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Original article

# Free fatty acid-induced miR-22 inhibits gluconeogenesis via SIRT-1-mediated PGC-1α expression in nonalcoholic fatty liver disease

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ABSTRACT

*Background and aims:* Free fatty acids (FFAs) are one of the important regulators of the progression of nonalcoholic fatty liver disease. The FFAs are shown to modulate the metabolic status of the liver by modulating several cellular pathways in hepatocytes. Here, we elucidated the role of miR-22 in modulating FFAs-mediated gluconeogenesis.
*Methods:* Huh7 and WRL68 cells were transfected with nonspecific miRNA, miR-22 premiRs or anti-miR-22 oligos followed by incubation with palmitic acid, oleic acid, and linoleic acid (300 μM each) for 48 and 72 h after transfection. The expression of miR-22 was performed using real-time polymerase chain reaction and Western blots were performed for SIRT-1, PGC-1α, PEPCK, and glucose-6-phosphatase. Three groups of C57BL/6 mice (6 mice per group) were fed with standard diet, choline sufficient L-amino acid defined diet or choline-deficient L-amino acid defined (CDAA) diet for 6, 18, 32, or 54 weeks. Triglycerides content was measured in the serum. Expression of miR-22 and the protein expression of gluconeogenic enzymes were analyzed in the tissue samples.
*Results:* Incubation of miR-22-transfected cells with FFAs inhibited the expression of SIRT-1, PGC-1α, PEPCK, and glucose-6-phosphatase, while miR-22 expression was increased. These changes were reversed when the cells were transfected with anti-miR-22 oligos. CDAA-fed mice showed the significant increase in triglycerides content and miR-22 expression, while there was an inhibition of SIRT-1, PGC-1α, PEPCK, and glucose-6-phosphatase expression in CDAA-fed mice.
*Conclusions:* These data confirm that FFAs inhibited gluconeogenesis via miR-22-mediated inhibition of SIRT-1, which in turn inhibited PGC-1α in hepatic cells.

## 1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is one of the major health concerns in the world and is usually associated with obesity and insulin resistance [1,2]. An increasing percentage of NAFLD has been reported in children and one-third of adults of the developed countries [3]. Among these, 20–25% of individuals may develop nonalcoholic steatohepatitis (NASH), cirrhosis, liver failure, and sometimes hepatocellular carcinoma (HCC) [4]. Due to the changing dietary habits, many people are developing NAFLD, but well-defined cellular and molecular mechanism(s) leading to NAFLD are not yet known.

One of the major functions of the liver is to maintain glucose homeostasis by switching from glucose storage to export. The liver stores glucose in the form of glycogen and converts the excess glucose into fatty acids in the fed state. Conversely, in the fasted state, the liver induces a gluconeogenic pathway utilizing fatty acids and some amino acids [5]. Thus, the liver effectively switches between the fed and the fasting gene expression programs, which requires a highly coordinated action of several transcription factors and coactivators. Most of these transcription factors are coactivated by peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), which plays an important role in maintaining this molecular switch. Sirtuin-1 (SIRT-1) is a nicotinamide

*Abbreviations:* NAFLD, Nonalcoholic fatty liver disease; HCC, Hepatocellular carcinoma; CDAA, Choline-deficient l-amino acid diet; CSAA, Choline-sufficient l-amino acid diet; NS, Nonspecific; PEPCK, Phosphoenolpyruvate carboxykinase; G6P, Glucose-6-phosphatase; PGC1α, Peroxisome proliferator-activated receptor gamma coactivator 1-alpha; SIRT-1, Sirtuin 1; MED1, Mediator complex subunit 1; PVDF, Polyvinylidene fluoride; SDS-PAGE, Sodium dodecyl sulphate - polyacrylamide gel electrophoresis.

