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Original Article

Understanding the early onset of intracellular lipid accumulation induced by oleic and palmitic acids in HepG2 cells

Esther Jebarani Elangovan, Rihana Habeeb Rakuman, Adaikkalam Vellaichamy*

Centre for Biotechnology, Anna University, Chennai 600 025, Tamil Nadu, India

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Abstract

Increasing hepatic lipid accumulation is the primary cause of non-alcoholic fatty liver disease (NAFLD), which has become an emerging health concern globally. Many studies have used the HepG2 cell-based in vitro model of NAFLD to investigate intracellular lipid accumulation after several hours of exposure to free fatty acids (FFAs). However, the molecular mechanisms underlying the early onset of lipid accumulation are yet to be unveiled. In this study, we examined oleic acid (OA) and palmitic acid (PA)- induced lipid accumulation in HepG2 cells at early time points, i.e., in minutes. Using Oil Red O staining and fluorescence microscopy imaging, we observed a time-dependent increase in intracellular lipid accumulation in cells treated with 0, 0.25, 0.5, and 1.0 mM FFAs. Notably, significant lipid droplet formation was detected within 15 min of OA treatment at 0.5 and 1.0 mM concentrations compared to the control, whereas PA did not elicit such an early response. Gene expression analysis revealed upregulation of genes related to lipid metabolism (SREBF1, PDK4, and G6PC), beta-oxidation (CPT1a) and cholesterol synthesis (HMGCR) at the early time point. Additionally, immunoblot analysis showed increased expression of Fatty acid synthase (FASN), which is a well-known marker of lipogenesis. In summary, our findings indicate that OA induces lipid accumulation more robustly than PA at early time points, providing insights into the molecular changes at the onset of NAFLD progression.

Keywords: Oleic acid, Palmitic acid, Lipid accumulation, Early onset, NAFLD.

1. Introduction

The liver plays a vital role in key metabolic processes such as the uptake of FFA, lipogenesis, and lipolysis, managing lipid elimination through oxidation and secretion. Dysregulation of lipid metabolism can lead to several metabolic disorders, including obesity and NAFLD [1]. The prevalence of NAFLD is rapidly increasing in Asia (~29%) and is projected to affect 55.7% of the global population by 2040 [2,3]. Currently, nearly one-third of patients with NAFLD are under 30 years of age, putting them at a higher risk of developing liver cancer [4]. The primary hallmark of NAFLD is lipid accumulation in the hepatocytes, which can lead to non-alcoholic steatohepatitis (NASH), chronic cirrhosis, and ultimately liver cancer [5]. Therefore, a thorough understanding of the molecular mechanisms of lipid accumulation from its early onset is essential.

Fatty acids (FAs) are stored as lipid droplets (LDs) that accumulate as fat-storing organelles within hepatocytes. The LDs act as an energy source during starvation and cellular stress conditions [6]. These droplets are highly dynamic, undergo continuous fusion and fission, and interact with other cellular organelles to maintain lipid homeostasis. Structurally, LDs consist of a core made of triglycerides and sterol esters, surrounded by a phospholipid monolayer and associated proteins [7,8].

Palmitic acid (PA) and oleic acid (OA) are the most abundant FAs in hepatic triglycerides. Many studies have used PA, OA, and mixtures of PA/OA to develop steatotic cell-line models [9,10]. PA constitutes 20-30% of triglycerides (TG) and phospholipids (PL); participates in protein S-palmitoylation and de novo lipogenesis (DNL) [11]. However, excessive PA uptake causes lipotoxic effects, such as inflammation, ER stress, autophagy, mitochondrial oxidative damage, and cell death [12]. OA can be obtained from the diet or can be synthesized in the body from stearic acid via stearoyl-CoA 9-desaturase [13]. OA has protective effects against various metabolic diseases by improving lipid metabolism and reducing lipotoxic effects caused by PA and other FAs [14,15].

Several studies have examined the effects of OA and PA on HepG2 cells at various time points, ranging from 1 to 48 h [10]. Eynaudi et. al., reported a 30-fold increase in LD accumulation accompanied by the upregulation of PLIN5 and MFN2 proteins just after 2 h of OA treatment [16]. Another study in which HUH-7 cells treated with OA showed that free fatty acid receptor 4 (FFAR4) plays a role in the early lipid droplet development, which is primar-

* Corresponding author.
E-mail address: adaiks98@yahoo.com (A. Vellaichamy).
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ily dependent on endogenous lipids [17]. Therefore, more research is needed to uncover the mechanisms underlying the early onset of lipid accumulation, especially with an elevated exposure to FAs, as in the case of NAFLD. The current investigation aimed to study the effects of OA and PA on HepG2 cells during early time points towards understanding the development of cellular stress leading to NAFLD.

