SPANDIDOS PUBLICATIONS

EXPERIMENTAL AND THERAPEUTIC MEDICINE 11: 1487-1492, 2016

# Low simvastatin concentrations reduce oleic acid-induced steatosis in $HepG_2$ cells: An *in vitro* model of non-alcoholic fatty liver disease

MOHAMMAD J. ALKHATATBEH[1], LISA F. LINCZ[2] and RICK F. THORNE[3]

[1]Clinical Pharmacy Department, Faculty of Pharmacy, Jordan University of Science and Technology, Irbid 22110, Jordan;
[2]Hunter Haematology Research Group, Calvary Mater Newcastle Hospital, Waratah, NSW 2298;
[3]School of Environmental and Life Sciences, University of Newcastle, Ourimbah, NSW 2258, Australia

Received August 30, 2015; Accepted January 15, 2016

DOI: 10.3892/etm.2016.3069

**Abstract.** Non-alcoholic fatty liver disease (NAFLD) is an inflammatory condition caused by hepatic lipid accumulation that is associated with insulin resistance, diabetes and metabolic syndrome. Although statins should be used with caution in liver diseases, they are increasingly investigated as a possible treatment for NAFLD. The present study recreated an *in vitro* model of NAFLD using $HepG_2$ cells exposed to oleic acid (OA), which was used to quantify OA-induced lipid accumulation in $HepG_2$ cells treated with various concentrations of simvastatin. In addition, the effect of simvastatin on $HepG_2$ cell morphology and microparticle generation as a marker of cell apoptosis was assessed. OA-induced lipid accumulation was quantified by Oil Red O staining and extraction for optical density determination. Stained lipid droplets were visualized using phase contrast microscopy. Furthermore, $HepG_2$ cell-derived microparticles were counted by flow cytometry subsequent to staining for Annexin V. $HepG_2$ cells treated with 0-1 mM OA showed dose-dependent lipid accumulation. Treatment of $HepG_2$ cells with increasing concentrations of simvastatin followed by treatment with 1 mM OA showed that low simvastatin concentrations (4-10 $\mu$M) were able to reduce lipid accumulation by ~40%, whereas high simvastatin concentrations (20 and 30 $\mu$M) induced apoptotic changes in cell morphology and increased the production of Annexin $V^+$ microparticles. This suggests that low simvastatin doses may have a role in preventing NAFLD. However, further investigations are required to confirm this action *in vivo* and to determine the underlying mechanism by which simvastatin reduces hepatic steatosis.

*Correspondence to:* Dr Mohammad J. Alkhatatbeh, Clinical Pharmacy Department, Faculty of Pharmacy, Jordan University of Science and Technology, P.P. Box (3030), Irbid 22110, Jordan
E-mail: khatatbeh@just.edu.jo

*Abbreviations:* NAFLD, non-alcoholic fatty liver disease; OA, oleic acid; $HepG_2$, human hepatocellular carcinoma cell line

*Key words:* non-alcoholic fatty liver disease, oleic acid, simvastatin, steatosis, microparticles

## Introduction

The liver is a vital organ that is highly susceptible to fat accumulation, resulting in a condition known as fatty liver disease or hepatic steatosis (1). Although chronic alcohol consumption is a major cause of fatty liver disease (2), non-alcoholic fatty liver disease (NAFLD) is also common and is strongly associated with central obesity, insulin resistance, hyperlipidemia and the metabolic syndrome (3). Insulin resistance causes increased lipolysis and thus leading to high levels of plasma free fatty acids (FFAs), as well as increased FFA uptake by hepatocytes, which results in the formation of intracellular lipid droplets (4). Hepatic lipid accumulation can progress from simple steatosis to non-alcoholic steatohepatitis (NASH), which includes hepatocellular injury, inflammation and fibrosis (5). In addition, further severe complications may occur, such as liver cirrhosis and hepatocellular carcinoma (6).

In order to prevent and treat NAFLD, lifestyle changes including weight reduction and increased physical activity are considered as the first-line approach (7). Although pharmacologic therapy is mainly directed toward increasing insulin sensitivity using insulin-sensitizing medications, such as metformin and pioglitazone (8), the use of other medications is increasingly investigated. Medications used to treat dyslipidemia, such as gemfibrozil (a triglyceride lowering agent) and statins (HMG-CoA reductase inhibitors that reduce cholesterol synthesis in the liver), are among the agents investigated (9). Gemfibrozil has been found to be beneficial in the treatment of patients with NASH as it was able to significantly reduce the elevated levels of hepatic aminotransferases when compared with the control group (10). Similarly, atorvastatin was efficacious in the treatment of patients with NAFLD and dyslipidemia as it was effective in reducing hepatic aminotransferases and improving lipid profile (11,12). Although statins are administered with caution in patients with elevated aminotransferases due to the risk of statin-induced hepatotoxicity, this concern is not clinically important as statin-associated hepatic adverse effects are of low incidence, reversible and dose-dependent (13). Thus, statins should not be contraindicated in patients with NAFLD and elevated liver enzymes as they are promising medications for these conditions (9).

