

Clofibrate Attenuates ROS Production by Lipid Overload in Cultured Rat Hepatoma Cells

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ABSTRACT - PURPOSE: To investigate the effect of clofibrate on inducing liver fatty acid binding protein (FABP1) following a high-fat load in a hepatocyte cell culture model. **METHODS:** Rat hepatoma cells (CRL-1548) were treated with a fatty acid (FA) mixture consisting of oleate:palmitate (2:1) in the presence of 3% albumin. Cells were treated with 0, 0.5, 1, 2, or 3 mM FA for 24 and 48 hr, or further treated with 500 μ M clofibrate (CLO) to induce FABP1 levels. Cytotoxicity was determined using the WST-1 assay. Intracellular lipid droplets were quantitated following staining with Nile Red. Dichlorofluorescein (DCF) was used to assess the extent of intracellular reactive oxygen species (ROS). **RESULTS:** Cell viability decreased ($p < 0.01$) with an increase in lipid concentration. Intracellular lipid droplets accumulated significantly ($p < 0.001$) with an increase in long-chain fatty acid load, which was associated with a statistical increase ($p < 0.05$) in ROS levels. Early clofibrate treatment showed significant increases in intracellular FABP1 levels with significant decreases in ROS levels ($p < 0.05$). Silencing FABP1 expression using siRNA revealed that FABP1 was the main contributor for the observed intracellular ROS clearance. **CONCLUSIONS:** Characteristic cellular damage resulted from released ROS following a high fat load to hepatoma cells. The damage was attenuated through early treatment with clofibrate, which may act as a hepatoprotectant by inducing FABP1 expression and in this manner, suppress intracellular ROS levels.

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INTRODUCTION

A high fat diet may result in non-alcoholic fatty liver disease (NAFLD), which is the most common cause of chronic liver disease in many Western countries (1-3) and is increasing in prevalence in many Asian countries (4). NAFLD encompasses a spectrum of hepatic pathologies that have the potential to progress to end-stage liver disease. A major histological hallmark feature of NAFLD is the lipid accumulation in the form of lipid droplets (LDs) within hepatocytes (5). Although the exact pathogenesis of NAFLD is still under investigation, the loss of metabolic competency may be attributed to oxidative stress (6), which causes increased lipid peroxidation products (7), peroxidation of lipids in the hepatocyte membrane (8), cytokine over-production (9), decreased antioxidant capacity (10), and impaired hepatic function of specific antioxidant enzymes (11). To counteract oxidative

stress, antioxidant therapies have been introduced to help patients fight against NAFLD (12-14).

One potential therapeutic antioxidant is liver fatty acid-binding protein (FABP1), a 14.4 kDa protein abundantly found in the cytosol and nucleus of hepatocytes (15, 16). FABP1 cDNA stably transfected Chang cells showed a high resistance toward a hydrogen peroxide challenge (17). The cyclic redox reactions involving the reducing methionine groups in FABP1 act as the endogenous scavengers of reactive oxygen species (ROS) (18). Moreover, clofibrate could up-regulate the expression of FABP1 in 1548 rat hepatoma cells in a time- and dose-dependent manner (19, 20), resulting in a reduction in ROS induced by hydrogen peroxide (19, 20).

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Since an enhanced oxidative stress is associated with a high-fat intake, it is reasonable to investigate whether FABP1 can be utilized as an endogenous antioxidant in cells incubated with a high fat load. Thus, the hypothesis to be tested in this study was that FABP1 could protect hepatocytes against free radicals generated following a high fat-load using cell cultures. The aims of the present study were to establish and characterize cell cultures following a high fat load and evaluate the cytoprotective effects of inducible FABP1 on hepatocellular antioxidant status following clofibrate modulation of FABP1 expression.

METHODS

Materials

HyClone classical liquid media Minimum Essential Medium with Earle's Balanced Salts (MEM/EBSS) was purchased from Thermo Fisher Scientific (Nepean, Ontario, CA). Oleate, palmitate, fatty-acid-free bovine serum albumin, fetal bovine serum, pyruvate, L-glutamine, penicillin, streptomycin, trypsin-EDTA, Nile Red, 2,7-dichlorofluorescein diacetate (DCFDA), 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI), mouse anti- β -actin primary antibody, and poly-L-lysine hydrochloride were purchased from Sigma-Aldrich (Oakville, Ontario, CA). Rat hepatoma cell line 1548 was purchased from American Type Culture Collection (ATCC, Rockville, MD). Donkey anti-rabbit IgG, sheep anti-mouse IgG and enhanced chemiluminescence (ECL Plus) Western blotting kit were purchased from GE Healthcare (Pittsburgh, USA). FABP1 polyclonal antibody was generated in our laboratory as previously reported (21). Cell Proliferation Reagent WST-1 was purchased from Roche (Laval, Quebec, CA).

Cell Culture Condition

Rat hepatoma cell line 1548 was grown in MEM/EBSS supplemented with 100 U/mL penicillin, 100 μ g/mL streptomycin, 10% fetal bovine serum, 4 mM L-Glutamine and 0.011% sodium pyruvate in a humidified, 37°C incubator in an atmosphere of 95% air and 5% CO₂. Cells were maintained in 75 cm² culture flask (Corning, MA, USA) until required.

Fatty Acid and Clofibrate Treatment

Cells were seeded at a density of 2.5 x 10⁴ cells/well (for WST-1, intracellular lipid droplet quantification

and dichlorofluorescein (DCF) assay, 96-well plates) and 1 x 10⁵ cells/well (for Nile Red Staining, 12-well plates) in MEM/EBSS medium and incubated overnight for adherence. The next day cell culture medium was replaced with freshly prepared medium containing the fatty acid mixture oleate:palmitate (2:1) in presence of 3% fatty-acid-free bovine serum albumin. Cells were treated with 0, 0.5, 1, 2, and 3 mM FA mixture for 24 and 48 hr at 37°C in a humidified incubator in an atmosphere of 95% air and 5% CO₂. Clofibrate was used to increase levels of FABP1 in treated cell cultures. Concentration of clofibrate was based on its effective dose reported previously (18, 20, 22). Clofibrate (500 μ M) was dissolved in dimethyl sulfoxide (DMSO) and later added to the medium (DMSO < 0.1% v/v in final volume). Control cells were incubated with DMSO alone. Four different cell treatments were performed in the current study as summarized in Table 1, including 1-day FA treatment, 2-day FA treatment, early clofibrate intervention and late clofibrate intervention.

