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The Inhibitory Effect of Biochanin A on Hepatic Cholesterol Biosynthesis in High Glucose-Induced Steatosis in $HepG_2$ Cells

Negar Karimi[1], Darya Ghadimi*[2], Mojtaba Fathi [3,4]

[1] M.Sc Student of Clinical Biochemistry, Biochemistry Department, School of Medicine, Zanjan University of Medical Sciences, Zanjan, Iran

[2] Assistant Professor of Clinical Biochemistry, Biochemistry Department, School of Medicine, Zanjan University of Medical Sciences, Zanjan, Iran

[3] Associate Professor of Clinical Biochemistry, Biochemistry Department, School of Medicine, Zanjan University of Medical Sciences, Zanjan, Iran

[4] Associate Professor of Clinical Biochemistry, Zanjan Metabolic Disease Research Center, Zanjan University of Medical Sciences, Zanjan, Iran

***Corresponding authors:** Darya Ghadimi, **Address**: Biochemistry Department, School of Medicine, Zanjan University of Medical Sciences, Mahdavi St., Zanjan, Iran, **Email**: d.ghadimi@zums.ac.ir

Tel: +982433420651

Abstract

***Background & Aims*:** Non-alcoholic fatty liver disease (NAFLD) results from fat accumulation in the liver (liver fat >5% of liver weight). The excess of lipids in hepatic steatosis primarily consists of triacylglycerol and cholesterol esters. De novo hepatic lipogenesis from excessive dietary carbohydrate intake is the most consistent underlying pathogenic agent of NAFLD. Hypercholesterolemia that mostly associates with NAFLD has been recognized as the most important risk factor for the development of coronary heart disease (CHD). In other words, reducing the hepatic cholesterol synthesis in NAFLD patients prevents risk of developing atherosclerosis and CHD. HMGCR is the rate-controlling enzyme pathway, responsible for cholesterol biosynthesis. De novo cholesterol synthesis by inducing the expression of HMGCR; activates the SREBP2. PPARα activation significantly lowers hepatic SREBP2 and HMGR mRNA levels. The aim of this study was to investigate the effect of Fenofibrate and Biochanin A, as PPARα agonists, on mRNA levels of SREBP2 and HMGR in $HepG_2$ cells exposed to high glucose concentration.

Materials & Methods: $HepG_2$ cells were used in this study. The induction of steatosis was performed by high glucose concentration. Cytotoxicity of Glucose, Fenofibrate, and Biochanin A were assessed in separate experiments for $HepG_2$ cells. Some biochemical parameters such as intracellular total cholesterol, HMGCR, ALT, and AST activity were measured. SREBP2 and HMGR mRNA levels were examined by real-time RT-PCR.

Results: Results of our study indicated an inhibitory effect of Fenofibrate and Biochanin A on the mRNA levels of SREBP2 and HMGR in HepG2 cells which were treated by high glucose concentration. Additionally, a decreased level of intracellular total cholesterol concentration was accompanied by decreased HMGCR activity.

Conclusion: Based on the findings of the present study, it can be concluded that Biochanin A could be a useful agent in the prevention of de novo hepatic cholesterol synthesis and development of hypercholesterolemia; which is the main cause of CHD.

***Keywords*:** NAFLD, SREBP2, HMG-CoA Reductase, Biochanin A, Fenofibrate

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Introduction

Hepatic Steatosis results from fat accumulation in the liver (liver fat >5% of liver weight). Non-alcoholic fatty liver disease (NAFLD) is a metabolic condition that encompasses a wide spectrum of chronic liver diseases ranging from simple steatosis to non-alcoholic steatohepatitis (NASH), fibrosis and cirrhosis; in the presence of less than 20 g of alcohol ingestion per day (1). The prevalence of NAFLD is now estimated to be approximately 15–30% worldwide and has been recognized as a global public health problem (2). The increasing prevalence of the disease worldwide is particularly worrying since NAFLD patients are at increased risk for dyslipidemia and cardiovascular diseases (3). Obesity (70% -80% of subjects), type 2 diabetes mellitus (30%–80% of subjects) and metabolic syndrome (88% of subjects) are recognized as the most important risk factors of NAFLD (4, 5).

The excess of lipids in hepatic steatosis primarily consists of triacylglycerols (TAGs) and cholesterol esters (CEs). The pathophysiology of NAFLD is multifactorial which results in fat accumulation in hepatocytes. De novo hepatic lipogenesis (DNL) from non-lipid precursors; like excessive dietary carbohydrate intake, is the most consistent underlying pathogenic agent (6-8). DNL is an essential biosynthetic pathway in the liver, the biochemical process of synthesizing fatty acids and cholesterol from acetyl-CoA subunits that are produced from carbohydrate catabolism. Glucose most commonly supplies carbon units for DNL contributing to the lipids that are stored and secreted by hepatocytes. In other words, a high-carbohydrate diet can increase rates of DNL. Importantly this leads to an accumulation of DNL products, such as fatty acyl chains and cholesterol (9, 10).

In hepatocytes, under fasted states, glucose is metabolized into pyruvate through glycolysis in the cytoplasm, and the mitochondrial pyruvate dehydrogenase complex then catalyzes the oxidative decarboxylation of pyruvate to produce acetyl-CoA. In the mitochondria, citrate synthase then catalyzes the condensation of the acetyl moiety of acetyl-CoA with oxaloacetate to yield a six-carbon citrate molecule to generate ATP through the TCA cycle and oxidative phosphorylation in the mitochondria. In the fed state, acetyl-CoA is used to synthesize fatty acids and cholesterol through DNL. High acetyl-CoA and citrate amounts are a signature of a fed state and promote its utilization for lipid synthesis (11, 12). Citrate, a signal that building blocks and energy are abundant, activates the carboxylase. Acetyl-CoA Carboxylase is an essential rate-limiting enzyme in fatty acid metabolism (12, 13). On the other hand, HMG-CoA (3-hydroxy-3-methylglutaryl-CoA) is the precursor for cholesterol synthesis. HMG-CoA is formed by condensation of acetyl-CoA and acetoacetyl-CoA, catalyzed by HMG-CoA synthase. HMG-CoA reductase (HMGCR) catalyzes the production of mevalonate from HMG-CoA, in which the HMGCR reaction is the rate-limiting step for cholesterol synthesis (14).

Dyslipidemia that mostly associates with NAFLD has been recognized as the most important risk factor for the development of coronary heart disease (CHD). It is widely accepted that the lipid-lowering strategies for NAFLD and CHD are similar. The main feature of dyslipidemia in patients with NAFLD is an atherogenic lipid profile, consisting of low high-density lipoprotein cholesterol (HDL-C), and an increase in very-low-density lipoprotein (VLDL) and low-density lipoprotein cholesterol (LDL-C) (15, 16). Atherosclerotic cardiovascular disease (ASCVD) and its clinical manifestations, such as myocardial infarction (MI) and ischemic stroke, are the leading cause of mortality worldwide. The most extensively studied agent is the level of LDL-C. There is a growing body of evidence that suggests cholesterol-rich LDL particles are directly implicated in the development of ASCVD (17). In other words, reducing the hepatic cholesterol synthesis in NAFLD patients prevents developing ASCVD.

HMGCR is the rate-controlling enzyme pathway, responsible for cholesterol biosynthesis. HMGCR is a transmembrane protein, containing 8 domains that is anchored in the membrane of the endoplasmic reticulum. The expression of the HMG-CoA reductase gene (HMGR) is regulated by the sterol regulatory

element-binding protein2 (SREBP2). De novo cholesterol synthesis by inducing the expression of HMGR; activates SREBP2. When the cellular cholesterol level is increased, SREBP2 remains in the endoplasmic reticulum (ER). On the other hand, when the cellular cholesterol level is decreased, SREBP2 is translocated from the ER to the Golgi, a step that is essential for converting to a transcription factor. Inside the nucleus, SREBP2 binds to a sterol regulatory element DNA segment to stimulate the expression of the HMGR gene. It would appear, that in the context of NAFLD, the conventional feedback regulation of cholesterol on hepatic cholesterol synthesis may be dysfunctional. Lipid accumulation in hepatocytes usually associates with peroxidation of fatty acids and production of free radicals which leads to oxidative stress. Inflammation initiates in response to oxidative stress and inflammatory mediators accumulate in liver. SREBP2 is activated by cytokines. In NAFLD, chronic inflammation activates SREBP2 continuously. Taken together, the transcriptional regulation of de novo cholesterol synthesis might be disrupted by lipid accumulation (18).