Through a review of the relevant literature using PubMed (http://www.ncbi.nlm.nih.gov/pubmed) and English as a

search language, the majority of studies were found to clinically evaluate the effect of statin use on reducing the elevated hepatic enzyme levels in patients with NAFLD (14-17).

A previous study evaluated the effect of statin therapy on hepatic lipid accumulation by comparing the liver density measured by computerized tomography prior to and following statin use for a certain period of time (12). Changes in the density reflected alterations in hepatic lipid accumulation in response to statin therapy. However, to the best of our knowledge, the efficacy of statins in reducing hepatic intracellular lipid accumulation has not been previously assessed *in vitro*. Therefore, the aim of the present study was to evaluate the effect of simvastatin on hepatic intracellular lipid accumulation on an *in vitro* model of NAFLD. A human hepatocellular carcinoma cell line ($HepG_2$) was exposed to oleic acid (OA), which is a monounsaturated omega-9 fatty acid, and this served as a model of NAFLD (18,19). Specifically, the study aimed to visualize and quantify OA-induced lipid accumulation in $HepG_2$ cells treated with various concentrations of simvastatin.

## Materials and methods

*Cell line*. $HepG_2$ cells were a gift of Professor David Morris at the Department of Surgery at St. George Hospital Clinical School (New South Wales, Australia). The cell line was originally obtained from the European Collection of Authenticated Cell Cultures (Salisbury, UK).

*Cell culture method*. $HepG_2$ cells were maintained in Dulbecco's modified Eagle's medium that contained 2 mM L-glutamine (both purchased from Lonza Australia Pty., Ltd., Mount Waverley, Australia) and 4.5 g/l glucose (Sigma-Aldrich, Castle Hill, Australia), and was supplemented with 10% fetal bovine serum (FBS; Sigma-Alrdich), 0.1% penicillin/streptomycin (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 24 mM sodium hydrogen carbonate and 25 mM HEPES [also known as 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] (both purchased from Lonza Australia Pty., Ltd.). Cells were incubated at 37˚C in a humidified atmosphere of 5% $CO_2$ in air (v/v). Cell growth and induction of cell death was monitored by phase contrast microscopy (Olympus CKS; Olympus Corporation, Tokyo, Japan) and by detecting the generation of $HepG_2$ cell-derived Annexin $V^+$ microparticles. Digitized images were generated using a SPOT CCD camera (Diagnostic Instruments, Inc., Sterling Heights, MI, USA) using SPOT version 2.1.2 software.

*Cell culture treatments*. $HepG_2$ cells were grown in 96-well plates at a density of $1x10^4$ cells/well until ~70% confluence was reached. Next, cells were deprived from FBS for 24 h before treatment with 0-10, 20 and 30 $\mu$M simvastatin (Sigma-Aldrich). Oleic acid (OA; Sigma-Aldrich) was dissolved at a concentration of 12 mM in phosphate-buffered saline (PBS; 137 mM NaCl, 10 mM phosphate, 2.7 mM KCl and pH 7.4) that contained 11% fatty acid-free bovine serum albumin (BSA; MP Biomedicals, Santa Ana, CA, USA) by sonification at a frequency of 10 KHz with two 5 min pulses (Soniprep 150 fitted with an exponential probe; Thermo Fisher Scientific, Inc.) prior to shaking at 37˚C for 15 h (20) using an OM10 Orbital Shaking Incubator (Ratek Instruments Pty, Ltd., Boronia, Australia). OA solution was filtered using a 0.22 $\mu$m filter and stored at 4˚C prior to use. $HepG_2$ cells were treated with increasing concentrations of OA solution (0-1 mM) for 24 h to determine the optimal concentration that induces cellular OA accumulation. To determine the effect of simvastatin on OA-induced $HepG_2$ cell steatosis, cells were treated with increasing concentrations of simvastatin (0-30 $\mu$M) for 24 h before treatment with 1 mM OA. Simvastatin was activated prior to use with NaOH as previously described (21), and according to the manufacturer's instructions.