Table 1. FA and clofibrate treatments in the present study.

	Day 1	Day 2	Day 3	Day 4
1-day FA	FA			
2-day FA	FA	FA		
Early CLO	FA	FA+CLO	CLO	
Late CLO	FA	FA	CLO	CLO

siRNA in vitro Transfection

Transfection of rat FABP1 siRNA (synthesized by GE Healthcare Dharmacon Inc., Ottawa ON, Canada) was conducted using Lipofectamine™ 2000 Transfection Reagent (Thermo Fisher Scientific) according to the manufacture's protocol and previous study (19). The RNA sense sequence of rat L-FABP siRNA was 5'-r(GAUGGAGGGUGACAAUAAA)dTdT-3'. The RNA antisense sequence of rat L-FABP siRNA was 5'-r(UUUAUUGUCACCCUCCAUC)dTdT-3'. Briefly, 8 x 10⁴ 1548 cells were seeded in each well of a 24-well plate containing antibiotic-free medium 24 hour prior to transfection. A working siRNA solution (40 pmol) was prepared in culture medium and incubated with cells for 24 hr before challenged with various FA medium (or in early clofibrate intervention, 1 day before the challenge and second day during the challenge). Subsequently, intracellular lipid and ROS quantitation as well as

FABP1 Western blot were performed as described below.

Intracellular Lipid Droplets Observation

Nile Red is a fluorescent dye that stains lipid droplets (23) while DAPI is a commonly used dye for cell nucleus staining. After 48 hr treatment with fatty acids, cell morphology was observed using a Nikon Diaphot inverted optical microscope. Nile Red and DAPI fluorescent staining further assessed the intracellular accumulation of lipid droplets. Cells were seeded at a density of 1×10^5 cells/well and cultured on sterilized cover slips coated with 0.2 mg/ml Poly-L-Lysine in 12-well plates. After 48 hr FA treatment, cells were washed and fixed in 4% formaldehyde in PBS at room temperature for 30 min. Intracellular lipid droplets were stained with 1 μ g/ml Nile Red in PBS and cell nucleus was stained with 5 μ g/ml DAPI in PBS at room temperature for 15 min in the dark. After the final PBS wash, the cover slips were mounted onto glass slides with 20 μ L self-made anti-fade mounting medium (0.1% P-phenylenediamine and 90% glycerol in PBS, adjusted to pH 8.0-9.0 with 0.2 M sodium bicarbonate buffer, pH 9.2) and observed using a fluorescent microscope.

Assessment of Intracellular Lipid Droplets

Following FA treatment, cells in each well of the 96-well plate were rinsed with 200 μ L warm PBS. Nile Red (2 μ g/ml) and DAPI (1 μ g/ml) were prepared in PBS to co-stain cells. Cells in each well were stained with 100 μ L of the co-stain solution at 37°C for 10 min in the dark. After washing cells with warm PBS once, additional 200 μ L PBS was added onto each well. Fluorescence was analysed using a Wallac 1420 multilable counter (Perkin Elmer; Woodbridge, Ontario, CA) with excitation set at 485 nm and emission at 535 nm for Nile Red while excitation was set at 355 nm and emission at 460 nm for DAPI. Nile Red intensity in each well was normalized by the corresponding DAPI signal.

Cell Viability Assay

The cell proliferation reagent WST-1 is based on the metabolic activity of viable cells. WST-1 (10 μ L/well) was added to cells that had already been treated with FA (0, 0.5, 1, and 2 mM) for 5 days followed by 1 or 2 hr incubation with WST-1 reagent according to manufacturer's criteria to acquire the best sensitivity. Cell viability was measured using an ELx 808 Ultra Microplate Reader (BIO-TEK

Instrument. INC; Winooski, VT, USA) with absorbance being set at 450 nm.

Protein Extraction and Western Blot

After treatment, control and treated cells in culture flasks were washed and harvested. Protein extraction was performed using PBS (supplemented with 1mM PMSF, 5 μ g/ml aprotinin, 10 μ g/ml leupeptin) and sonicated using a Fisher Sonic Dismembrator (Model 300, FisherScientific, USA). After centrifugation, supernatants were collected and aliquoted for BCA protein quantitation (Pierce) following manufacture's protocol.

The protein sample (60 μ g) mixed with sample loading buffer (0.0625 M Tris-HCl, 10% glycerol, 2% SDS, 0.1% bromophenol blue, 5% β -mercaptoethanol, pH 6.8) was resolved on a 12% SDS-PAGE gel at a constant voltage of 90 Volts using a Bio-Rad Miniprotein II Electrophoresis Cell. The resolved proteins were transferred onto Immunogoblin-P PVDF membranes (EMD Millipore, Toronto Canada). After blocking with 5% powdered skim milk in tris-buffered saline (TBS), FABP1 was detected by a polyclonal anti-sera raised against rat FABP1 as described previously (21) in TBS (at 1:200 dilution) containing 5% skim milk, followed by a secondary donkey anti-rabbit IgG antibody at 1:5,000 dilution in TBS containing 0.1% Tween-20 (TBST) and 2% skim milk. Housekeeping gene β -actin production was detected following a similar procedure. Mouse anti- β -actin primary antibody was used at 1:2,000 dilution in TBST for primary incubation and sheep anti-mouse IgG antibody was used at 1:1,000 dilution for detection. Bands were visualized using an enhanced chemiluminescence Western blotting kit (ECLPlus system, GE Healthcare). The densitometry of the target protein bands were determined using ImageJ software (Frederick, USA).

Assessment of Intracellular ROS

Fluorescence intensity of DCF from reactive oxygen species (ROS) formed in cell cultures was assessed using the Wallac 1420 multilable counter and a DCF fluorescence assay (24). Briefly, 10 mM (4.87 mg/ml) stock solution of 2,7-dichlorofluorescein diacetate (DCFDA) was prepared freshly in ethanol and stored at -20°C until required and diluted to 100 μ M with the FBS-free media prior to each study. Following incubation with fatty acids, cells were washed twice with warm FBS-free media to remove the extracellular albumin and incubated with 100 μ M

DCFDA at 37 °C in the dark for 30 min. After washing once with pre-warmed PBS, cells were lysed using 90% DMSO in PBS. The DCF fluorescent intensity in each well was measured using a fluorescence plate reader with excitation set at 485 nm and emission at 535 nm. Cells were then lysed and total protein in each well quantitated via Bradford assay (Bio-Rad). DCF intensity in each well was normalized with the corresponding total protein (μg).