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adenosine dinucleotide (NAD)-dependent deacetylase that removes acetyl groups from many histone and nonhistone proteins [6]. SIRT1 can deacetylate a variety of substrates and hence is involved in a broad range of physiological functions, including control of gene expression and metabolism [7–9]. A number of SIRT1 substrates have been reported, including peroxisome proliferator-activated receptor-gamma (PPARγ) and PGC-1α (PPARγ coactivator) [6–9]. SIRT-1 activates PGC-1α by deacetylating it, and once activated, PGC-1α enhances gluconeogenesis [10] and fatty acid oxidation [11].

The pathogenesis of NAFLD is associated with increased fat deposition in liver [12]. Insulin resistance causes the accumulation of triglycerides (TG) in the liver [13]. Mitochondrial β-oxidation is upregulated significantly to accommodate the lipid influx and insulin action during fasting [14]. Induction of fatty acid oxidation is required for gluconeogenesis, which occurs mostly in liver mitochondria and is constitutively upregulated during insulin resistance [15]. However, the exact mechanisms that regulate these changes under pathological conditions, such as NAFLD, are not known.

It was reported that 23 miRNAs were differentially expressed in NASH as compared to healthy liver [16]. MiRNAs are 21–23 nucleotide long small noncoding RNA molecules [17,18], which can regulate various cellular activities [19,20]. In NAFLD, miR-27b was shown to act as a regulatory hub for lipid metabolism [21]. Using choline-deficient amino acid defined (CDAA) diet-induced NAFLD mice model, miRNA-23a was shown to inhibit gluconeogenic enzymes in liver tumors by inhibiting both glucose-6-phosphatase (G6P) and PGC-1α [22]. Increasing number of evidences suggest that the expression of SIRT-1 may also be regulated by miRNAs [23, 24]. Previously, we have shown that miR-22 inhibited the expression of SIRT-1 in hepatic cells [25]. Several studies have shown that miR-22 is one of the factors responsible for modulating various cancers, including liver cancer [26], and it is also considered as tumor suppressor and silencer. In addition, miR-22 exerts several other biological functions including modulation of arterial smooth muscle cell proliferation, cardiac and vascular remodeling, modulating inflammatory response, and regulation of immune system [27–29]. However, it was not known whether miR-22 is involved in free fatty acid (FFA)-mediated inhibition of gluconeogenesis. In the present study, we elucidated the role of miR-22 in FFA-mediated regulation of gluconeogenesis in hepatic cells.

## 2. Materials and methods

### 2.1. Cell culture and FFA treatment

A human hepatoma cell line (Huh7) and WRL68 cells (National Center for Cell Science, Pune, India) were used in this study. Cell culture and FFAs treatment were done as described previously [30].

### 2.2. Transfection experiments

The transfection of cells with miR-22 pre-miRs, anti-miR-22 oligos, or nonspecific miRNA (NS-miR) (Sigma-Aldrich, St Louis, MO, USA) was performed as mentioned in previous study [30]. The transfected cells were collected after 48 h or 72 h of transfection for RNA or protein isolation, respectively.

### 2.3. CDAA diet-induced NAFLD mice model

The study was approved by Institutional Animal Ethics Committee, South Asian University, New Delhi. All the experiments were performed in accordance with the relevant guidelines and regulations. All the animal experiments were done as mentioned in materials and methods of previous study [30].

### 2.4. RNA isolation, cDNA synthesis, and RT-PCR

The total RNA in the Huh7cell was isolated using Trizol Reagent as per the manufacturer's instructions (Thermo Fisher Scientific, Cat. No. 15596026). The total RNA from mice liver tissue was isolated by homogenizing frozen liver tissue in Trizol reagent and then used for RNA isolation. Single-strand cDNAs were synthesized from 20 ng total RNA using miRCURY LNA™ RT Kit (Qiagen, Maryland, USA, Cat# 339340). Real-time polymerase chain reaction (RT-PCR) for miR-22 and 5S RNA (Qiagen) was performed using SYBR Green master mix (Qiagen) in a ViiA 7 Real-Time PCR system (Applied Biosystems, Thermo Fisher Scientific). 5S RNA was used as a control in all the RT-PCR experiments, and the relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method as described previously [25].