*Oil Red O staining*. A stock solution of 0.35% Oil Red O (BDH Chemicals, Poole, England) in isopropanol was prepared, filtered twice using a 0.22 $\mu$m filter and diluted in double-distilled $H_2O$ ($ddH_2O$; 3:2) prior to use. To detect and quantify cellular lipid accumulation, OA-treated $HepG_2$ cells were gently washed with PBS and fixed using 4% paraformaldehyde for 1 h at room temperature (RT). Subsequently, the cells were washed twice using $ddH_2O$ and stained with Oil Red O solution for 20 min at RT. In order to remove the background staining, the cells were washed for 5 min with 60% isopropanol solution. Lipid droplet accumulation was detected by watching under the microscope. To quantify intracellular lipid accumulation, Oil Red O stain was extracted using pure isopropanol and the optical density was detected at 510 nm using a Spectramax 250 Plate reader; data analysis was performed using SoftMax Pro version 5.0 software (both purchased from Molecular Devices (UK), Ltd. (Wokingham, UK).

*Microparticle quantification*. $HepG_2$ cell-derived microparticles were stained and quantified as previously described (22,23). The technique was performed according to the guidelines established by the International Society of Thrombosis and Haemostasis on the standardization of platelet-derived microparticle enumeration by flow cytometry (24), along with modifications suggested in the manufacturer instructions of the BD FACSCanto flow cytometer (BD Biosciences, San Jose, CA, USA). The flow cytometer was calibrated to set the lower microparticles' detection limit as previously described (22). $HepG_2$ cells in culture media were centrifuged at 400 x g for 5 min at room temperature to remove cellular debris. Subsequently, 10 $\mu$l samples were incubated at RT for 30 min with Annexin V-APC (eBioscience, Inc., San Diego, CA, USA) to detect phosphatidylserine microparticle expression as a marker of vesicles derived from apoptotic cells. Appropriate dilutions were made using calcium-rich binding buffer as supplied by Annexin V-APC manufacturer. Absolute microparticle numbers were also determined as previously described (22) using TruCount counting tubes (BD Biosciences).

*Statistical analysis*. Data for continuous variables are expressed as the mean ± standard deviation of three independent experiments for each treatment. Data was analyzed using the Student's t-test (SPSS version 18.0; IBM SPSS, Amronk, NY, USA). $P<0.05$ was considered to indicate a statistically significant difference.

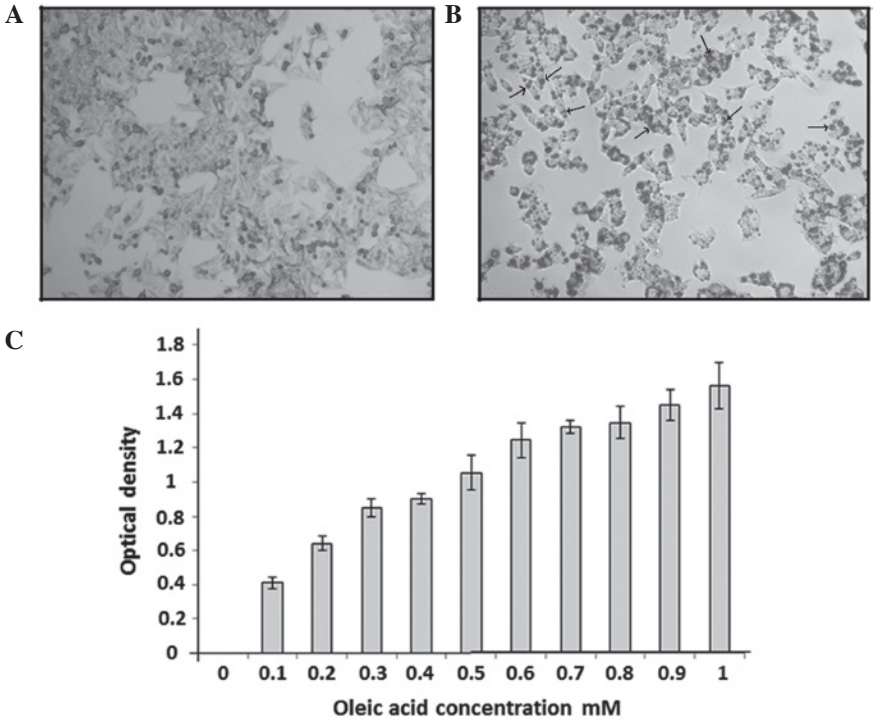


Figure 1. $HepG_2$ cells as a model of NAFLD. (A) Phase contrast microscopy image (magnification, x400) of untreated $HepG_2$ cells shows a lack of lipid accumulation revealed by a negative staining for Oil Red O. (B) Phase contrast microscopy image (magnification, x400) of $HepG_2$ cells treated with 1 mM OA shows positive staining for Oil Red O, which reveals lipid accumulation intracellularly. (C) Quantification of OA accumulation in $HepG_2$ cells treated with increasing concentrations (0-1 mM) of OA for 24 h, followed by Oil Red O staining and extraction for optical density measurement at 510 nm. OA accumulation was dose-dependent. Bar graphs represent the mean ± standard deviation of three experiments in duplicates. NAFLD, non-alcoholic fatty liver disease.