STATISTICAL ANALYSES

Data are presented as mean \pm SEM. The n value refers to number of replicates performed for each study. Statistical analyses were carried out by *t* test (unpaired) where 2 groups were compared while a one-way ANOVA was used for multiple comparisons followed by a Bonferroni post-test using GraphPad Prism 5.0. Statistical differences with $p < 0.05$ were taken as significant.

RESULTS

Cell Morphology

Intracellular lipid accumulation was detected by both optical microscopy observation (Figure 1) and Nile

Red staining (Figure 2) after 48 hrs incubation in medium containing the FA mixture. As FA concentration increased, intracellular lipid droplets increased in a dose-dependent manner. Lipid droplet density and diameter increased in the cytoplasm when cells were exposed to a high fat environment.

Intracellular Neutral Lipid Accumulation Assessment

Similarly, the FA-treated groups showed a dose-dependent accumulation of lipids with and without FABP1 siRNA treatment (Figure 3). In the absence of siRNA Treatment, 0.5, 1, and 2 mM FA groups showed $224.3 \pm 56.9\%$ (mean \pm SEM), $339 \pm 49.6\%$, and $927 \pm 158.4\%$ accumulation of lipid, respectively, compared to 0 mM group without siRNA treatment ($p < 0.05$ for 0.5 mM group and $p < 0.001$ for other two). Cells treated with FABP1 siRNA, showed similar increased trend of FA dose-dependent lipid accumulation of $222.5 \pm 65.9\%$, $290.3 \pm 70.5\%$, and $785.3 \pm 124.3\%$ ($p < 0.001$ only for 2 mM group in comparison to siRNA treated 0 mM group). siRNA treatment did not result in any significant differences in lipid accumulations in all groups.

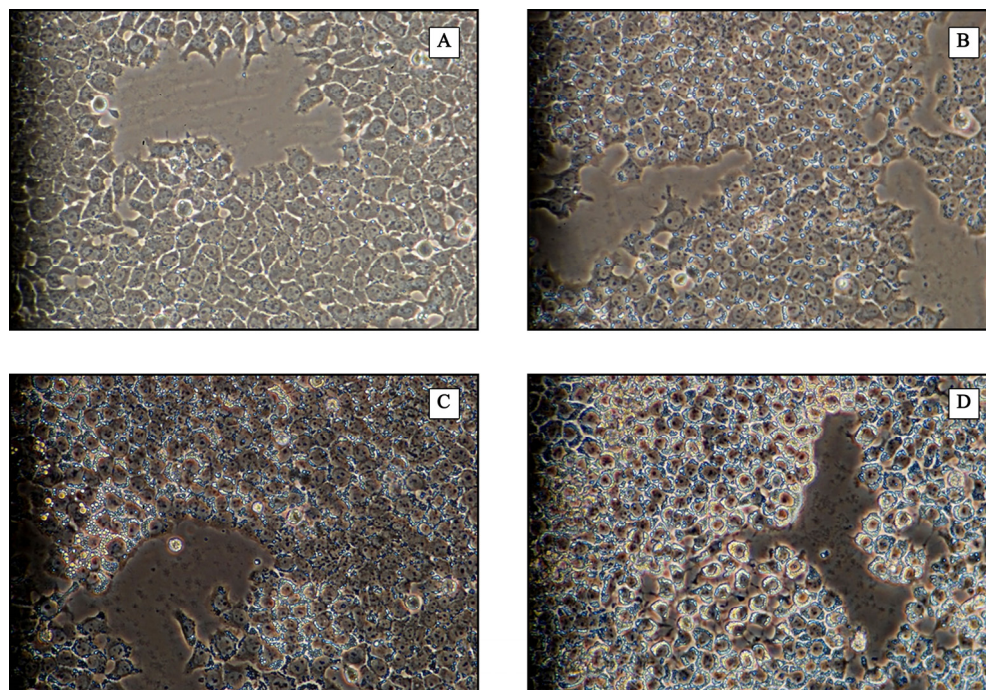


Figure 1. Optical microscopy observations of 1548 cells treated with various concentrations of FA. Cells were grown in MEM/EBSS medium containing oleate and palmitate (2:1) mixture in presence of 3% BSA either untreated (A), or cells treated with 0.5 mM FA (B), or 1 mM FA (C), or 2 mM FA (D). Cell morphology greatly changed when treated with different concentrations of FA (20X magnification).