Peroxisome proliferator-activated receptors (PPARs) are a group of ligand inducible transcription factors that play important role in expression of proteins that are involved in lipid metabolism. Compelling evidence demonstrates that hepatic PPARα is activated by fatty acids generated via de novo lipogenesis. Whereas PPARα is mostly known for its ability to regulate lipogenesis. A functional PPRE was identified in the promoter of SREBP2 gene. Recent studies indicated that PPARα activation significantly lowers the levels of hepatic SREBP2 and HMGR mRNA. Therefore, PPARα activation could prevent NAFLD development (19, 20).

PPARα is the main target of Fibrate drugs, a class of amphipathic carboxylic acids, such as Fenofibrate. They are most frequently used for hypercholesterolemia treatment (21). Isoflavonoids are a cluster of flavonoid phenolic compounds. They are well known as phytoestrons because of biological effects via the estrogen receptor. In addition to estrogenic effects, some of other biological effects have been known for isoflavonoids; such as activation of PPARα(16). Biochanin A (BCA) is one of the better-known soy-derived isoflavonoids that has attracted the attention of scientists because of its beneficial effects on lipid metabolism (22, 23). BCA is extracted from red clover, soy, and some legumes. In recent studies, the effect of BCA on lipogenesis was approved, however, research on the relationship between Fenofibrate, BCA, glucose and NAFLD is inadequate. As a result, PPARα agonists such as Fenofibrate and BCA, might activate PPARα as a transcription factor and significantly lower hepatic SREBP2 and HMGR mRNA levels.

This study was designed to investigate the effect of different concentrations of Fenofibrate, BCA, and glucose on SREBP2 and HMGR mRNA levels in $HepG_2$ cells and how these chemical agents affect the hepatic cholesterol homeostasis feedback system of NAFLD.

Material and Methods

Cell culture and treatment:

The hepatoma cell line $HepG_2$, which originated from the American Type Culture Collection (ATCC), were obtained from the Pasteur Institute of Iran (IPI) (Tehran, Iran). The cells were cultured in Eagle's Minimum Essential Medium (EMEM, Biosera, LM-E1141) supplemented with 10% Fetal Bovine Serum (FBS, Biochrom, S 0115) and 100 U/ml Penicillin-Streptomycin (Gibco, 15-140-122). The cells were grown on cell culture flasks (Corning, CLS3289) in an incubator with a humidified atmosphere (95% air /5% CO2 v/v) at 37 ˚C. Steatosis was induced by incubating the hepatocytes with 50 and 100 mM of D-Glucose (Sigma, G8769) for 24 h**.** Biochanin A (BCA, Sigma, D2016) and Fenofibrate (Feno, Sigma, F6020-5g) were dissolved in 75% Dimethyl Sulfoxide (DMSO, Sigma, S-002-M) and passed through the 0.22 μm filter (Whatman UNIFLO 25 syringe filter, WHA9913-2502) then added to cell culture media.

MTT assay:

Cytotoxicity of Glucose, Fenofibrate, and Biochanin A were assessed in separate experiments for HepG2 cells. $HepG_2$ cells (10×10^5), cultured in 24-well

plates in EMEM, were exposed to Glucose at 25, 50, 75, 100, 125, 150, 175, 200, 225, 250 mM for 24 h; then they were exposed to Fenofibrate and Biochanin A separately at 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 µM for 24 h. Cell viability was assessed by measuring the reduction of Methylthiazolyldiphenyl-tetrazolium bromide (MTT, Sigma, M5655-100MG). Mitochondrial dehydrogenates of living cells reduce the tetrazolium ring, yielding a blue formazan product that can be measured photometrically. The optical densities obtained are directly proportional to the number of living cells. The cytotoxic effect of a sample is assessed by the percentage of living cells present in the sample, in relation to the cells treated only with the solutions. After treatment, the medium was replaced by a solution of 1 mg MTT/ml in EMEM medium. After 4 h of incubation, the liquid was aspirated and the insoluble formazan which was produced by living cells dissolved in DMSO. The optical densities of the obtained solutions were measured at 570 nm.

Steatosis induction:

Based on MTT assay results in Fig1, Glucose at 50 and 100 mM concentrations had no significant cytotoxic effects to induce steatosis in $HepG_2$ cells. $HepG_2$ cells were exposed to 50 and 100 mM concentrations of Glucose for 24 h. After that, for steatosis induction confirmation; a quantitative Oil Red O staining assay was used.

Oil red O staining:

HepG2 cells were seeded at a density of (10×10^6) on 8-well plates and exposed to Glucose. After 24 h, cells were rinsed with cold Phosphate Buffered Saline (PBS), fixed in 4% paraformaldehyde in PBS (v/v) for 30 minutes, and stained with Oil Red O (0.2% w/v, 2-propanol 40 %) for 40 minutes. Images of cells were captured using an optic microscope and dye is eluted with 2-propanol, and absorption of the elute is measured photometrically at 510 nm**.**

Total lipid extraction from $HepG_2$ cells:

$HepG_2$ cells were seeded at a density of (10×10^6) on 8-well plates and exposed to Glucose, Fenofibrate and Biochanin A in different concentrations (Fig 5 and Fig 9). After 24 h, to extract intracellular total lipid from $HepG_2$ cells, Bligh and Dyer method was used. The lipid extraction method by Bligh and Dyer is based on a solvent system consisting of chloroform /methanol /water 2:2:1 (v/v/v).

Determination of intracellular total Cholesterol concentration:

After lipid extraction from $HepG_2$ cells; total Cholesterol concentration was determined in cell lysates using a total cholesterol assay kit (Cell Biolabs, STA-384). The assay is based on the enzyme driven reaction that quantifies both cholesterol esters and free cholesterol. Cholesterol esters are hydrolyzed via cholesterol esterase into cholesterol, which is then oxidized by cholesterol oxidase into the ketone cholest-4-en-3-one plus hydrogen peroxide. The hydrogen peroxide is then detected with a highly specific colorimetric probe. Cholesterol concentration was reported as mg/g protein of cell lysates. Cell lysates were prepared on ice using RIPA lysis buffer system (Santa Cruz, Sc-24948). HepG2 cells were seeded at a density of (10×10^6) on 8-well plates and exposed to Glucose, Fenofibrate, and Biochanin A in different concentrations (Fig 5 and Fig 9). After 24 h, cells were homogenized by 0.6 ml of RIPA buffer and 10 µl protease inhibitor (abcam, ab65621). Homogenates were centrifuged at 4˚C (14000 g, 10minutes×2). The insoluble fat layer was removed and the supernatants were collected. Total protein concentration was measured using Bicinichoninic acid (BCA) assay (Santa Cruz, Sc-202389).

Determination of ALT and AST activity:

$HepG_2$ cells were seeded at a density of (10×10^6) on 8-well plates and exposed to Glucose, Fenofibrate and Biochanin A in different concentrations (Fig.6,7 and Fig.10,11). Following the 24 h treatment, cell culture media from each well of plates was collected, centrifuged at 3000×g for 10 min and the upper layer was collected. ALT and AST activities were measured using a kit (Zist Chimie, Iran) based on the colorimetric method of Reitman and Frankel. ALT and AST activity was reported as U / L.