## Results

*OA induces dose-dependent $HepG_2$ cellular lipid accumulation*. High levels of plasma FFAs are implicated in the pathogenesis of NAFLD as they can accumulate in hepatocytes to form lipid droplets (4). This condition was recreated *in vitro* by treating $HepG_2$ cells with increasing concentrations of OA (0-1 mM). Intracellular lipid droplets were negatively stained for Oil Red O dye in the OA-untreated $HepG_2$ cells (Fig. 1A) and positively stained in the OA-treated cells (Fig. 1B). As shown in Fig. 1C, lipid accumulation in OA-treated cells was dose-dependent and the concentration of 1 mM OA was considered as an optimal concentration for the induction of lipid accumulation in $HepG_2$ cells as a model of NAFLD.

*Low simvastatin concentrations reduce OA-induced steatosis in $HepG_2$ cells*. To evaluate the effect of simvastatin on $HepG_2$ cellular lipid accumulation, cultured $HepG_2$ cells were exposed to increasing concentrations of simvastatin (0-10 $\mu$M) for 24 h before treatment with 1 mM OA. Subsequently, Oil Red O staining and extraction for optical density determination were performed in order to quantify the OA accumulation intracellularly. Fig. 2 shows that simvastatin was able to reduce OA accumulation in $HepG_2$ cells in a dose-dependent manner over a concentration range of 1-4 $\mu$M. Further increase in simvastatin dose (up to 10 $\mu$M) was not able to reduce OA accumulation more than ~40%. This effect was also confirmed by visualizing Oil Red O staining using phase contrast microscopy (data not shown).

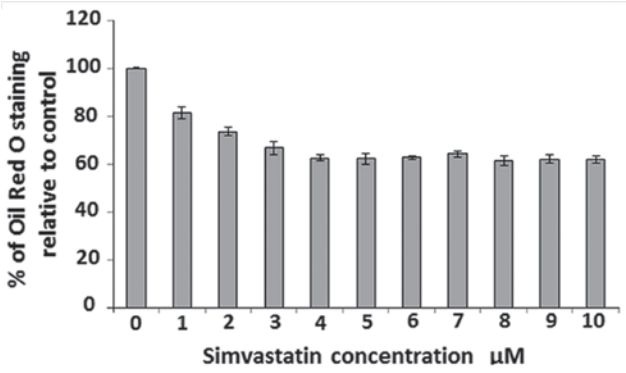


Figure 2. Low simvastatin concentrations reduce OA-induced steatosis in $HepG_2$ cells. Quantification of OA accumulation in $HepG_2$ cells exposed to increasing concentrations of simvastatin (0-10 $\mu$M) for 24 h before treatment with 1 mM OA and staining for Oil Red O. Bar graphs show that OA accumulation was reduced in a dose-dependent manner in cells exposed to 1-4 $\mu$M simvastatin. Doses between 5-10 $\mu$M simvastatin have similar effect on reducing OA-induced lipid accumulation. Bar graphs represent the percentage of OA accumulation relative to simvastatin-untreated $HepG_2$ cells (control group). Percentages represent the mean ± standard deviation of three experiments in duplicates.

*High simvastatin concentrations induce the release of $HepG_2$ cellular microparticles and morphological characteristics of cell apoptosis*. Microparticles are small (<1 $\mu$m in diameter) vesicles that originate from the plasma membranes of cells undergoing apoptosis (25). To evaluate the effect of simvastatin on $HepG_2$ cell growth and induction of cellular apoptosis, cultured $HepG_2$ cells were treated with low-dose (4 $\mu$M) and high-dose (20 and 30 $\mu$M) simvastatin for 24 h