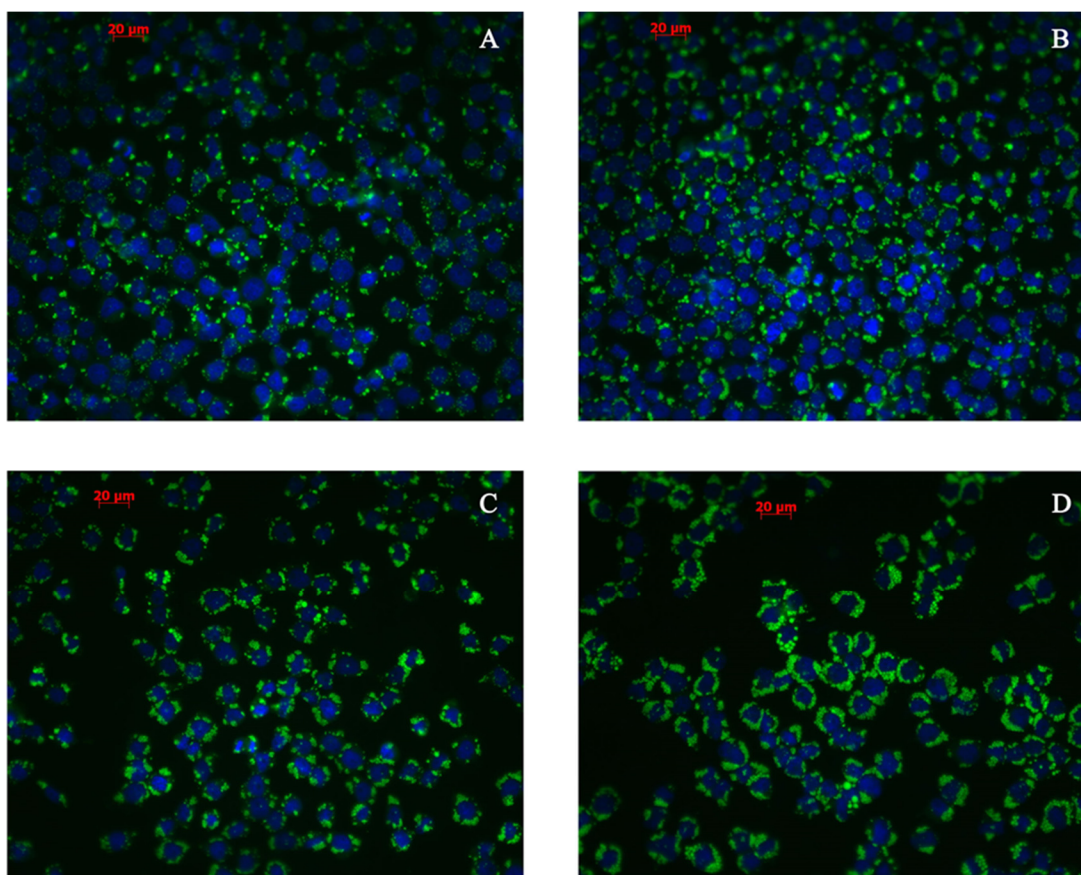


Figure 2. Fluorescent microscopy observations of 1548 cells treated with various concentrations of FA, stained with Nile Red and DAPI. Cells were grown in MEM/EBSS medium containing oleate and palmitate (2:1) mixture in presence of 3% BSA in untreated (A), or treated with 0.5 mM FA (B), 1 mM FA (C), or 2 mM FA (D). Cytoplasmic lipid droplets were stained in green, surrounding the nucleus of the cells, which was stained in blue by DAPI. The green signal in control cells (A) was thought to be endogenous background (20X magnification).

Cellular Viability

Lipotoxicity induced by FA in the rat hepatoma cells using the cell proliferation reagent WST-1 is shown in Figure 4. FA treatment induced a dose-dependent lipotoxicity in 1548 cells. After the first day of treatment, cell viability in the 2 mM FA group was greatly reduced. Nearly half of the cells were nonviable because of the high fat load. There was no viability loss in the 0.5 and 1 mM FA treated groups. On the second day of FA treatment, 1548 cells showed a $4.7 \pm 8.6\%$, $35.9 \pm 4.8\%$, and $74.4 \pm 1.5\%$ (mean \pm SEM) decrease in viability at concentrations of 0.5 mM, 1 mM, and 2 mM of FA, respectively. FA treatment with the 1 mM and 2 mM groups statistically reduced cell viability ($p < 0.01$). There was no statistical difference between the 0.5 mM FA treated and control groups. Moreover, there were no statistical differences between treated groups from 2-

day though 5-day, suggesting that a 2-day incubation of FA is sufficient to induce lipotoxicity in 1548 cells.

FABP1 Levels

Western blot analysis was used to detect expression of FABP1 in control and FA treated cells (Figure 5). One-day FA treatment did not induce any statistical changes in FABP1 expression in any high fat treated experimental group (Figure 5A). Statistical increases in expression of FABP1 occurred only at the 1 mM FA group following 2-day high fat treatment (Figure 5B, $p < 0.05$). FABP1 expression in the 2 mM FA treated group was not statistically different from the control group (Figure 5B). After clofibrate administration, FABP1 expressions in all groups increased, of which 1 mM showed statistical increases in FABP1 expression in two FA-clofibrate

combination treatment groups (Figure 5, C and D; $p < 0.01$). Employment of FABP1 siRNA treatment significantly reduced FABP1 expressions in all FA treated groups in 2D FA treatment (Figure 5E) and early clofibrate intervention (Figure 5F). No statistical difference in FABP1 expressions were observed in all FA and FABP1 siRNA treated groups compared to the 0 mM group (Figure 5, E and F).

Intracellular Free Radical Levels

The diacetate ester of DCF (DCFDA) was employed to measure free radical levels as it easily diffuses into cells and is hydrolyzed by intracellular esterases to the fluorescent product DCF. Figure 6 shows that FA significantly enhanced oxidative stress in a dose-dependent manner in the absence of any CLO interventions. One-day treatment of various FA (1D FA groups in Figure 6 A-D) resulted in $151.3 \pm 5.0\%$, $266.2 \pm 21.2\%$, and $331.7 \pm 19.4\%$ increased ROS levels in the 0.5 mM, 1 mM, and 2 mM FA treated groups, respectively compared to the 0 mM group. ROS production was further increased after two-day FA (2D FA groups in Figure 6A-D) treatment leading to $310.4 \pm 21.3\%$, $514.6 \pm 43.2\%$, and $688.5 \pm 48.3\%$, in the 0.5 mM, 1 mM, and 2 mM

FA treated groups respectively. After early clofibrate treatment (early groups in Figure 6A-D) cellular ROS levels were lowered to $223.8 \pm 17.1\%$, $349.1 \pm 27.5\%$, and $445.3 \pm 30.0\%$ in the 0.5 mM, 1 mM, and 2 mM FA treated groups, respectively. When clofibrate was introduced as a late intervention (late groups in Figure 6A-D), ROS levels were reduced at a lesser extent; $307.6 \pm 20.9\%$, $483.8 \pm 41.0\%$, and $585.8 \pm 37.5\%$ in the 0.5 mM, 1 mM, and 2 mM FA treated groups, respectively. Statistically decreased total ROS level was observed in early clofibrate treatment but not late clofibrate treatment (Figure 6, C and D). Furthermore, since early clofibrate intervention demonstrated better protective outcome, we evaluated ROS generation in the absence of FABP1 by knocking down FABP1 expression using siRNA treatment (Figure 7). Significantly increased intracellular ROS level ($p < 0.001$) were observed in all FA groups with FABP1 silencing even with early clofibrate intervention. Up to 1.8, 2.2, and 1.9-fold of ROS elevations were shown in 0.5, 1, and 2 mM FA groups, respectively. Similarly, increased ROS levels were also observed in 2D FA groups with FABP1 siRNA silencing.