### 2.5. Western blot experiments

The total cell lysate was prepared by adding MPER mammalian protein extraction reagent (Thermo Fisher Scientific, Cat No. 78501) containing protease inhibitor cocktail (100:1 ratio; Thermo Fisher Scientific, Cat No. 78430) followed by sonication. The MPER reagent with protease inhibitor cocktail was added to the liver tissue and homogenized. The homogenized lysate was centrifuged at 12,000 g for 15 min at 4 °C, and the protein in supernatant was quantitated using bicinchoninic acid protein assay kit (Pierce Cat No. 23227). Total proteins from Huh7 cells or liver tissues (40 μg) or WRL-68 cells (80 μg) were separated using 10% sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto polyvinylidene fluoride membranes. Polyvinylidene fluoride membranes were blocked using 5% nonfat dry milk for 1 h and then incubated with primary antibodies against SIRT1, PGC1α, phosphoenolpyruvate carboxykinase (PEPCK), G6P, or β-actin overnight at 4 °C on a rocker. After washing with tris buffered saline with 0.1% tween-20 (TBS-T), the membranes were incubated with horse radish peroxidase (HRP)-conjugated secondary antibodies for 1 h. The membranes were again washed and developed using Clarity™ western ECL Substrate (BioRad, Hercules, CA, USA, Cat. No. 170-5061). The band intensities were quantified using Image J software (NIH, USA), and the relative intensity of the protein to β-actin was calculated.

### 2.6. Triglyceride assay

Serum was isolated from the blood of mice after centrifugation at 2000*g* for 10 min at room temperature. It was used for the quantification of triglycerides using Triglyceride Quantification Colorimetric/Fluorometric Kit (BioVision, Milpitas, CA, USA, Catalog #K622) as per the manufacturer's protocol. Briefly, 3 μL serum was diluted to 50 μL with triglyceride assay buffer. Diluted serum was incubated with 2 μL of lipase for 20 min at room temperature to convert triglyceride into glycerol and fatty acid. Then, it was mixed with 50 μL triglyceride reaction mixture (triglyceride assay buffer 46 μL, triglyceride probe 2 μL, and enzyme mix 2 μL) and incubated at room temperature for 60 min. The absorbance was taken at 570 nm, and the TG content was calculated.

### 2.7. H&E staining and oil red O staining

Morphology of liver tissues was examined using hematoxylin and eosin (H&E) staining. The mouse liver tissues were embedded into optimal cutting temperature compound (OCT) and sectioned into slices of 5 μm thickness. Part of the sectioned tissues were fixed with 4% paraformaldehyde and then stained with H&E as per standard procedures [31]. Other part of the tissue sections were fixed using 4% paraformaldehyde for 10 min, washed with phosphate buffered saline (PBS), and then incubated with Oil Red O stain for 30 min followed by 1 min of counterstaining with hematoxylin. After washing, the sections were visualized under the microscope.