Determination of HMG-COA Reductase activity:

HepG2 cells were seeded at a density of (10×10^6) on 8-well plates and exposed to Glucose, Fenofibrate and Biochanin A in different concentrations (Fig.8 and Fig.12). Following the 24 h treatment, cells were homogenized by 0.6 ml of RIPA buffer and 10 µl protease inhibitor (abcam, ab65621). Homogenates were centrifuged at 4˚C (14000 g, 10minutes×2). The insoluble fat layer was removed and the supernatants were collected. Total protein concentration was measured using Bicinichoninic acid (BCA) assay (Santa Cruz, Sc-202389) and HMG-COA Reductase activity was determined using a colorimetric assay kit (abcam, ab204701). HMG-COA Reductase activity was reported as µmol /min /mg protein.

RNA isolation:

$HepG_2$ cells were seeded at a density of (10×10^6) on 8-well plates and exposed to Glucose, Fenofibrate and Biochanin A in different concentrations (Fig 9 and Fig 13). Following the 24 h treatment, the cells were lysed by the addition of Trizol reagent (Invitrogen, 15596026). After homogenization, the addition of chloroform causes phase separation, where protein is extracted to the organic phase, DNA resolves at the interface, and RNA remains in the aqueous phase. In the next step, by adding isopropanol; salts tend to co-precipitate with DNA because salts are generally less soluble in isopropanol than in ethanol. Finally, using ethanol with a bit of water added (75%), salts dissolved and washed away while leaving most of the RNA/DNA behind, because the salts are more soluble in ethanol. The RNA was eluted in RNase/DNase-free water and stored at −80°C until analysis. The A260/A280 and A260/A230 ratio and RNA concentration were determined using a NanoDrop spectrophotometer. An A260/A280 ratio in the range of 1.8 to 2.0 and an A260/A230 ratio in the range of 2.0 to 2.2 were considered acceptable. The integrity of the RNA was also analyzed using denaturing gel electrophoresis. The integrity of the RNA is indicated by the presence of two distinct bands corresponding to the ribosomal 28S and 18S subunits, with the intensity of the larger 28S band approximately twice than that of the smaller 18S band.

cDNA synthesis:

cDNA was synthesized from total RNA with random hexamer primers using TaqMan reverse transcription reagents (Biofact, W2569-100).

Real-time RT-PCR:

Specific primers for each gene were designed using Primer3 software. PCRs were done in triplicate. Amplification was done for 40 cycles using Power SYBR Green PCR master Mix Kit (Biofact, DQ485-096). The mRNA level was normalized to the GAPDH mRNA level and subsequently expressed as fold changes relative to normal control $HepG_2$ cells (ΔΔCt method). Details of all primers used are provided in Table 1.

Table1. Specific primers for real-time PCR

Genes	Forward primer sequence (5´-3´)	Reverse primer sequence(5´-3´)
HMGCR	TCCCTGGGAAGTCATAGTGG	GGCCAGCAATACCCAAAATA
SREBP2	CATCCCTTGGGCCAGAAGTT	TCCTTGGCTGCTGACTTGATC
GAPDH	GGTGTTAAGGTGGTGGCTGT	GGGCTCTTTGCACTGGTAGA

Statistical Analysis:

All results were reported as mean ± SD of three separate experiments; each point represents a triplicate of each experiment. Statistically significant differences were tested by one-way analysis of variance (ANOVA).

Results

The Effect of Glucose on $HepG_2$ cells viability:

Fig 1 indicates the effect of different concentrations of Glucose on cell viability determined by an MTT assay. The inhibition of cell viability was expressed as a percentage of viable cells in experimental wells relative

to control wells. Values represent mean ± SD**.** Results are representative of three different experiments. Glucose concentration from 25-100 mM had no significant effect on cell viability; however, the concentration of 125 mM of Glucose reduced the cell viability percentage considerably(p-value = 0.04). Glucose reduced $HepG_2$ viability by 50% at 225 mM after 24-h treatment.

The Effect of Fenofibrate on $HepG_2$ cells viability:

As is shown in Fig 2, the effect of different concentrations of Fenofibrate on cell viability was determined by an MTT assay. The inhibition on cell viability was represented as a percentage of viable cells in experimental wells relative to control wells. Values represent mean ± SD**.** Results are representative of three different experiments. Fenofibrate concentration from 10-50 µM had no considerable effect on cell viability; however, the concentration of 60 µM of Fenofibrate reduced the cell viability percentage significantly (p-value = 0.01). Fenofibrate reduced $HepG_2$ viability by 50% at 90 µM after 24-h treatment.

The Effect of Biochanin A on $HepG_2$ cells viability:

Based on Fig3, the effect of different concentrations of Biochanin A on cell viability was determined by an MTT assay. The inhibition of cell viability was expressed as a percentage of viable cells in experimental wells relative to control wells. Values represent mean ± SD**.** Results are representative of three different experiments. BCA concentration from 10-40 µM had no considerable effect on cell viability; however, the concentration of 50 µM of BCA reduced the cell viability percentage notably (p-value = 0.03). BCA reduced $HepG_2$ viability by 50% at 90 µM after 24-h treatment.

The effect of Glucose and Fenofibrate on intracellular total Cholesterol concentration in $HepG_2$ cells:

Table 2 and Fig 5 demonstrate the effect of Fenofibrate and Glucose on intracellular total Cholesterol concentration in $HepG_2$ cells. Cholesterol concentration increased significantly in the cells treated with 50 and 100 mM Glucose compared to normal control, 40 and 50 µM of Fenofibrate treated cells (p-value ≤ 0.005). Cholesterol concentration decreased considerably in the cells treated with 50 mM Glc + 50 µM Feno and 100 mM Glc + 50 µM Feno compared to cells treated with 50 and 100 mM Glucose (p-value ≤ 0.01). However; Cholesterol concentration of normal control cells was notably lower than treated cells (p-value < 0.005).

The effect of Glucose and Fenofibrate on ALT and AST activity in culture media of $HepG_2$ cells:

As is shown in Fig 6 and Fig7, ALT and AST activity increased significantly in culture media of cells treated by Glucose at both concentrations of 50 and 100 mM in comparison to normal control cells and cells cultured with 40 and 50 µM of Fenofibrate (p-value ≤ 0.005). It should be noted that ALT and AST activity decreased notably in media of cells cultured with 50 mM Glc + 50 µM Feno and 100 mM Glc + 50 µM Feno compared to cells treated with 50 and 100 mM of Glucose (p-value ≤ 0.01). As can be inferred from Table 2, ALT and AST activity in culture media of normal control cells were considerably lower than the cells mentioned above (p-value < 0.003).

The effect of Glucose and Fenofibrate on HMG-COA Reductase activity in $HepG_2$ cells:

Fig 8 indicates the effect of Glucose and Fenofibrate on HMG-COA Reductase activity in $HepG_2$ cells. Glucose at both concentrations of 50 and 100 mM increased HMG-COA Reductase activity in $HepG_2$ cells significantly compared to normal control cells and cells exposed to 40 and 50 µM of Fenofibrate (p-value ≤0.005). In cells exposed to Fenofibrate and Glucose 50 mM Glc + 50 µM Feno and 100 mM Glc + 50µM Feno; HMG-COA Reductase activity decreased considerably in comparison to cells exposed to Glucose (p-value ≤ 0.005). Moreover, HMG-COA Reductase activity in normal control cells was notably lower than that of treated cells (p-value < 0.01).

The effect of Glucose and Biochanin A on intracellular total Cholesterol concentration in $HepG_2$ cells:

Table 3 and Fig 9 indicate the effect of Biochanin A and Glucose on intracellular total Cholesterol concentration in $HepG_2$ cells. Cholesterol concentration increased considerably in the cells treated with 50 and 100 mM Glucose in comparison with normal control , 30 and 40 M of BCA treated cells (p-value $\leq$ 0.001). Cholesterol concentration decreased significantly in the cells which treated by (50 mM Glc + 40 µM BCA) and (100 mM Glc + 40 µM BCA) compared to cells treated with 50 and 100 mM Glucose (p-value $\leq$ 0.02). However; Cholesterol concentration of normal control cells was significantly lower than mentioned above-exposed cells (p-value < 0.003).