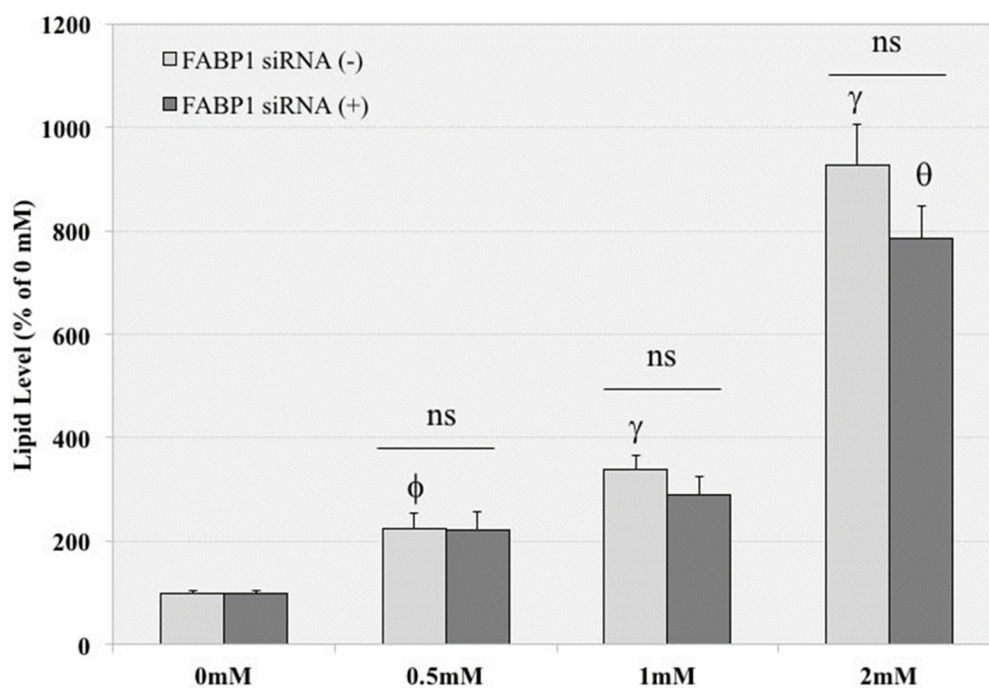


Figure 3. Lipid accumulation in 1548 cells following 2-day treatment with differing concentrations of FA with or without FABP1 siRNA. Cells were cultured in black 96-well plates. Nile Red fluorescence (485 nm excitation/535 nm emission) was normalized by DAPI signal (355 nm excitation/460 nm emission) in each well and results are expressed as % of control. All treated groups had statistically increased intracellular lipid content compared to control group. Values are mean \pm SEM, $n=4$, ϕ , $p < 0.05$; γ , $p < 0.001$ (compared to 0 mM group without FABP1 siRNA); θ , $p < 0.001$ compared to 0 mM group with FABP1 siRNA. ns, no statistically difference.

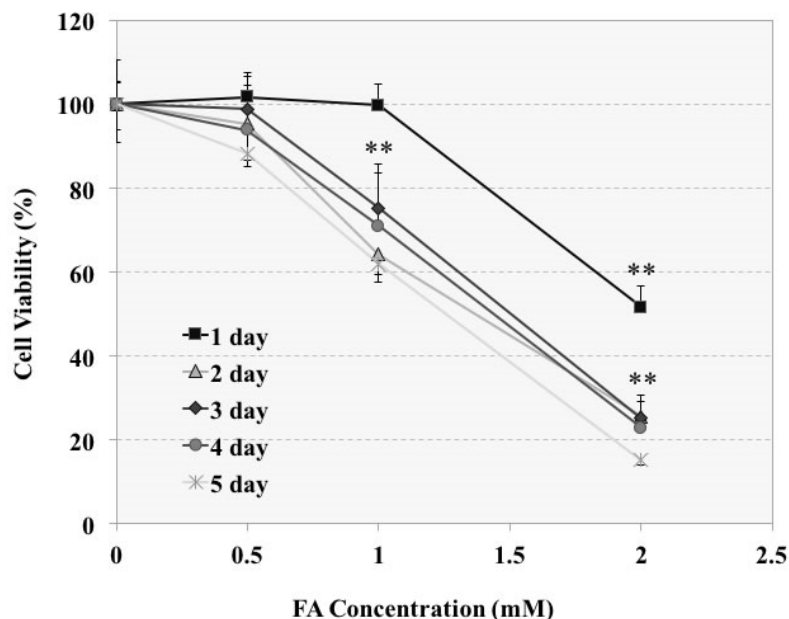


Figure 4. Cell viability of 1548 cells during the 5-day FFA treatment using WST-1 cell proliferation assay. Significant loss of cell viability was observed on the first day FFA treatment in 2 mM group comparing to control (** $p < 0.01$). Loss of cell viability in 1 mM was found on the second day treatment, which is also statistical significant comparing to control (** $p < 0.01$). There were no significant different between 0.5 mM and control group in 2-day treatment. All FFA treated groups showed no further significant change in cellular viability from day 2 to day 5. Values are mean \pm SEM, $n = 10$.

DISCUSSION

Establishment of Cell Cultures for Studying FABP1 in NAFLD

Well-defined cell culture NAFLD models enable researchers to study the effect of lipid overload upon hepatocytes, the main characteristics of NAFLD, within a short period of time by directly exposing cells to high concentrations of fatty acids. The rationale for using oleic and palmitic acids to induce a high fat load in hepatocytes was that these FA are the most abundant FA in liver triglycerides in NAFLD patients (25). Saturated fatty acids such as palmitate have been shown to be responsible for the induction of lipoapoptosis in NAFLD (26, 27), while unsaturated fatty acids such as oleate may render hepatocytes vulnerable to oxidative stress (28). The extent of lipid accumulation (Figure 3, ~3.4-fold increase in 1 mM group without siRNA treatment) observed in the present study was comparable to that reported in other cell culture models using primary hepatocytes from NAFLD patients (3.8-fold) (29). Abolishment of FABP1 expression could result in a decline of lipid accumulation in cytosol due to the reduction of fatty acid uptake (30), which may

explain the observed decreased lipid accumulation in FABP1 siRNA treated groups (Figure 3).

Lipototoxicity

Lipoapoptosis is a characteristic feature of NASH (31) that is specifically induced by saturated fatty acids (32, 33). The dose-dependent pattern of decreased cell viability observed in H4IIE-derived 1548 cells may be attributed to the palmitate-induced endoplasmic reticulum (ER) stress, eventually resulting in calcium-mediated mitochondrial cytochrome c release and the activation of the intrinsic apoptotic pathway (34, 35). In addition, Gomez-Lechon et al reported an oleate:palmitate ratio of 2:1 over a concentration range of 0-2 mM was associated with similar time- and dose-dependent cytotoxicity in isolated human hepatocytes and HepG2 cells (29), providing the rationale for choosing the FA dose range and ratio in the current study to induce a lipid overload in 1548 cells. Our data demonstrated a similar decreasing cell viability pattern (29). The statistical decrease in cell viability after exposure to 1 mM FA on the second day (Figure 4) suggested that hepatoma cell lines may be more sensitive to FA and less tolerant of lipid overload induced lipoapoptosis.