2. Materials and methods

2.1. Cell culture and treatment

The HepG2 cell line was obtained from the National Centre for Cell Science, Pune, India. Cells were cultured in Dulbecco modified Eagle's medium (AL007A; Himedia) containing 10% fetal bovine serum (RM10416; Himedia) and 1% penicillin-streptomycin (15140122; Gibco) in a constant 5% CO2 atmosphere at 37 °C. After 80% confluence, the cells were serum-starved for 24 h and treated with 0, 0.25, 0.5, and 1.0 mM concentrations of PA (P0500; Sigma), OA (O1008; Sigma), and PA/OA combined (1:1) for 15, 30, and 60 min duration. The stock solutions were prepared by conjugating PA or OA with FFA-free BSA (TC348; Himedia). The working concentrations of FFA were chosen based on the previous NAFLD model of in-vitro studies [9,10].

2.2. Cell viability assay

HepG2 cells were seeded at a density of 6 x 10^3 cells per well in a 96-well plate. Treatment with PA, OA, and PA/OA was given at concentrations of 0, 0.25, 0.5, and 1.0 mM for 0, 15, 30, and 60 min duration. The cell viability was assessed using the MTT assay. In brief, after removing the cell culture medium, the cells were incubated with 0.5 mg/ml of MTT reagent (TC191; Himedia) for 1h at 37 °C. Then, the formazan crystals formed were dissolved in DMSO, and the absorbance was measured at 570 nm using a microplate reader (BioTek Instruments).

2.3. Lipid accumulation and quantification

Following an early time-course treatment of HepG2 cells with FAs (at 0, 15, 30, and 60 min durations) in a 24-well plate, the cells were washed twice with 1X PBS and fixed with 4% paraformaldehyde (GRM3660; Himedia). The intracellular lipid droplets were stained with 3 mg/ ml Oil Red O (ORO) (TC256; Himedia) for 30 min. The unbound ORO stain was removed with 1X PBS wash, followed by nuclear counterstaining with DAPI (D9542; Sigma). Fluorescent images were captured at 40X magnification using a Nikon Eclipse Ts2 microscope (Nikon Instruments Inc.). Quantification of intracellular lipid accumulation was performed using images captured in phase-contrast mode and analyzed using ImageJ software.

2.4. Real-time PCR analysis

After 30 min of OA exposure (1.0 mM) in 60 mm cell culture dishes, total RNA was extracted from approximately 3 x 10^6 cells using Tri-reagent (T9424; Sigma). 1.0 µg of RNA from each treatment group was reverse-transcribed to make cDNA using the iScript cDNA synthesis kit (1708891; Bio-Rad). Quantitative real-time PCR was performed on a Step-One Plus Real-time PCR system using a SYBR Fast Universal Kit (KK4600; Kapa Biosystems). The expression levels of the target genes, viz., sterol regulatory element-binding transcription factor 1 (SREBF1), pyruvate dehydrogenase kinase 4 (PDK4), glucose-6-phosphatase (G6PC), and carnitine palmitoyltransferase I alpha (CPT1a) and 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR), were assessed by using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the internal control.