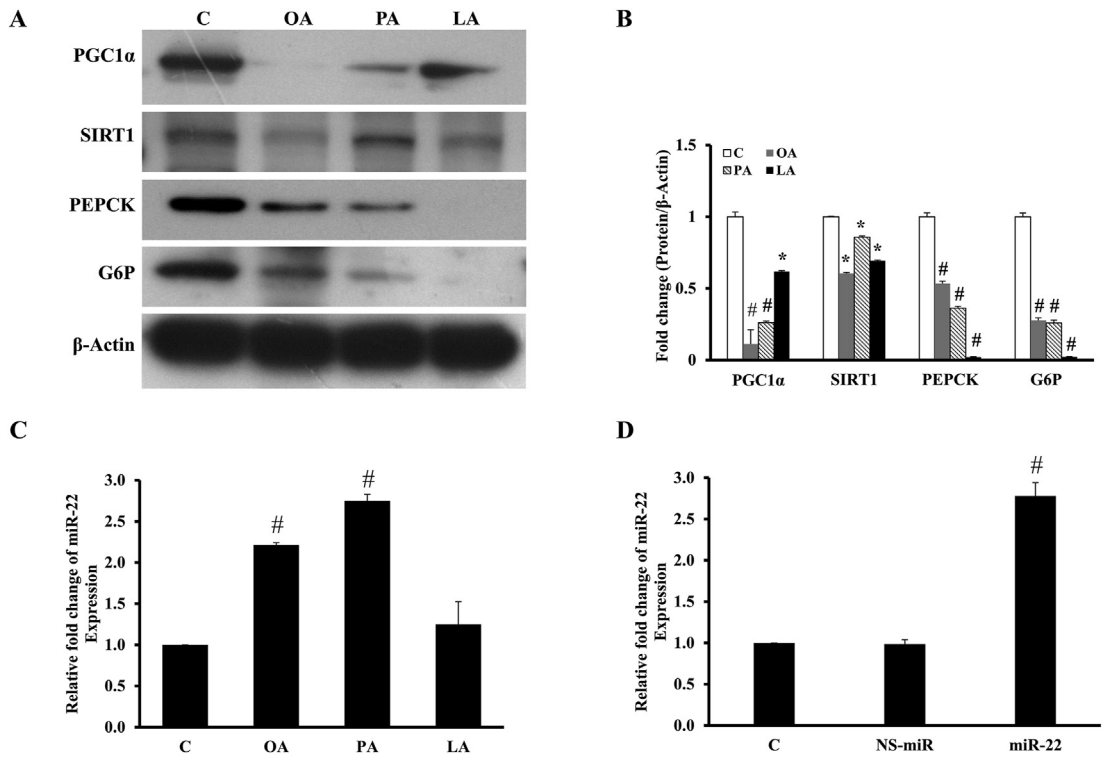


**Fig. 1. Effect of FFAs on PGC-1α, SIRT-1, PEPCK, G6P and miR-22 expression**. Huh7 cells were incubated with FFAs (OA, PA and LA; 300 μM each) for 48 h or 72 h s for RNA or protein isolation respectively. (A) The representative Western blot results show the expression of PGC1α, SIRT1, PEPCK, G6P, and β-actin. Lane 1, control; lane 2, OA-treated; lane 3, PA-treated, and lane 4, LA-treated cells. (**B**) The band intensities were quantified and the fold change was calculated ($n = 3$; $^*p \leq 0.05$ and $\#p \leq 0.01$). (C) RT-PCR was performed using total RNA for the expression of miR-22. 5s rRNA was used as internal control ($n = 3$; $\#p \leq 0.01$). (D) Huh7 cells were transfected with miR-22 premiRs and the intracellular expression of miR-22 was determined ($n = 3$; $\#p \leq 0.01$). FFAs, free fatty acids; G6P, glucose-6-phosphatase; LA, linoleic acid; OA, oleic acid; PA, palmitic acid; PEPCK, phosphoenolpyruvate carboxykinase; PGC1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; RT-PCR, real-time polymerase chain reaction; SIRT-1, sirtuin 1.

### 2.8. Statistical analysis

All the in vitro experiments were performed in duplicates and repeated at least three times. The standard deviation was calculated. The mean and standard deviation were calculated from the *in vivo* mice data of each group (6 mice per group). Unpaired Student's *t*-test or one-way ANOVA was used to calculate the difference between two groups or among the groups, respectively. The difference was considered significant only when $p \leq 0.05$.

## 3. Results

### 3.1. FFAs inhibit gluconeogenic enzymes and upregulate miR-22 expression

Incubation of Huh7 cells with FFAs (oleic acid [OA], palmitic acid [PA], or linoleic acid [LA]; 300 μM each) for 72 h significantly inhibited the expression of PGC-1α, SIRT1, PEPCK, and G6P as determined by Western blots (Fig. 1A and B) ($n = 3$; $^*p \leq 0.05$ and $\#p \leq 0.01$). Expression of miR-22 increased 2.25-fold and 2.75-fold in cells incubated with OA and PA, respectively (Fig. 1C) ($n = 3$; $\#p \leq 0.01$). However, no significant effect of LA on miR-22 expression was observed (Fig. 1C).