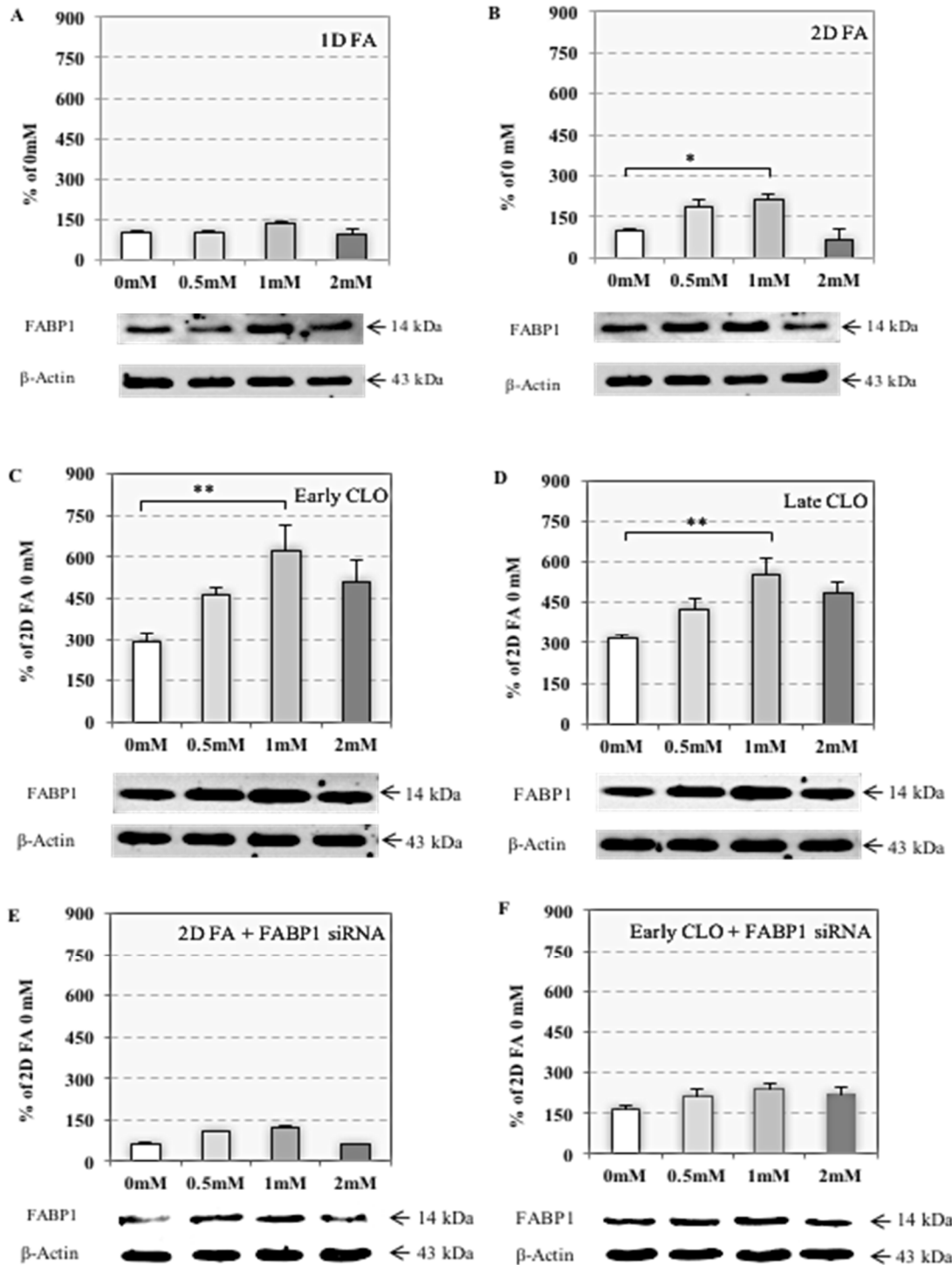


Figure 5. FABP1 expression following clofibrate and FABP1 siRNA treatments. Cells treated with (A) 1-day FA, or (B) 2-day FA, or (C) early clofibrate treatment (1-day FA followed by co-treatment of FA and clofibrate on the second day followed by clofibrate-only treatment on the third day), or (D) late clofibrate treatment (2-day FA followed by 2-day clofibrate treatment), or (E) 2-day FA in the presence of FABP1 siRNA and, (F) early clofibrate treatment in the presence of FABP1 siRNA. Bottom - Histogram representing integrated density values for FABP1 from four different experiments. CLO, clofibrate. Band densities were quantified and expressed as mean \pm SEM. * $p < 0.05$, ** $p < 0.01$.