The effect of Glucose and Biochanin A on ALT and AST activity in culture media of $HepG_2$ cells:

As it can be inferred from Fig 10 and Fig 11, ALT and AST activity increased notably in culture media of cells exposed to Glucose at both concentrations of 50 and 100 mM; compared to normal control cells and cells cultured with 30 and 40 µM of BCA (p-value $\leq$ 0.005). Additionally, ALT and AST activity decreased significantly in media of cells cultured with 50 mM Glc + 40 µM BCA and 100 mM Glc + 40 µM BCA in comparison to cells exposed to 50 and 100 mM of Glucose (p-value $\leq$ 0.01). As shown in Table 3, ALT and AST activity in culture media of normal control cells were considerably lower than treated cells (p-value < 0.001).

The effect of Glucose and Biochanin A on HMG-COA Reductase activity in $HepG_2$ cells:

Fig 12 demonstrates the effect of Glucose and Biochanin A on HMG-COA Reductase activity in $HepG_2$ cells. Glucose increased HMG-COA Reductase activity at 50 and 100 mM in $HepG_2$ cells notably compared to normal control cells and cells treated with 30 and 40 µM of BCA (p-value $\leq$ 0.003). In cells treated with BCA and Glucose (50 mM Glc + 40 µM BCA and 100 mM Glc + 40µM BCA); HMG-COA Reductase activity decreased significantly compared to cells treated with Glucose (p-value $\leq$ 0.005). Additionally, HMG-COA Reductase activity in normal control cells was notably lower than that of exposed cells (p-value < 0.003).

The Effect of Glucose and Fenofibrate on SREBP2 and HMGCR mRNA levels:

Gene expression was quantified by real-time quantitative PCR analysis. The gene expression levels were normalized to GAPDH mRNA levels. Values represent mean ± SD. Results are representative of 3 independent experiments with n = 3. Quantitative real-time PCR showed that (Fig.13, 14), 50 and 100 mM of Glucose increased the mRNA levels of HMGCR and SREBP2 significantly compared to normal control, 40 and 50 µM of Fenofibrate treated cells (p-value $\leq$ 0.003). Treatment with Fenofibrate (Glc 50 mM + Feno 50 µM and Glc 100 mM + Feno 50 µM) significantly decreased the expression of HMGCR and SREBP2 in comparison with cells treated by Glucose at both concentrations of 50 and 100 mM (p-value $\leq$ 0.01). It should be noted that HMGCR and SREBP2 mRNA levels in normal control cells were notably lower than that of treated cells (p-value < 0.002).

The Effect of Glucose and Biochanin A on SREBP2 and HMGCR mRNA levels:

Gene expression was quantified by real-time quantitative PCR analysis. The gene expression levels were normalized to GAPDH mRNA levels. Values represent mean ± SD. Results are representative of 3 independent experiments with n = 3. It can be inferred from Fig 15 and 16 that 50 and 100 mM of Glucose increased the mRNA levels of HMGCR and SREBP2 considerably in comparison with normal control, 40 and 50 µM of BCA exposed cells (p-value $\leq$0.003). BCA (Glc 50 mM + BCA 40 µM and Glc 100 mM + BCA 40 µM) significantly decreased the expression of HMGCR and SREBP2 in comparison with cells exposed by Glucose at both concentrations of 50 and 100 mM (p-value $\leq$ 0.005). Moreover, HMGCR and SREBP2 mRNA levels in normal control cells were significantly lower than exposed cells (p-value < 0.03).

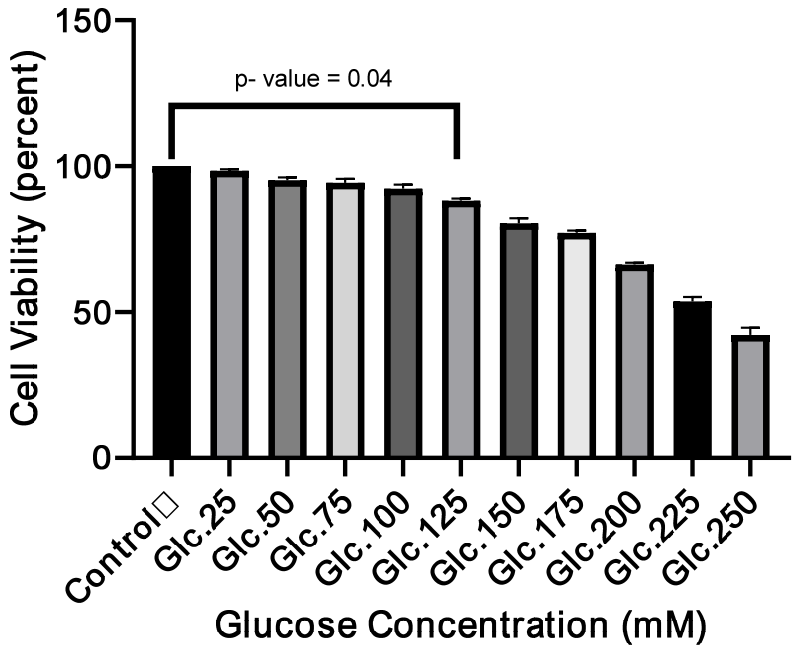

Fig 1. MTT assay results for hepG2 cells exposed to different concentration of Glucose from 0-250 mM

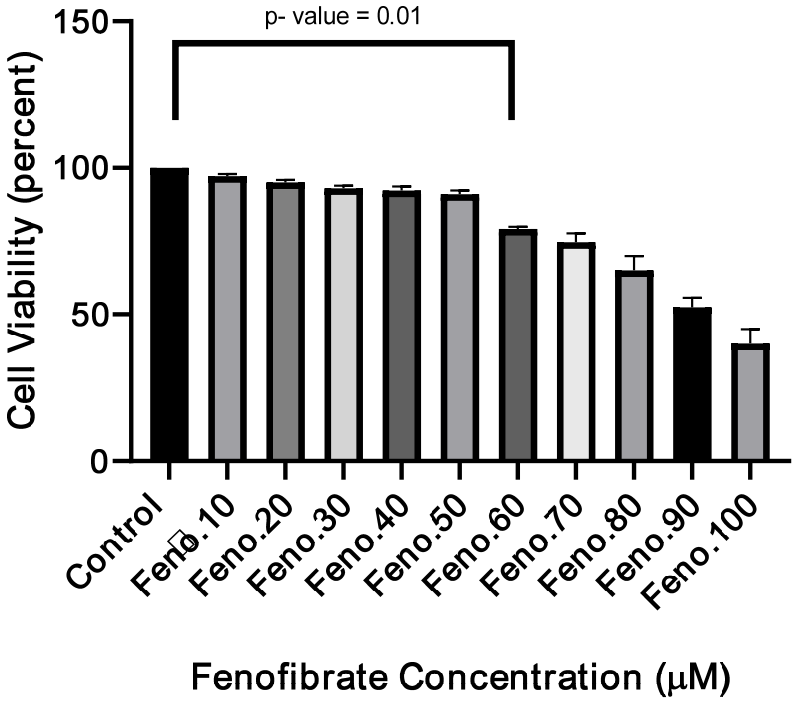

Fig 2. MTT assay results for hepG2 cells exposed to different concentration of Fenofibrate from 0-100 μM

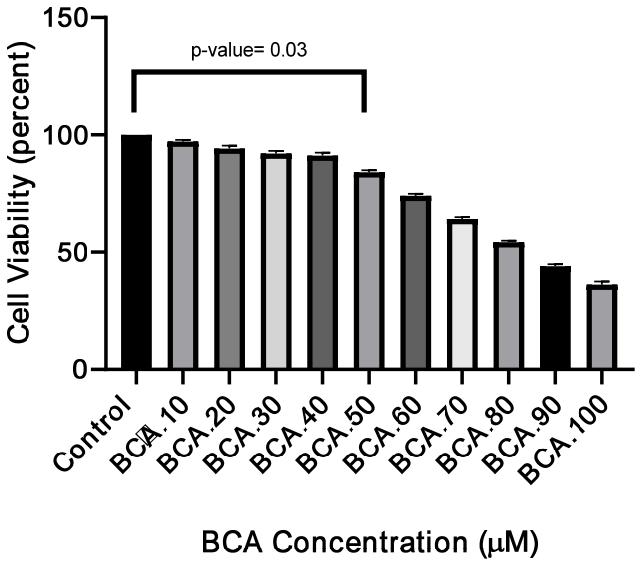

Fig 3. MTT assay results for hepG2 cells exposed to different concentration of Biochanin A from 0-100 μM