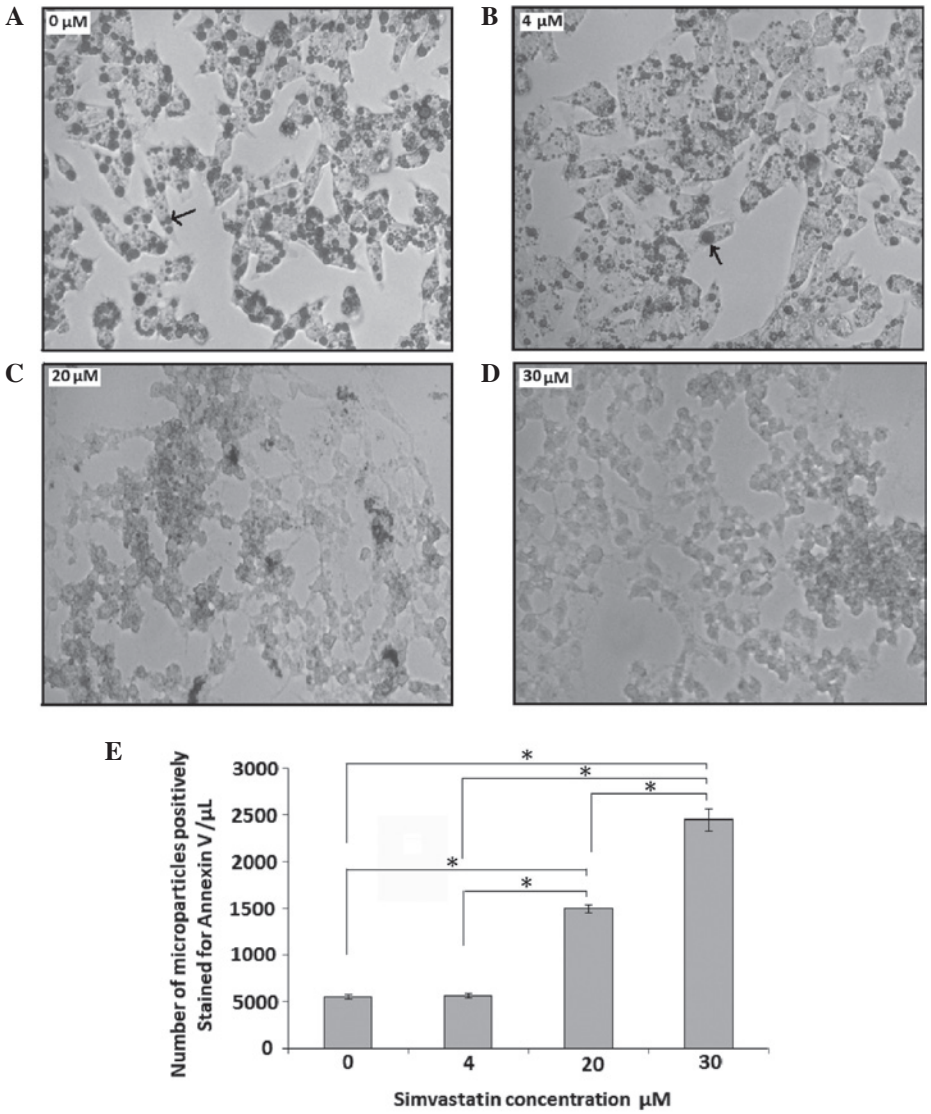


Figure 3. Effect of simvastatin on $HepG_2$ cell morphology and microparticle generation. Phase contrast microscopy images (magnification, x400) show the cellular morphology of $HepG_2$ cells treated with (A) 0 $\mu$M, (B) 4 $\mu$M, (C) 20 $\mu$M and (D) 30 $\mu$M simvastatin for 24 h, followed by treatment with 1 mM OA and staining for Oil Red O. Cells treated with 20 and 30 $\mu$M simvastatin showed apoptotic morphological changes including cell shrinkage and blebbing of cell membranes with reduced staining for Oil Red O when compared with cells treated with 0 and 4 $\mu$M simvastatin. (E) Bar graphs represent the average number of cell-derived microparticles positively stained for Annexin V per $\mu$l of cell culture media of $HepG_2$ cells treated with 0, 4, 20 and 30 $\mu$M simvastatin. Low-dose simvastatin (4 $\mu$M) did not induce microparticle generation, whereas high simvastatin doses (20 $\mu$M and 30 $\mu$M) induced generation of a larger number of microparticles compared with those released in the control (0 $\mu$M simvastatin). $^*P<0.05$. Bar graphs represent the mean ± standard deviation of three experiments.

and compared with the untreated control (0 $\mu$M simvastatin; Fig. 3A-E). Samples from cell culture media were analyzed by flow cytometry to count the number of Annexin $V^+$ microparticles released by the $HepG_2$ cells. As shown in Fig. 3, low-dose simvastatin (4 $\mu$M) did not induce any change in cell morphology (Fig. 3B) and did not induce an increase in the number of Annexin $V^+$ microparticles compared with that in the control group (Fig. 3E). By contrast, treatment high simvastatin doses (20 and 30 $\mu$M; Fig. 3C and D, respectively) induced changes in cellular morphology and significantly increased the generation of cell-derived Annexin $V^+$ microparticles compared with the control ($P<0.05$; Fig. 3E). As revealed by phase contrast microscopy, $HepG_2$ cells treated with 20 or 30 $\mu$M simvastatin prior to treatment with 1 $\mu$M OA showed reduced staining for Oil Red O compared with cells treated with 4 $\mu$M and the control cells (Fig. 3A-D).