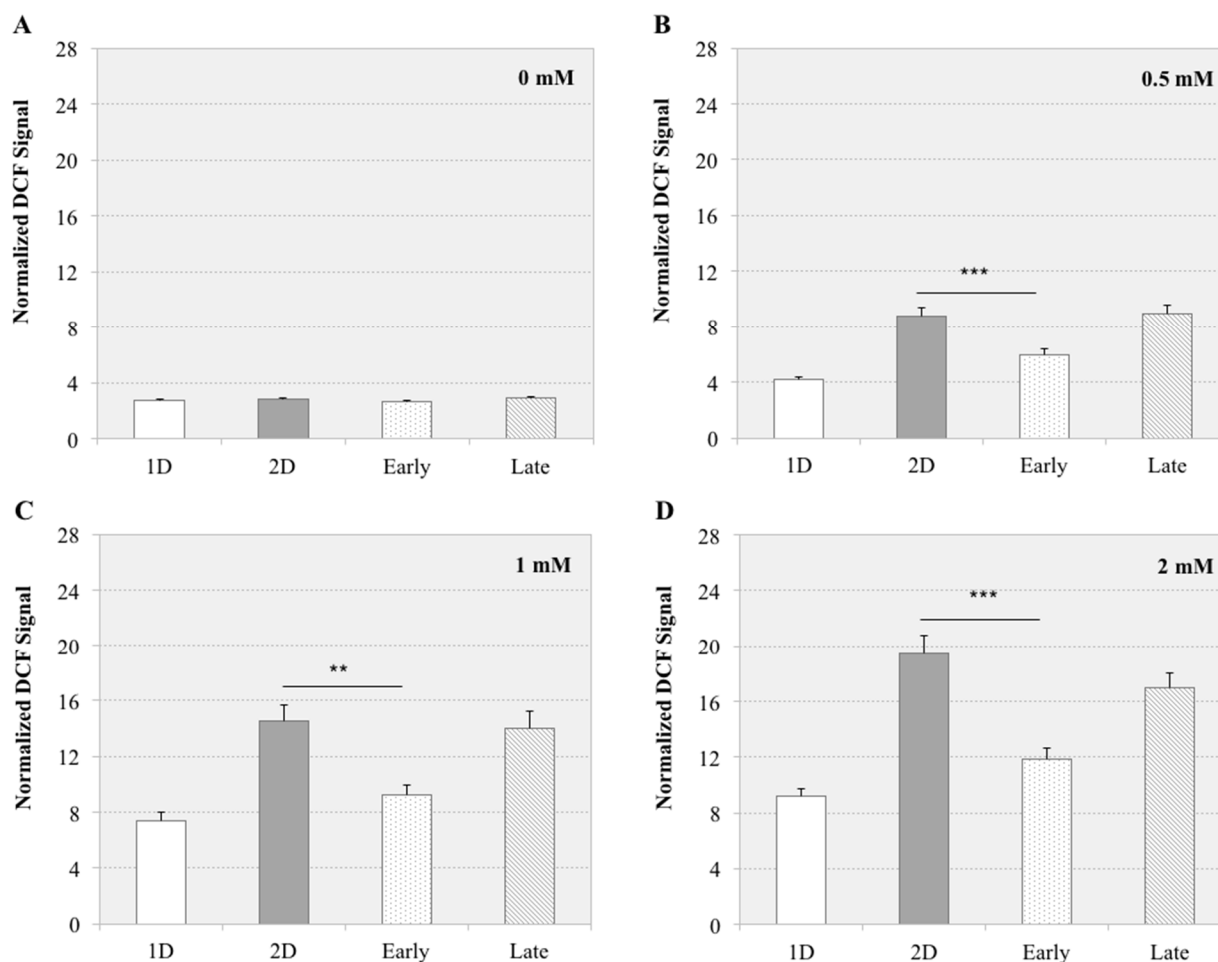


Figure 6. Comparison of ROS production with or without clofibrate interventions. Comparison of (A) 0 mM FA treated cells with (B) 0.5 mM FA treated, (C) 1 mM FA treated and (D) 2 mM FA treated cells. Cells were exposed to 1D / 2D FA treatment, or 2D FA treatment followed by early / late clofibrate intervention. DCF fluorescence was recorded by fluorescence plate reader with excitation 485 nm and the emission 535 nm. Results are normalized by the total protein (μg) in each well and expressed as DCF signal per μg of total protein. ROS production in all FA-treated groups were compared to control (0 mM, no FA treated) in each experiment. Early - early clofibrate intervention; Late - late clofibrate intervention. Values are mean \pm SEM, $n = 4$, ** $p < 0.01$, *** $p < 0.001$.

Intracellular ROS Generation

ROS have been found to be the main contributor to hepatic lipid peroxidation. Elevated cytochrome P450 2E1 (CYP2E1) activity (36) and palmitate-induced mitochondrial ROS over-production (37) may contribute to the augment of intracellular ROS in the current study. Although, palmitic and oleic acids have been reported to induce CYP2E1 expression in primary human hepatocyte cultures (38), there is no direct evidence indicating inducible expression of CYP2E1 in 1548 cells. However, FAO cells, similarly derived from the same hepatoma H-35 strain like the 1548 cells (39), were reported to constitutively express inducible CYP2E1 (40, 41).

Thus, it could be argued that induced CYP2E1 activity may be one of the possible contributors for the elevated ROS production in the current lipid overload model, but further work is needed in this area. Elevated ROS production may be closely correlated to the accumulation of FA induced mitochondrial dysfunction. Nakamura et al found a 58% increase in intracellular ROS production induced by palmitate at 0.25 mM within 8 hr of treatment. The increased ROS was dose-dependent (37) and considered to be due to the impaired flux of electrons into the mitochondrial respiratory chain resulting in an abnormal reduction of oxygen. In the present study, the 1548 rat hepatoma cells were

challenged with a higher concentration of palmitate, implying that a similar mechanism may be responsible for the observed time- and dose-dependent ROS over-production.

Enhanced FABP1 Levels Contribute to Reduced Intracellular ROS

The various residues in proteins differ in their susceptibility to oxidation by ROS (42). Amongst them, the sulphur containing residues cysteine and methionine are the most easily oxidized amino acids by ROS (43). In cells, methionine participates in oxidation processes through redox cycling reactions, the so-called "methionine regeneration", and thus implementing its repair over oxidative modified proteins (44). FABP1 is an attractive endogenous intracellular antioxidant by containing multiple methionine groups in its amino acid sequence. The rat 1548 cell line was chosen because it contains more methionine residues than human FABP1 (18, 45). Using the 1548 cell line together with clofibrate treatment gives rise to very high levels of inducible intracellular antioxidant capacity since clofibrate increases FABP1 mRNA half-life by up to 25 hrs and induces FABP1 expression through enhanced transcription of the FABP1 gene via activation of PPAR α (19) and the hepatocellular level of FABP1 itself is relatively high (close to 0.4 mM) (46). We previously demonstrated over-expressing FABP1 (17) or inducing FABP1 with clofibrate (20) was able to elevate intracellular antioxidant capacity against ROS without significant changes of superoxide dismutase, glutathione peroxidase and catalase activities (19, 20). In the current study, we intended to evaluate the ROS-scavenging role of FABP1 as an inducible intracellular antioxidant under a high long-chain fatty acid environment. Two different combined treatments of FA and clofibrate in 1548 cells were compared in terms of cytosolic ROS level alteration and FABP1 expression. Early clofibrate intervention (but not late) significantly attenuated ROS formation in all 2-day FA-treated groups (Figure 6, B, C and D) associated with more than a 2-fold increased FABP1 expression (Figure 5, B and C). To investigate if FABP1 being the most significant contributor for the intracellular ROS clearance, we knocked down FABP1 expression in the presence of clofibrate during the early intervention (Figure 5F). With FABP1 silenced, significant elevations of ROS were observed in all FA-treated groups even with early clofibrate intervention (Figure 7, B, C and D). Therefore, the