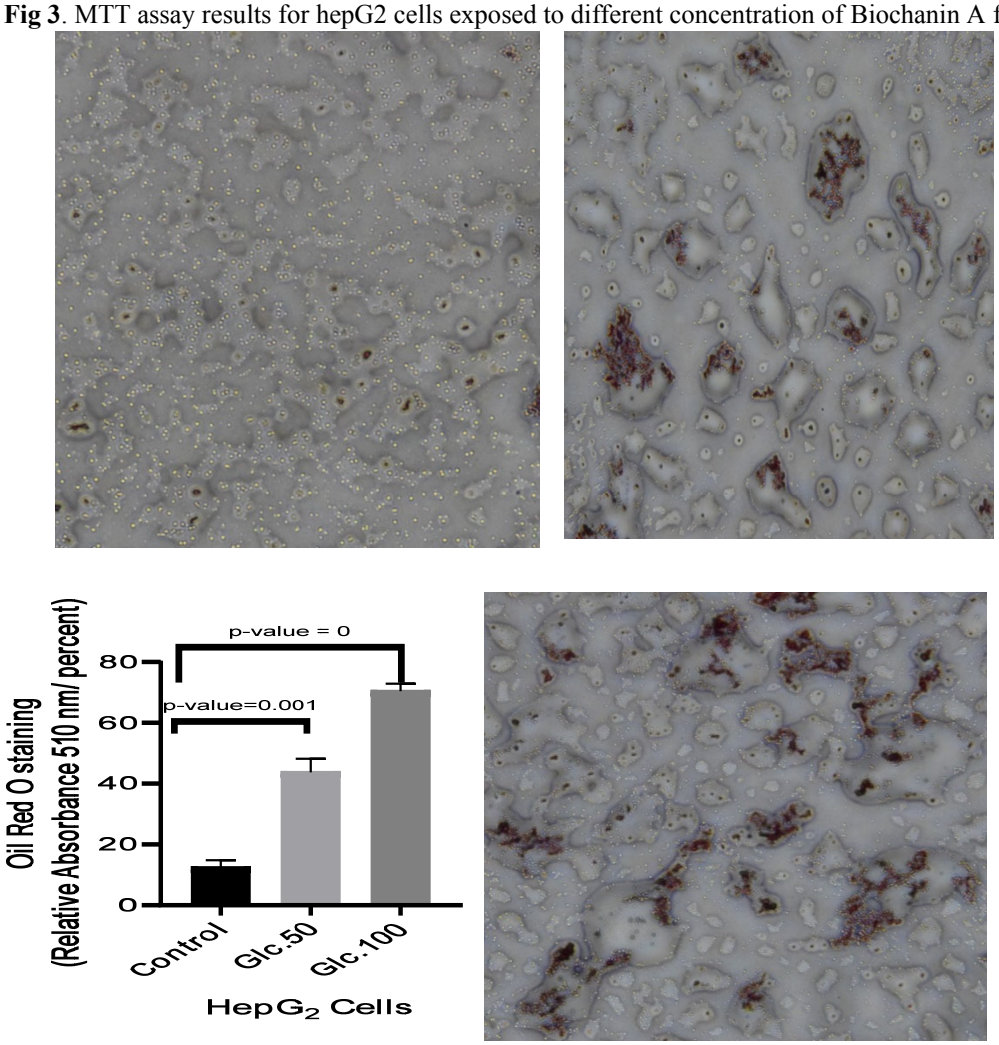

Fig 4. Oil Red O staining assay results for hepG2 cells exposed to different concentration of Glucose, 50 and 100 mM. Red spots represent fatty vacuoles.

Table 2. Effects of Fenofibrate on HepG2 cells

	Control	Feno.40	Feno.50	Glc.50	Glc.100	Glc.50+ Feno.40	Glc.50+ Feno.50	Glc.100+ Feno.40	Glc.50+ Feno.50	p-value
Cholesterol Concentration (mg/g protein)	35.21± 0.70	29.18± 0.73	32.09± 0.85	51.05±0.92	68.13± 1.21	44.04± 0.93	39.07± 0.89	62.11± 0.83	58.17± 0.75	0.001
ALT (U/L)	66.57± 1.5	64.24± 1.38	68.14± 1.03	86.14±1.03	97.17± 0.75	75.10± 0.84	70.07± 1.00	86.04± 0.94	83.07± 1.00	0.012
AST (U/L)	41.15± 1.03	45.53 ± 1.50	45.10 ± 0.84	67.19±1.05	78.11 ± 1.01	59.26 ± 1.09	56.29 ± 1.46	66.14± 0.79	59.07± 0.88	0.014
HMG-COA Reductase (µmol / min /mg protein)	2.01± 0.12	1.91 ± 0.10	2.00 ± 0.11	2.52± 0.13	2.81 ± 0.11	2.44 ± 0.10	2.11 ± 0.11	2.66± 0.15	2.30± 0.11	0.003

Each value represents the Mean±SD.

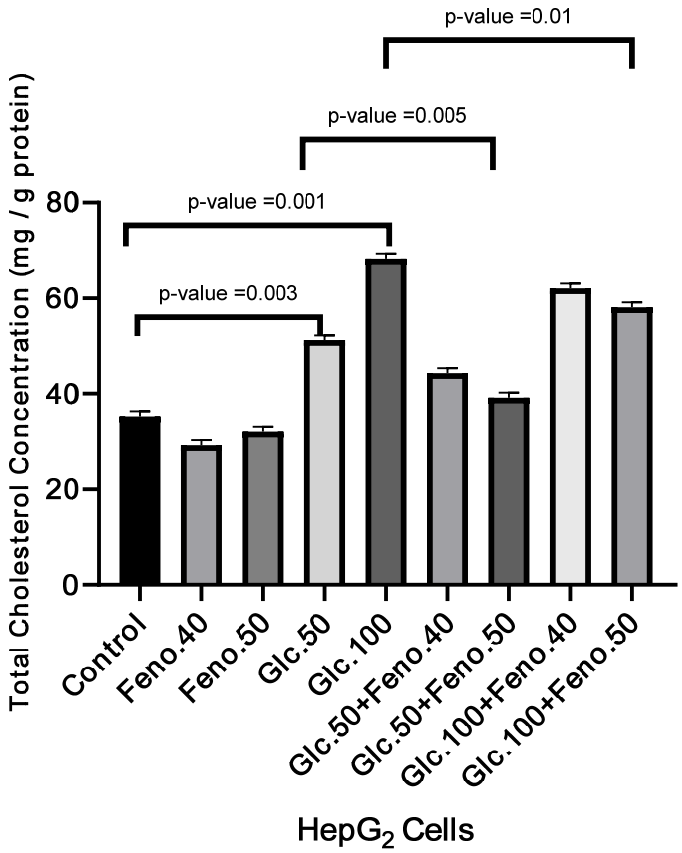

Fig 5. Total Cholesterol assay results for hepG2 cells exposed to different concentration of Glucose (Glc, mM) and Fenofibrate (Feno, µM) for 24 hours