2.5. Immunoblot analysis

Cells were seeded in 100 mm culture dishes and treated with control (i.e., 0.25% and 0.5% BSA) and OA (0.25 and 0.5 mM) for 30 min. After washing twice with ice-cold 1X TBS, cell lysates were prepared in 250 µL of RIPA buffer (R0278; Sigma) containing 1.0 mM PMSF (10837091001; Roche). Lysates were sonicated on ice using an ultrasonic probe sonicator for 10 s and repeated three times to ensure homogenization. Following centrifugation at 12000 rpm for 20 min at 4 °C, the supernatant containing soluble proteins was collected. The concentration of protein in the sample was measured using the Bradford assay (5000205; Bio-Rad) by taking absorbance at 595 nm in a microplate reader. Proteins (20 µg) from each treatment group were separated on a 10% SDS-PAGE gel and transferred onto a 0.45 µm PVDF membrane (10600023; Amersham) at 20V for 1 h. Membranes were then blocked with 5% skimmed milk powder in TBST (0.1% Tween-20) for 1 h and incubated with primary antibody for fatty acid synthase (FASN, 1:1000 dilution, Cat #C20G5; Cell Signaling Technology - CST) and GAPDH (1:1000 dilution, Cat #0411; Santacruz Biotechnology) overnight at 4 °C. After three washes, the membranes were incubated for 1 h at room temperature with anti-rabbit (1:5000 dilution, Cat #7074P2; CST) and anti-mouse (1:5000 dilution, Cat #7076P2; CST) secondary antibodies, respectively. Protein bands were visualized using an ECL substrate (SuperSignal™ West Femto Maximum Sensitivity Substrate, Cat #34095; Pierce) and imaged via Chemdoc System (Bio-Rad). Band intensities were quantified with ImageLab software (Bio-Rad).

2.6. Statistical analysis

Statistical analysis of the data obtained from MTT assay, intracellular lipid quantification, and quantitative RT PCR was performed using Student's t-test available from the Microsoft Excel tool (Microsoft Office version 16.17). The outcome values with $p<0.05$ were treated as statistically significant.

3. Results

3.1. Effects of FFA on cell viability at early time points

The cytotoxic effects of PA, OA, and their combination (PA/OA at 1:1 ratio) at various concentrations were studied (Figure 1). At 1.0 mM concentration, OA maintained approximately 90% of cell viability until the 60-minute time point. We found that even at 15 min of treatment with PA, significant reduction in cell viability (i.e., 40% cell death) was observed.

The combined PA/OA treatment showed a cytotoxic effect similar to that of PA alone at the 30-minute time point. This effect is observed even at 60 min (Figure 1).

3.2. Lipid droplet formation at early time points

The intracellular lipid accumulation profile obtained in response to FFA treatment at early time points is given in Figure 2A. It is observed that with 15 min of treatment, the 0.25 mM OA increased the intracellular lipid accumu-

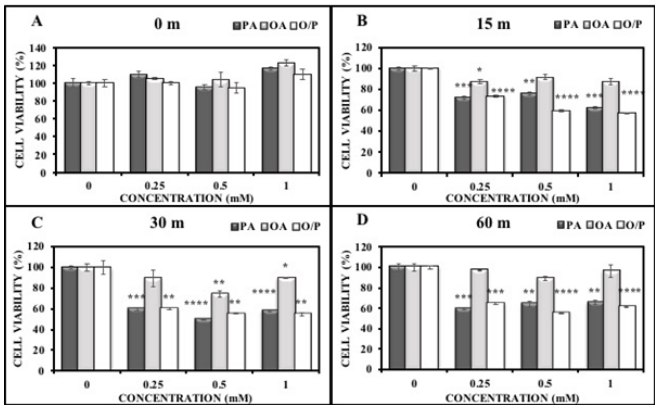

Fig. 1. Cell viability of HepG2 cells treated with Oleic and Palmitic acids. MTT assay was performed on HepG2 cells treated with PA, OA, and PA/OA, each at 0.25, 0.5, and 1.0 mM concentrations at A) 0 min, B) 15 min, C) 30 min, and D) 60 min time points. The data are presented as means ± SD of three replicates. *= treatment vs control, p<0.05; **= treatment vs control, p<0.01; ***= treatment vs control p<0.001; ****=treatment vs control, p<0.0001.

lation when compared to its control. The OA-dependent lipid accumulation increased with an increase in treatment concentrations and an increase in treatment time points, i.e., maximal at 1mM treatment for 60 min. However, treatment with PA led to only a minimal increase in intracellular lipids, even at 1.0 mM treatment given for 60 min. It is interesting to note that PA/OA combination treatment showed fluorescence levels comparable to OA treatment (Figure 2A).

Lipid droplet quantification data obtained from the phase-contrast microscopy analysis are in line with the above-observed fluorescence microscopy results. At 15 minutes of treatment with OA, 0.25 mM and 1.0 mM treatment concentrations led to 1.9- and 2.4-fold increase in intracellular lipids, respectively. Additionally, with 1.0 mM OA treatment, a 2.4 and 3.6-fold increase of lipids was observed, respectively, at 15 and 60 min time points. However, the concentration and time-dependent increase of lipid accumulation was not apparent in treatments with PA (Figure 2B). As expected [18], the lipid accumulation analysis performed with a 24 h treatment with 1.0 mM OA revealed large-sized intracellular droplets as against the smaller-sized droplets observed at the early time points (Figure 2C). It is to be noted that the variability in lipid accumulation is more accurately measured using the phase contrast microscopy data when compared to the fluorescence microscopy data.