## Discussion

OA-induced lipid accumulation in $HepG_2$ cells may act as an *in vitro* model for studying NAFLD. Consistent with other studies (18,26), the results of the present study demonstrated that lipid accumulation in $HepG_2$ cells in response to OA treatment was dose-dependent and can be easily quantified using an Oil Red O colorimetric technique. This technique is based on the staining of intracellular lipid droplets by Oil Red O, followed by stain extraction and measurement of optical density that is proportional to the intracellular lipid content. In agreement with the findings of Cui *et al* (18), the present study also revealed that $HepG_2$ intracellular lipid quantification using the aforementioned staining technique provided reliable measurements of intracellular lipid-droplet levels. Assay results were able to reflect the dose-dependent uptake of OA and appeared entirely consistent with lipid droplets stained with Oil-Red O,

as visualized using microscopy. In addition, intracellular Oil Red O-stained lipid droplets can be directly visualized using phase contrast microscopy. Therefore, quantification of OA-induced $HepG_2$ cell steatosis may act as a valuable model to study the pathogenesis of NAFLD and assess the effect of possible treatments for hepatic steatosis.

In the current study, pretreatment of $HepG_2$ cells with low simvastatin doses (4-10 $\mu$M) was able to reduce the OA-induced intracellular lipid accumulation by ~40%. By contrast, pretreatment with high simvastatin doses (20 and 30 $\mu$M) induced $HepG_2$ cellular apoptosis that was detected by changes in cell morphology and production of cell-derived Annexin $V^+$ microparticles. To the best of our knowledge, the present study quantified fatty acid-induced lipid accumulation in human hepatocellular carcinoma cells in response to simvastatin treatment for the first time. Previous similar studies have clinically assessing the efficacy of statin therapy in the treatment of patients with NAFLD by evaluating their effect on reducing elevated hepatic enzymes including aminotransferases and γ-glutamyl transferase (14-17). Recently, de Keyser *et al* have also shown that statin therapy for >2 years was associated with a lower prevalence of hepatic steatosis among overweight subjects (27). This protective effect was considered to be associated with the ability of statins to improve the lipid profile by inhibiting the HMG-CoA reductase pathway and by acting as anti-inflammatory, anti-oxidant and immune-modulatory agents (27-29). However, no previous study has shown any direct effect for statins on hepatic lipid accumulation at the cellular level. At the organ level, statins, which can reduce elevated serum triglyceride concentrations (30), may decrease hepatic lipid accumulation in overweight and obese subjects by reducing the serum levels of triglycerides and fatty acids (4).

Using an *in vitro* model of NAFLD to quantify cellular lipid accumulation in response to simvastatin treatment is considered the main strength of the current study in comparison with other clinical studies. Although the mechanism through which simvastatin can reduce intracellular $HepG_2$ lipid accumulation was not determined in the current study, this model can be used in the future to study possible mechanisms or to investigate other possible treatments for NAFLD. However, there are certain limitations in the present study. Using $HepG_2$ cells to investigate the effect of simvastatin or other HMG-CoA reductase inhibitors on intracellular lipid accumulation may be inappropriate or should be used carefully, since certain previous studies have demonstrated that simvastatin can induce growth inhibition and apoptosis in $HepG_2$ cells (31,32). However, this effect was dependent on the concentration of simvastatin and the duration of cell exposure to simvastatin. For instance, Huang *et al* (31) and Kah *et al* (32) have shown that treatment of $HepG_2$ cells with 8 or 10 $\mu$M simvastatin for 72 h induced ~50% decrease in cell viability. Huang *et al* (31) have also shown that lower simvastatin doses (2 and 4 $\mu$M) for shorter period of time (24 h) induced ~20% decrease in $HepG_2$ cell viability. In the current study, $HepG_2$ cell viability in response to simvastatin treatment was not measured as an indicator of cell apoptosis; by contrast, the number of Annexin $V^+$ microparticles in the culture media was detected as an indicator of cell apoptosis, beside examining the cell morphology under a microscope as shown in Fig. 3. Furthermore, treatment of $HepG_2$ cells with low simvastatin dose (4 $\mu$M) for 24 h in the current study was able to reduce lipid accumulation by ~40% without increasing the number of Annexin $V^+$ microparticles or changing cell morphology, which suggests that this dose reduces lipid accumulation without inducing cell apoptosis. However, treatment of $HepG_2$ cells with high doses of simvastatin (20 and 30 $\mu$M) for 24 h was found to induce increased generation of Annexin $V^+$ microparticles and changes in cell morphology that were suggestive of cell apoptosis (Fig. 3). These results are consistent with the findings of Relja *et al* (33), which showed that $HepG_2$ cell apoptosis was induced by high doses of simvastatin (32 and 64 $\mu$M).