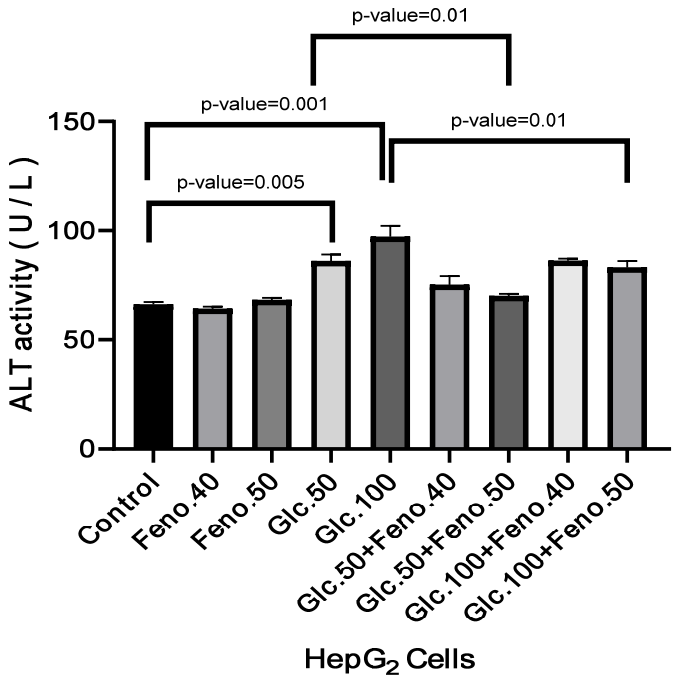

Fig 6. ALT activity assay results for hepG2 cells exposed to different concentration of Glucose (Glc, mM) and Fenofibrate (Feno, μM)

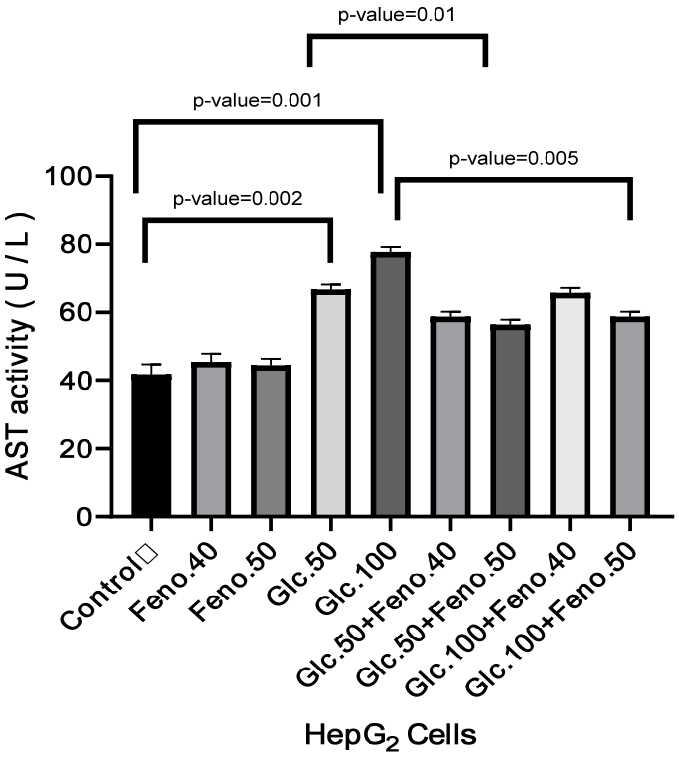

Fig 7. AST activity assay results for hepG2 cells exposed to different concentration of Glucose (Glc, mM) and Fenofibrate (Feno, μM)

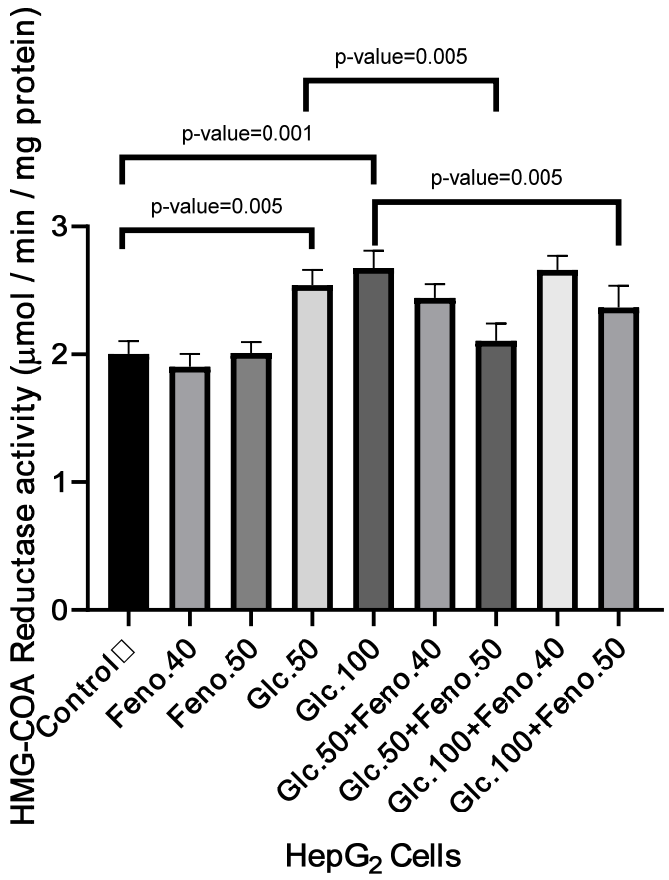

Fig 8. HMG-COA Reductase activity assay results for hepG2 cells exposed to different concentration of Glucose (Glc, mM) and Fenofibrate (Feno, µM)

Table 3. Effects of Biochanin A on HepG2 cells

	Control	BCA.30	BCA.40	Glc.50	Glc.100	Glc.50+ BCA.30	Glc.50+ BCA.40	Glc.100+ BCA.30	Glc.50+ BCA.40	p-value
Cholesterol Concentration (mg/g protein)	39.03± 0.94	37.19± 0.73	39.21± 0.70	51.14±0.79	71.11± 0.84	45.24± 0.66	41.21± 0.70	64.14± 0.79	62.08± 0.87	0.022
ALT (U/L)	70.21± 1.06	68.10± 1.01	71.11± 1.01	91.04± 1.00	104.10± 1.05	84.07± 0.89	79.18± 1.04	92.07± 1.00	87.10± 1.01	0.034
AST (U/L)	42.11± 1.01	41.19± 1.05	44.26± 1.09	65.24±1.08	81.09± 1.01	59.21± 1.06	56.24± 1.08	71.11± 1.01	64.06± 1.00	0.018
HMG-COA Reductase (µmol / min /mg protein)	1.90± 0.09	1.88± 0.10	1.95± 0.10	2.64± 0.10	2.88± 0.10	2.33± 0.11	2.28± 0.01	2.69± 0.10	2.70± 0.10	0.015

Each value represents the Mean±SD.

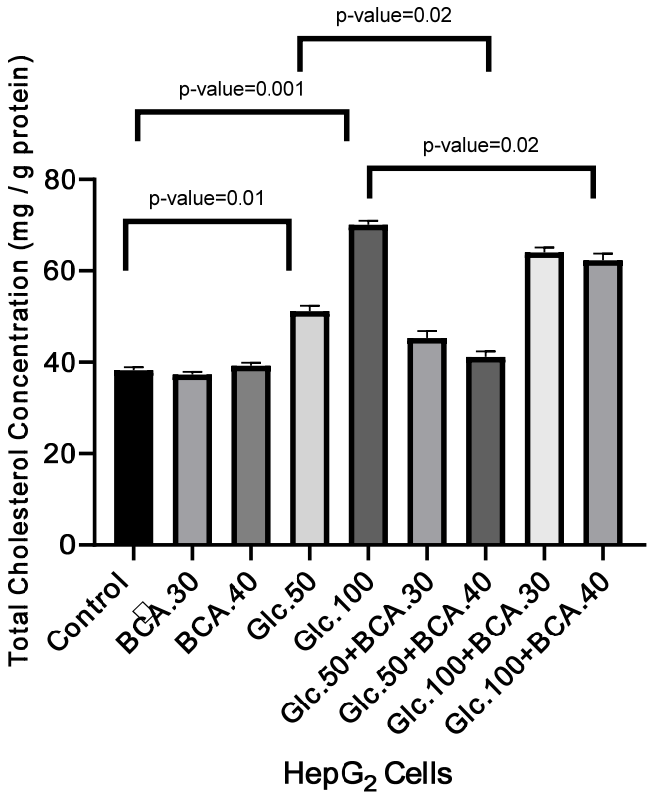

Fig 9. Total Cholesterol assay results for hepG2 cells exposed to different concentration of Glucose (Glc, mM) and Biochanin A (BCA, µM)