3.3. Effects of OA on gene expression changes at early time points

The mRNA expression levels of lipid metabolism-associated genes were measured between control and OA (1.0 mM) treated cells at the 30-minute time point. Significant upregulation was observed in genes involved in de novo lipogenesis, viz., SREBF1, PDK4, and G6PC, showing 2.4-, 4.7-, and 4.3-fold increase when compared to their controls (Figure 3A-C). Carnitine palmitoyltransferase I alpha (CPT1a), which is essential for the first step in beta-oxidation, was also found to be upregulated 3.0-fold at this early time point (Figure 3D). Moreover, OA treatment for 30 min increased the expression of HMGCR (2.1-fold), which is responsible for cholesterol synthesis (Figure 3E).

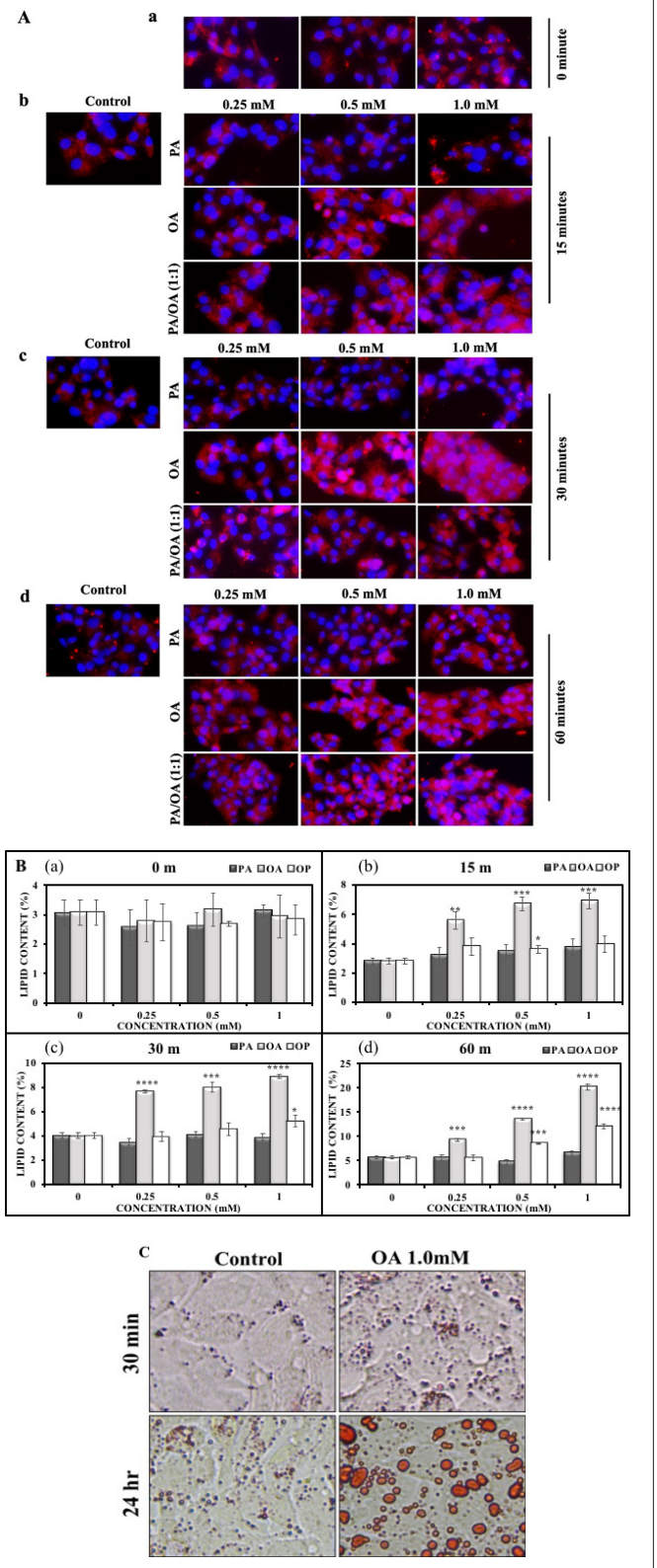

Fig. 2. Assessment of Lipid accumulation by using Oil Red O stain. A) HepG2 cells were treated with PA, OA, and PA/OA (1:1) of various concentrations (0.0, 0.25, 0.5, 1.0 mM) at early time course a) 0 min, b) 15 min, c) 30 min, and d) 60 min, and were visualized under a fluorescent microscope after staining with ORO and DAPI. B) (a-d) Quantification of intracellular lipid droplets using phase-contrast microscopy images acquired at 0, 15, 30, and 60 min time points was performed using ImageJ software. C) Comparison of control and OA-treated (1.0 mM) cells between 30 min and 24 h. The data are presented as means ± SD of three replicates. *= treatment vs control, p<0.05; **= treatment vs control, p<0.01; ***= treatment vs control p<0.001; ****=treatment vs control, p<0.0001.

3.4. Effects of FFA on a lipogenic protein marker

Immunoblot analysis was performed to assess the key lipogenic marker FASN within 30 min of OA treatment. Remarkably, FASN protein levels increased during the initial 30 min of 0.25 and 0.5 mM OA treatment, as shown in Figure 4A. Specifically, there was a 1.7-fold increase in band intensity with 0.25 mM concentrations of OA when compared to its control. In contrast, PA treatment showed no change in FASN protein expression even with 0.5 mM treatment for 30 min (Figure 4B). GAPDH was used as loading control and was used to normalize the FASN band intensities.

4. Discussion