Determining the underlying mechanism by which low simvastatin concentrations can reduce $HepG_2$ intracellular lipid accumulation was out of the scope of the current study. However, more research is required to determine how simvastatin can produce this effect and whether other statins have similar effects on hepatic lipid accumulation. Possible roles for statins that deserve further investigations include their effect on fatty acid uptake by hepatic cells and their effect on hepatic intracellular triglyceride formation, which is stored as lipid droplets.

In conclusion, the present study demonstrated that low simvastatin concentrations can reduce $HepG_2$ intracellular OA-induced lipid accumulation without inducing cell death, whereas high simvastatin concentrations induced $HepG_2$ cell apoptosis, as revealed by detecting $HepG_2$ cell-derived Annexin $V^+$ microparticles and changes in cell morphology. These findings support the results of previous clinical studies that encourage administration of statin therapy for the prevention of NAFLD (14-17). Additionally, the current study has shown that OA-treated $HepG_2$ cells may act as a model for studying other possible treatments for NAFLD.

## Acknowledgements

The corresponding author would like to thank Dr. Rick Thorne and Dr. Lisa Lincz as they did not hesitate to support this work by providing sufficient lab space, equipment, cell line, materials and advice to perform this study. The project was supported by the HMRI Research Grant (grant no. 10-08), funded by the Lions District 201 N3 Diabetes Foundation.

## References

1. Green CJ and Hodson L: The influence of dietary fat on liver fat accumulation. Nutrients 6: 5018-5033, 2014.
2. Baraona E and Lieber CS: Effects of ethanol on lipid metabolism. J Lipid Res 20: 289-315, 1979.
3. Souza MR, Diniz Mde F, Medeiros-Filho JE and Araújo MS: Metabolic syndrome and risk factors for non-alcoholic fatty liver disease. Arq Gastroenterol 49: 89-96, 2012.
4. Marchesini G, Brizi M, Morselli-Labate AM, Bianchi G, Bugianesi E, McCullough AJ, Forlani G and Melchionda N: Association of nonalcoholic fatty liver disease with insulin resistance. Am J Med 107: 450-455, 1999.
5. Pais R, Pascale A, Fedchuck L, Charlotte F, Poynard T and Ratziu V: Progression from isolated steatosis to steatohepatitis and fibrosis in nonalcoholic fatty liver disease. Clin Res Hepatol Gastroenterol 35: 23-28, 2011.
6. Onnerhag K, Nilsson PM and Lindgren S: Increased risk of cirrhosis and hepatocellular cancer during long-term follow-up of patients with biopsy-proven NAFLD. Scand J Gastroenterol 49: 1111-1118, 2014.

