornelissen, I., Chan, L., Terayama, K., Jr, R. V. DGAT1-dependent triacylglycerol storage by macrophages protects mice from diet-induced insulin resistance and inflammation. *The Journal of clinical investigation* 2010; 120(3): 756–767.

[24]. Li, Z. Z., Berk, M., McIntyre, T. M., & Feldstein, A. E. Hepatic lipid partitioning and liver damage in nonalcoholic fatty liver disease: role of stearoyl-CoA desaturase. *Journal of Biological Chemistry* 2009; *248*(9): 5637–5644.

[25]. Yip, S. C., Saha, S., & Chernoff, J. PTP1B: a double agent in metabolism and oncogenesis. *Trends in biochemical sciences* 2010*; 35*(8): 442-449.

[26]. Zhang, S., & Zhang, Z. Y. PTP1B as a drug target: recent developments in PTP1B inhibitor discovery. *Drug discovery today* 2007; *12*(9): 373-381.

[27]. Waring, J. F., Ciurlionis, R., Clampit, J. E., Morgan, S., Gum, R. J., Jolly, R. A., & ... Jirousek, M. PTP1B antisense-treated mice show regulation of genes involved in lipogenesis in liver and fat. *Molecular and cellular endocrinology* 2003; *203*(1): 155-168.

[28]. Chu, J. H., Wang, H. Y., Chan, P. K., Pan, S. Y., Fong, W. F., & Yu, Z. L. Inhibitory effect of schisandrin B on free fatty acid-induced steatosis in L-02 cells. *World Journal of Gastroenterology: WJG* 2011; 17(19): 2379–2388.

[29]. Vernon, G., Baranova, A., & Younossi, Z. M. Systematic review: the epidemiology and natural history of non- alcoholic fatty liver disease and non- alcoholic steatohepatitis in adults. *Alimentary pharmacology & therapeutics* 2011; 34(3): 274-285.

[30]. Bellentani, S., Bedogni, G., & Tiribelli, C. Liver and heart: a new link? *Journal of hepatology* 2008; 49(2): 300-302.

[31]. Marra, F., Gastaldelli, A., Baroni, G. S., Tell, G., & Tiribelli, C. Molecular basis and mechanisms of progression of non-alcoholic steatohepatitis. *Trends in molecular medicine* 2008; 14(2): 72-81.

[32]. Ricchi, M., Odoardi, M. R., Carulli, L., Anzivino, C., Ballestri, S., Pinetti, A., & Lonardo, A. Differential effect of oleic and palmitic acid on lipid accumulation and apoptosis in cultured hepatocytes. *Journal of gastroenterology and hepatology* 2009; 24(5): 830-840.

[33]. C Chavez-Tapia, N., Rosso, N., & Tiribelli. In vitro models for the study of non-alcoholic fatty liver disease. *Current medicinal chemistry* 2011; 18(7): 1079-1084.

[34]. Yao, H. R., Liu, P. D., Cao, Y. B., He, T., Lin, L., & Shang, J. Lipotoxicity in HepG2 cells triggered by free fatty acids. *American journal of translational research* 2011; 3(3): 284-291.

[35]. Bashashati, A., Johnson, N., Khodabakhshi, A., Whiteside, M., Zare, H., Scott, D., Slack, G. B cells with high side scatter parameter by flow cytometry correlate with inferior survival in diffuse large B-cell lymphoma. *American journal of clinical pathology* 2012*;* 137(5): 805-814.

[36]. Cheng, Y., Wei, H., Sun, R., Tian, Z., & Zheng, X. Rapid method for protein quantitation by Bradford assay after elimination of the interference of polysorbate 80. *Analytical biochemistry* 2016; 494: 37-39.