abolished FABP1 up-regulation in the presence of clofibrate could be directly correlated to the increased intracellular ROS (Figure 7). Although late clofibrate intervention induced comparable FABP1 expressions (Figure 5D), less reduction of ROS was observed (Figure 6). This may imply that an early induction of FABP1 to achieve a lower ratio of FA load to FABP1 level might offer better protection against lipid induced free radical damage. Possible mechanisms include binding excessive lipids and their oxidation products and to target them to mitochondria and peroxisomes to stimulate their oxidation (47). Another plausible explanation as previously reported by our group was that FABP1 might act as an antioxidant (18), trapping the ROS thus inactivating them with the help of the methionine residues in FABP1, thus breaking the lipid peroxidation chain reaction. This notion was consistent with the finding that less lipid peroxidation products were observed after clofibrate-induced PPAR α induction in a methionine and choline deficient mouse NASH model (48).

In conclusion, an *in vitro* hepatocellular steatosis NAFLD model was established with lipid accumulation through overloading rat hepatoma cells with various concentrations of FA mixtures at the set proportion (2:1) of saturated (palmitic acid) to unsaturated (oleic acid) FA. This *in vitro* model demonstrated typical patterns of NAFLD including elevated lipid accumulation, increased lipotoxicity and augmented free radical generating properties. The present study shows and suggests that early pharmacological treatment with clofibrate (and drugs that induce FABP1 mRNA) intervention may improve hepatocellular steatosis through lowering intracellular ROS content with evidence of FABP1 as the main antioxidant contributor. This may be correlated to the induced FABP1 expression and antioxidant function of this protein. Thus, FABP1 may act as an effective antioxidant for the early stage of NAFLD to prevent the hepatocellular damage induced by oxidative stress. The finding that late intervention of clofibrate to induce FABP1 did not improve the outcome of free radical scavenging to the same extent compared to early intervention, supports the notion of using combination treatment of antioxidants and cytoprotectants as effective therapeutic modality to fight against NAFLD.

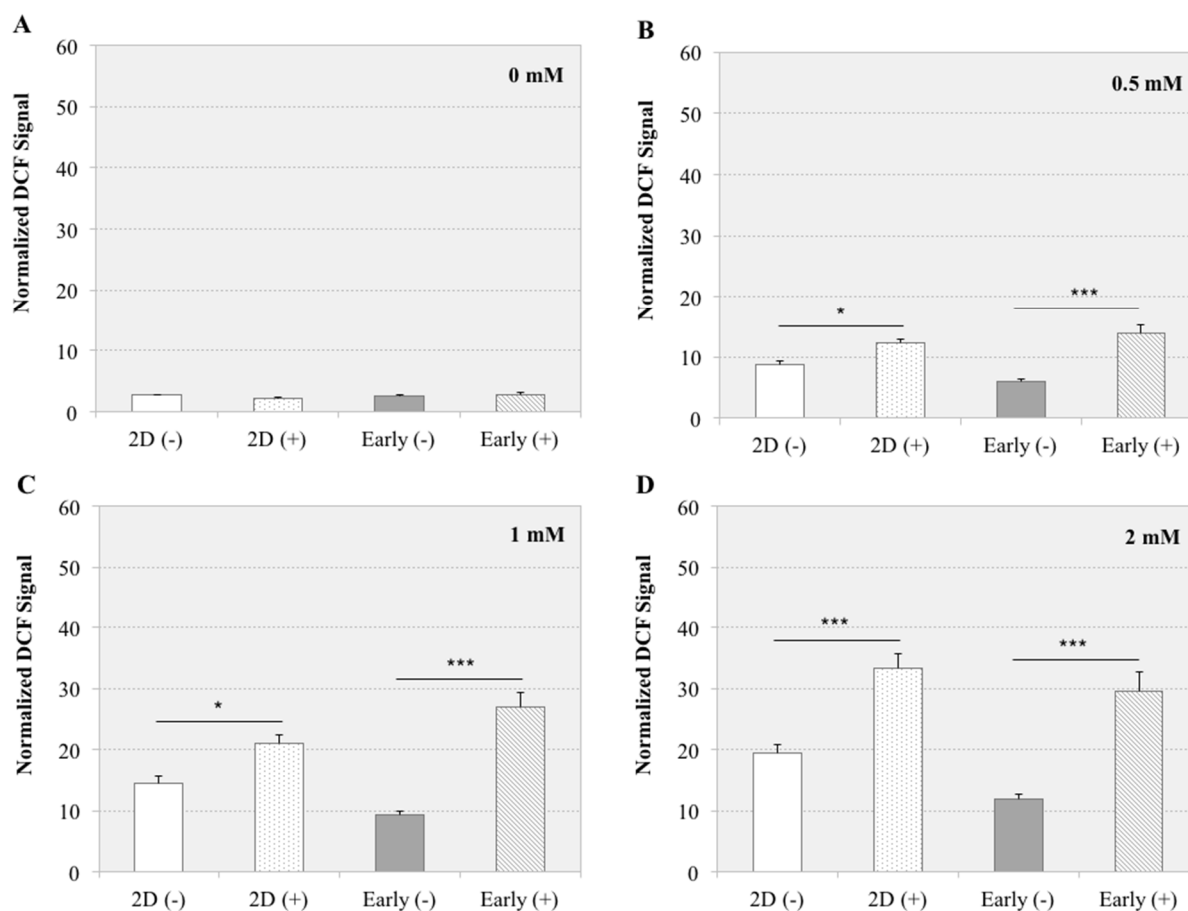


Figure 7. Comparison of ROS Production with or without FABP1 siRNA treatment. Comparison of (A) 0 mM FA treated cells with (B) 0.5 mM FA treated, (C) 1 mM FA treated and (D) 2 mM FA treated cells. Cells were exposed to 2D FA treatment or 2D FA treatment followed by early clofibrate intervention with (+) or without (-) FABP1 siRNA treatment. Results are normalized by the total protein (μg) in each well and expressed as DCF signal per μg of total protein. Normalized values were presented at the base of each bar. Dashed line indicates the basal line of normalized signal. ROS production in all FA-treated groups were compared to control (0 mM, no FA treated) in each experiment. Early - early clofibrate intervention. Values are mean \pm SEM, $n = 4$, * $p < 0.05$, *** $p < 0.001$.

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