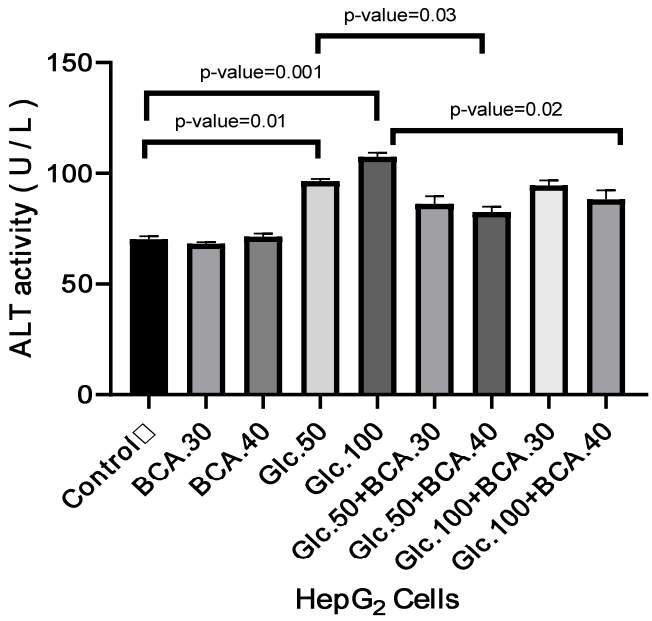

Fig 10. ALT activity assay results for hepG2 cells exposed to different concentration of Glucose (Glc, mM) and Biochanin A (BCA, µM)

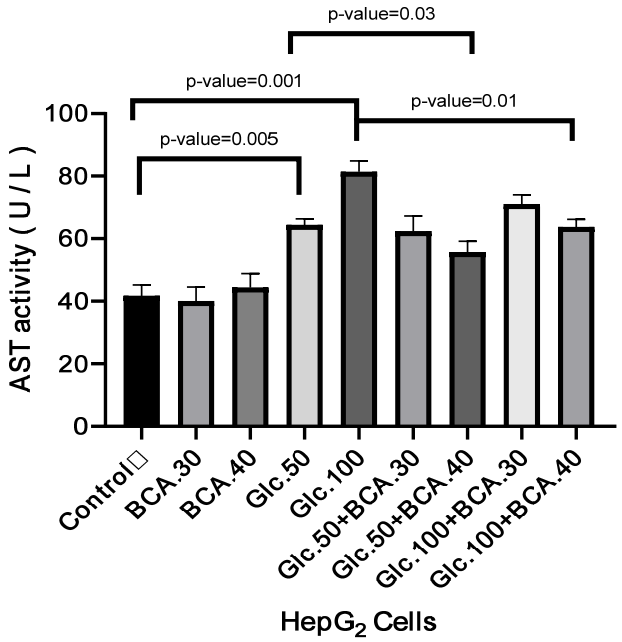

Fig 11. AST activity assay results for hepG2 cells exposed to different concentration of Glucose (Glc, mM) and Biochanin A (BCA, µM)

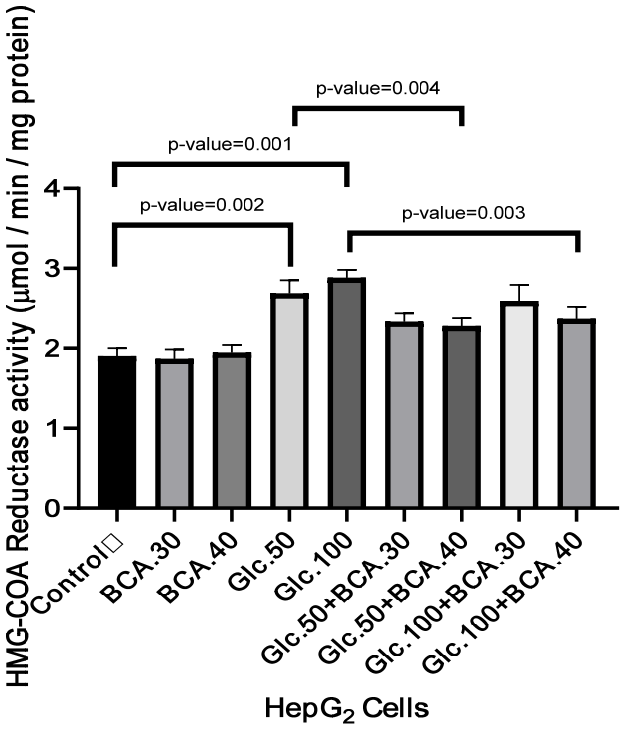

Fig 12. HMG-COA Reductase activity assay results for hepG2 cells exposed to different concentration of Glucose (Glc, mM) and Biochanin A (BCA, µM)

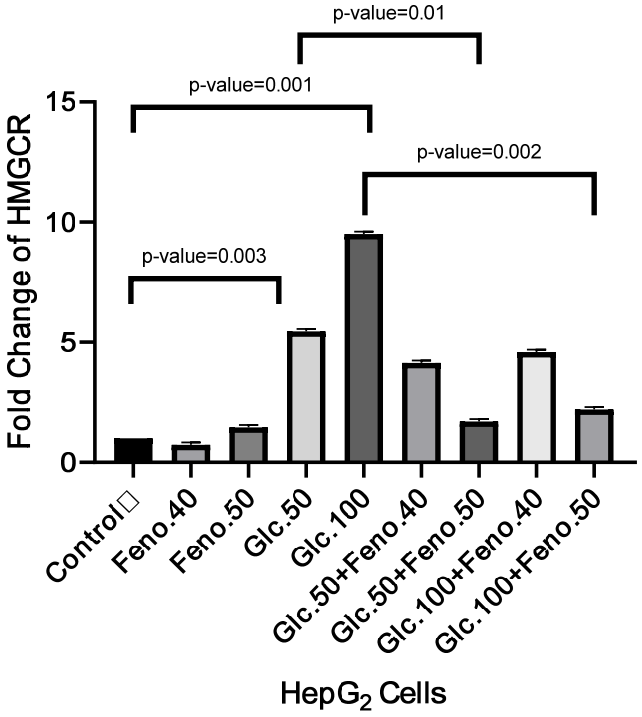

Fig 13. Relative mRNA concentration of HMGCR results for hepG2 cells exposed to different concentration of Glucose (Glc, mM) and Fenofibrate (Feno, µM)

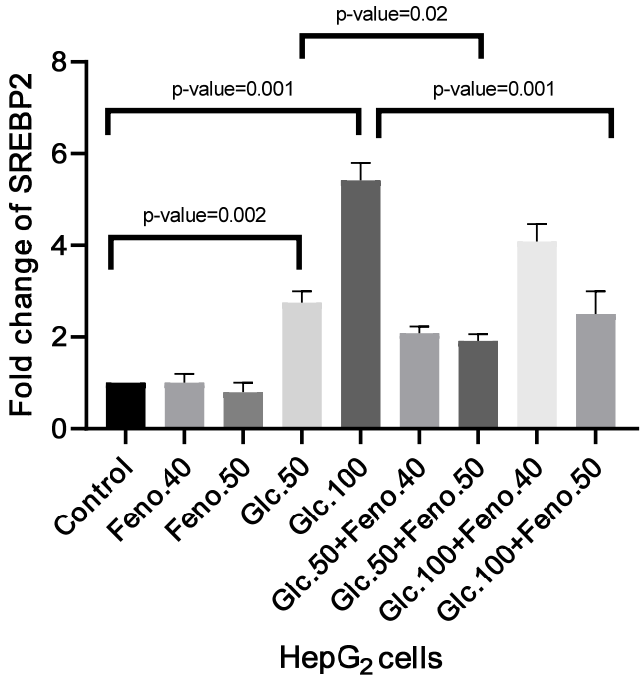

Fig 14. Relative mRNA concentration of SREBP2 results for hepG2 cells exposed to different concentration of Glucose (Glc, mM) and Fenofibrate (Feno, µM)