Our comparative early time point study involving PA and OA revealed clearly that PA exhibited greater cytotoxicity (up 40%) even at 15 min of treatment, which is consistent with findings from longer exposure times reported previously [16,19]. Treatment with the PA/OA combination led to significant cell death comparable to the effect of PA alone. This clearly indicates that the presence of an equal proportion of OA in the PA/OA combination did not improve cell viability (Figure 1). The strong cytotoxic effect of PA in the early time points may be due to the excessive glycolysis and mitochondrial fragmentation reported in a recent study with PA [20]. However, at longer exposure times, the OA has been reported to ameliorate the PA-induced cytotoxic effects [14].

Treatment with OA in our study led to the rapid accumulation of quantifiable lipid droplets within 15 min. In contrast, elevated PA treatment did not actively contribute to LD storage even at the 60-minute time point, and this could be attributed to several effects. Research findings reveal that PA treatment generates very small LDs within 2 h, accompanied by increased mitochondrial membrane potential and mitochondrial fragmentation, which promotes enhanced beta-oxidation [16,21]. Additionally, Per-oxisome proliferator-activated receptor gamma coactiva-tor 1-alpha (PGC-1α), a key regulator of FA oxidation [22], is found to be overexpressed in PA treatment; however, it is approximately 50% lower in OA treatment at 2 h [23]. Thus, at early time points, PA appears to be more involved in beta-oxidation than in lipid storage.

Our results showed that genes linked to fat metabolism were much more active with OA treatment at early time points. One such gene is SREBF1, a transcription factor that regulates lipid homeostasis [24]. Previous studies have demonstrated that SREBF1 mediates lipid accumulation during extended OA treatment periods in HepG2 cells [25]. Similarly, PDK4 was upregulated as early as 30 min in our study. The PDK4 gene regulates de novo lipogenesis, and knockout of this gene is reported to impair lipid uptake and synthesis [26]. Another gene, G6PC, was also upregulated with 30 min of OA treatment, and the G6PC-deficient mice are known to exhibit reduced lipid accumulation [27].

The qRT-PCR data revealed that CPT1a and HMGCR were upregulated in response to treatment with OA at early time points. The increase of CPT1a levels in HepG2 cells in response to FA for a 24 h time period is previously documented [28]. HMGCR, involved in cholesterol biosynthesis, is observed to be upregulated in HepG2 cells treated with OA for 24 h [29], as well as in NAFLD patients [30]. This provides clues that the OA-dependent early expressed genes may contribute to the development of NAFLD.