7. Centis E, Marzocchi R, Suppini A, Dalle Grave R, Villanova N, Hickman IJ and Marchesini G: The role of lifestyle change in the prevention and treatment of NAFLD. Curr Pharm Des 19: 5270-5279, 2013.
8. Angelico F, Burattin M, Alessandri C, Del Ben M and Lirussi F: Drugs improving insulin resistance for non-alcoholic fatty liver disease and/or non-alcoholic steatohepatitis. Cochrane Database Syst Rev: CD005166, 2007.
9. Tolman KG and Dalpiaz AS: Treatment of non-alcoholic fatty liver disease. Ther Clin Risk Manag 3: 1153-1163, 2007.
10. Basaranoglu M, Acbay O and Sonsuz A: A controlled trial of gemfibrozil in the treatment of patients with nonalcoholic steatohepatitis. J Hepatol 31: 384, 1999.
11. Hyogo H, Tazuma S, Arihiro K, Iwamoto K, Nabeshima Y, Inoue M, Ishitobi T, Nonaka M and Chayama K: Efficacy of atorvastatin for the treatment of nonalcoholic steatohepatitis with dyslipidemia. Metabolism 57: 1711-1728, 2008.
12. Kiyici M, Gulten M, Gurel S, Nak SG, Dolar E, Savci G, Adim SB, Yerci O and Memik F: Ursodeoxycholic acid and atorvastatin in the treatment of nonalcoholic steatohepatitis. Can J Gastroenterol 17: 713-718, 2003.
13. Calderon RM, Cubeddu LX, Goldberg RB and Schiff ER: Statins in the treatment of dyslipidemia in the presence of elevated liver aminotransferase levels: A therapeutic dilemma. Mayo Clin Proc 85: 349-356, 2010.
14. Eslami L, Merat S, Malekzadeh R, Nasseri-Moghaddam S and Aramin H: Statins for non-alcoholic fatty liver disease and non-alcoholic steatohepatitis. Cochrane Database Syst Rev 12: CD008623, 2013.
15. Egan M and Prasad S: PURLs: Statins for patients with nonalcoholic fatty liver? J Fam Pract 60: 536-538, 2011.
16. Nseir W and Mahamid M: Statins in nonalcoholic fatty liver disease and steatohepatitis: Updated review. Curr Atheroscler Rep 15: 305, 2013.
17. Dima A, Marinescu AG and Dima AC: Non-alcoholic fatty liver disease and the statins treatment. Rom J Intern Med 50: 19-25, 2012.
18. Cui W, Chen SL and Hu KQ: Quantification and mechanisms of oleic acid-induced steatosis in HepG2 cells. Am J Transl Res 2: 95-104, 2010.
19. Liu JF, Ma Y, Wang Y, Du ZY, Shen JK and Peng HL: Reduction of lipid accumulation in HepG2 cells by luteolin is associated with activation of AMPK and mitigation of oxidative stress. Phytother Res 25: 588-596, 2011.
20. Susztak K, Ciccone E, McCue P, Sharma K and Böttinger EP: Multiple metabolic hits converge on CD36 as novel mediator of tubular epithelial apoptosis in diabetic nephropathy. PLoS Med 2: e45, 2005.
21. Rossi J, Rouleau L, Tardif JC and Leask RL: Effect of simvastatin on Kruppel-like factor2, endothelial nitric oxide synthase and thrombomodulin expression in endothelial cells under shear stress. Life Sci 87: 92-99, 2010.
22. Alkhatatbeh MJ, Enjeti AK, Acharya S, Thorne RF and Lincz LF: The origin of circulating CD36 in type 2 diabetes. Nutr Diabetes 3: e59, 2013.
23. Robert S, Poncelet P, Lacroix R, Arnaud L, Giraudo L, Hauchard A, Sampol J and Dignat-George F: Standardization of platelet-derived microparticle counting using calibrated beads and a Cytomics FC500 routine flow cytometer: A first step towards multicenter studies? J Thromb Haemost 7: 190-197, 2009.
24. Lacroix R, Robert S, Poncelet P, Kasthuri RS, Key NS and Dignat-George F; ISTH SSC Workshop: Standardization of platelet-derived microparticle enumeration by flow cytometry with calibrated beads: Results of the International Society on Thrombosis and Haemostasis SSC collaborative workshop. J Thromb Haemost 8: 2571-2574, 2010.
25. Hargett LA and Bauer NN: On the origin of microparticles: From 'platelet dust' to mediators of intercellular communication. Pulm Circ 3: 329-340, 2013.
26. Gomez-Lechón MJ, Donato MT, Martínez-Romero A, Jiménez N, Castell JV and O'Connor JE: A human hepatocellular in vitro model to investigate steatosis. Chem Biol Interact 165: 106-116, 2007.
27. de Keyser CE, Koehler EM, Schouten JN, Visser LE, Hofman A, Janssen HL and Stricker BH: Statin therapy is associated with a reduced risk of non-alcoholic fatty liver in overweight individuals. Dig Liver Dis 46: 720-725, 2014.
28. Endo A: The discovery and development of HMG-CoA reductase inhibitors. J Lipid Res 33: 1569-1582, 1992.
29. Liao JK and Laufs U: Pleiotropic effects of statins. Annu Rev Pharmacol Toxicol 45: 89-118, 2005.
30. Stein EA, Lane M and Laskarzewski P: Comparison of statins in hypertriglyceridemia. Am J Cardiol 81: 66B-69B, 1998.
31. Huang X, Ma J, Xu J, Su Q and Zhao J: Simvastatin induces growth inhibition and apoptosis in HepG2 and Huh7 hepatocellular carcinoma cells via upregulation of Notch1 expression. Mol Med Rep 11: 2334-2340, 2015.
32. Kah J, Wüstenberg A, Keller AD, Sirma H, Montalbano R, Ocker M, Volz T, Dandri M, Tiegs G and Sass G: Selective induction of apoptosis by HMG-CoA reductase inhibitors in hepatoma cells and dependence on p53 expression. Oncol Rep 28: 1077-1083, 2012.
33. Relja B, Meder F, Wilhelm K, Henrich D, Marzi I and Lehnert M: Simvastatin inhibits cell growth and induces apoptosis and G0/G1 cell cycle arrest in hepatic cancer cells. Int J Mol Med 26: 735-741, 2010.