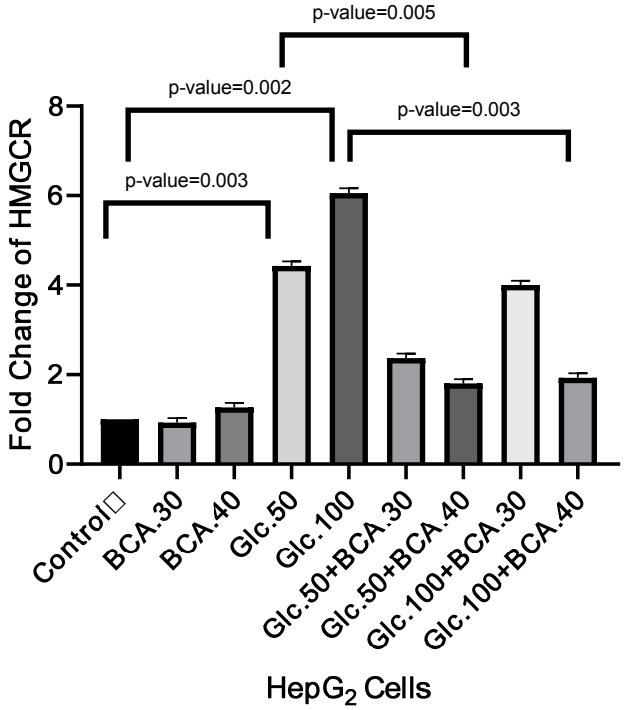

Fig 15. Relative mRNA concentration of HMGCR results for hepG2 cells exposed to different concentration of Glucose (Glc, mM) and Biochanin A (BCA, µM)

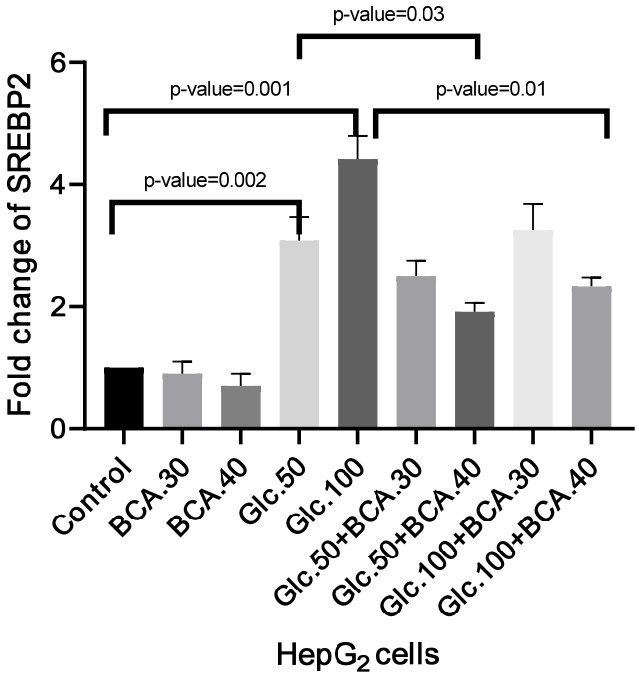

Fig 16. Relative mRNA concentration of SREBP2 results for HepG2 cells exposed to different concentration of Glucose (Glc, mM) and Biochanin A (BCA, µM)

Discussion

Results of the present study showed that treatment of $HepG_2$ cells with Glucose could induce steatosis which is confirmed by quantitative Oil Red O staining. Do et al. demonstrated that high Glucose concentration in $HepG_2$ cells could induce lipid accumulation (24). In the present study, intracellular total Cholesterol concentration in $HepG_2$ cells increased significantly because of exposure to Glucose. Based on the result of the present study, steatosis in $HepG_2$ cells is associated with increased mRNA levels of SREBP2 and HMGCR. Wu et al. indicated that in $HepG_2$ cells; the treatment activated the SREBP2 and also increased the mRNA and enzyme activity of HMGCR leading to intracellular cholesterol accumulation (18).

Assessing liver damage caused by lipid accumulation in the liver is important for the diagnosis of NAFLD. Therefore, in this study, AST and ALT activity were used as markers of liver damages. Levels of AST and ALT activity increased considerably in the cells exposed to high Glucose and with high intracellular total Cholesterol concentration, as compared to normal control cells with normal Cholesterol.

Primary NAFLD is now considered as the hepatic manifestation of the metabolic syndrome. Metabolic syndrome is a group of states that take place together, increasing the risk of type 2 diabetes mellitus and steatohepatitis. These states consist of high blood glucose and pressure, extra fat around the waist, and dyslipidemia (25). It has a high prevalence in developed countries; about 22.4 % in adult individuals (26). There is a body of growing evidence suggesting that obesity is the most important agent in developing metabolic syndrome (27). Recent studies have demonstrated that fat accumulation leads to inflammation. Inflammatory mediators in cells which have the receptor for insulin hormone targeted the insulin signaling pathway and caused to insulin resistance (6, 8). Hepatocytes and adipocytes are two important groups of cells that could develop insulin resistance; which is regarded as the most effective agent in the pathogenesis of hyperglycemia and hepatic steatosis (28). It seems that adipocytes, hepatocytes, and pancreatic β-islet cells are vertices of a triangle in progressing the metabolic disorders which lead to insulin resistance and its consequences. Also it should be noted that pathogenesis of insulin resistance is still controversial; 3 pathways are under the spotlight: insulin signaling, hepatic lipogenesis, and fatty acid β-oxidation (29, 30). Because of high-calorie diet and sedentary lifestyle especially in developed countries, researchers believe that the addition of supplement contains agents which could affect the 3 mentioned pathways above could improve lipid metabolism. PPARα regulates the expression of genes involved in lipid metabolism and is a major regulator of energy homeostasis. Fibrates are PPARα agonists and have been used to treat dyslipidemia for several decades because of their triglyceride-lowering and high-density lipoprotein cholesterol (HDL-C) elevating effects (31). Thus, natural PPARα agonists like Biochanin A and Genistein might decrease the progression of atherosclerosis by modulating metabolic risk factors.

Consistent with the result of our study; Zhang et al. reported that the effects of PPARα agonist on lipid metabolism in the liver involve inhibiting transcription and activation of SREBP2 and SREBP-dependent lipogenic and cholesterologenic gene expression, thereby resulting in a reduction of the triglyceride and cholesterol levels in the liver (32).

In summary, Fenofibrate and Biochanin A appear to alleviate steatosis in $HepG_2$ cells exposed to high Glucose concentration due to the following alterations in (1)reduced cholesterol concentration in $HepG_2$ cells, (2) decreased ALT and AST efflux from $HepG_2$ cells, (3) reduced SREBP2 and HMGR mRNA levels, and (4) decreased HMGCR activity in $HepG_2$ cells. We speculate that the progression of steatosis in the Fenofibrate and Biochanin A treated cells was prevented by the suppression of SREBP2 expression.

Conclusion

Results of our study indicated a dose-dependent inhibitory effect of Fenofibrate and BCA on the mRNA levels of SREBP2 and HMGCR in $HepG_2$ cells which were exposed to high Glucose concentration. Additionally, a decreased level of intracellular total

Cholesterol concentration was accompanied by decreased HMGCR activity. Based on the findings of our study, Biochanin A could be useful for the prevention of de novo hepatic Cholesterol synthesis and development of hypercholesterolemia which is the main cause of coronary heart disease.

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Conflict of interest

The authors declare no conflict of interest, financial or otherwise..

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