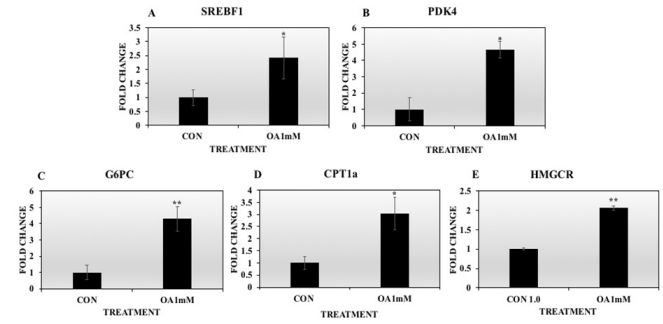

Fig. 3. Relative mRNA expression of transcription factors and enzymes involved in lipid homeostasis. Gene expression levels were measured by qPCR following treatment with 1.0 mM OA for the genes: A) SREBF1, B) PDK4, C) G6PC, D) CPT1a, E) HMGCR. The data are presented as means ± SD of three replicates. *= treatment vs control, $p<0.05$; **= treatment vs control, $p<0.01$.

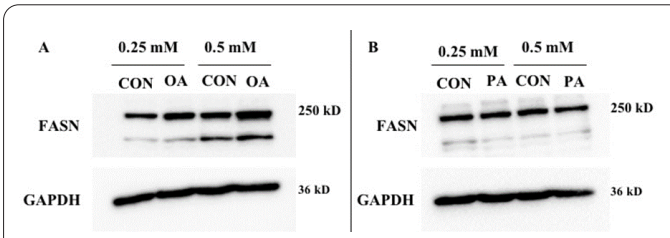

Fig. 4. OA-dependent upregulation of FASN at the early onset of lipid accumulation. Immunoblot analysis of HepG2 cells treated with increasing concentrations of (A) OA, and (B) PA at the 30-minute time point in HepG2 cells.

In our study, the fatty acid synthase, a key enzyme in fatty acid metabolism, is significantly increased within 30 min of OA treatment. Previous publications have reported that FASN is overexpressed in NAFLD patients [31]. It is conceivable that overexpression of FASN is due to the upregulation of SREBF1, which is a known regulator of FASN. Moreover, since the FASN is involved in the final stage of DNL, its significant upregulation at 30 min suggests that several genes upstream in the pathway may be activated at these early time points. Further studies involving the whole transcriptome or proteome level analyses will be more insightful in understanding the early onset of NAFLD development.

In conclusion, our study demonstrates that oleic acid rapidly induces lipid accumulation and activates key genes involved in lipid metabolism in HepG2 cells within the first 30 minutes of treatment. In contrast, palmitic acid triggers early cell death without significant lipid accumulation at these early time points. These findings highlight the distinct effects of the two free fatty acids on the early onset of intracellular lipid accumulation and provide more insights into the possible molecular events at the onset of NAFLD progression.

Abbreviations

NAFLD, Non-alcoholic fatty liver disease; FFAs, free fatty acids; OA, Oleic acid; PA, Palmitic acid; LD, Lipid droplets; FFAR4, Free fatty acid receptor 4; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DMSO Dimethyl sulfoxide; ORO, Oil Red O; PBS,

Phosphate Buffered Saline**;** DAPI, 4′,6-diamidino-2-phenylindole**;** cDNA complementary Deoxyribonucleic acid; SREBP1, Sterol regulatory element-binding transcription factor 1; PDK4, Pyruvate dehydrogenase kinase 4; G6PC, Glucose-6-phosphatase; CPT1a, Carnitine palmitoyltransferase 1 alpha; HMGCR, 3-hydroxy-3-methylglutaryl-CoA reductase; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; PMSF, Phenylmethylsulfonyl fluoride; TBST, Tris-buffered saline with Tween 20; FASN, Fatty acid synthase; CST, Cell signaling technology; ECL Enhanced Chemiluminescence**;** qPCR, Quantitative Real-time PCR; PGC-1α, Peroxisome proliferator-activated receptor gamma coactivator 1- alpha**;** DNL, de novo lipogenesis; RNA, Ribonucleic acid.

Conflict of interest
There is no conflict of interest.

Consent for publications
All authors have read and approved the final manuscript for publication.

Ethics approval and consent to participate
This research does not involve humans or animals.

Availability of data and material
All data are embedded in the manuscript

Authors' contributions
AV conceptualized and designed the study and supervised the work; EJE performed the experiments and analyzed the data; RHR assisted EJE in sample handling; AV and EJE wrote the manuscript.

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