### 3.2. Overexpression of miR-22 inhibits the expression of gluconeogenic enzymes in FFA-treated cells

Previously, we had shown that miR-22 targeted 3′-UTR of SIRT-1 and decreased its expression in hepatic cells [25], and Yang, Zhuo et al. had also shown that miR-22 suppresses the expression of SIRT1 by targeting its 3′-UTR [32]. Hence, to evaluate the effect of miRNA-22 on FFA-mediated inhibition of gluconeogenic enzymes, Huh7 cells were transfected with miR-22 premiRs. The results showed enhanced expression of intracellular miR-22 levels in these cells compared to control or NS-miR-transfected cells (Fig. 1D) ($n = 3$; $\#p \leq 0.01$).

Overexpression of miR-22 in Huh7 cells treated with or without FFAs resulted in a significant decrease in the expression of PGC-1α, SIRT1, PEPCK, and G6P levels compared to control or NS-miRNA-transfected cells (Fig. 2A and B). Quantification of the band intensities showed that both OA and PA decreased all these proteins, while LA decreased only PGC-1α and G6P expression (Fig. 2C) ($n = 3$; $^*p \leq 0.05$ and $\#p \leq 0.01$). Additionally, the effect of miR-22 overexpression on the levels of these proteins in WRL-68 cells was determined. The overexpression of miR-22 in WRL-68 cells treated with or without FFAs decreased the expression of PGC-1α, SIRT1, PEPCK, and G6P (Fig. 2D and E). The quantitative analysis of the bands showed that there was no further decrease in the expression of these gluconeogenic proteins by FFAs in miR-22-transfected cells (Fig. 2F) ($n = 3$; $^*p \leq 0.05$, $\#p \leq 0.01$ and $\#\#p \leq 0.001$).

### 3.3. Inhibition of miR-22 increases the expression of gluconeogenic enzymes in FFA-treated cells

To confirm the involvement of miR-22 in regulating gluconeogenesis, the Huh7 cells were transfected either with anti-miR-22 or NS-miR. The expression of miR-22 was measured, and a significant decrease in its expression was observed in anti-miR-22-transfected cells (Fig. 3A). Inhibition of miR-22 followed by incubation with FFAs resulted in a significant increase of gluconeogenic enzymes in Huh7 cells (Fig. 3B and C) ($n = 3$; $^*p \leq 0.05$; $\#p \leq 0.01$; $\#\#p \leq 0.001$). Next, the effect of inhibiting miR-22 in WRL-68 cells was determined, and an increase in the gluconeogenic enzymes was observed in anti-miR-22-transfected cells

incubated with or without FFAs (Fig. 3D and E). Quantification of the western blot results showed that the increase in expression of these enzymes was significant ($n = 3$; $*p \leq 0.05$ and $\#p \leq 0.01$) for all anti-miR-22-transfected cells, treated with or without FFAs (Fig. 3F). These results suggested that miR-22 is directly involved in modulating gluconeogenesis in hepatic cells.

### 3.4. Accumulation of lipid droplets increases in mice fed with CDAA diet

CDAA diet-induced NAFLD mice model was used to determine the expression of miR-22 and the expression of gluconeogenic enzymes in the liver of these mice. H&E staining revealed the presence of inflammation and ballooning in the liver tissue of mice fed with CDAA diet, while the liver tissue of standard or CSAA diet-fed mice showed normal histology. The extent of ballooning and inflammation in the mice liver tissue increased significantly as the duration of feeding increased from 6 to 54 weeks (Fig. 4A–D). Lipid accumulation also increased in the liver tissues of CDAA diet-fed mice (Fig. 5A), and it further increased with longer duration of feeding upto 54 weeks (Fig. 5B–D). Although there was a slight weight gain in the mice fed with CDAA diet, the increase in body weight was not significant.

### 3.5. Triglyceride content and expression of miR-22 increases in CDAA diet-fed mice

TG content was quantified in the serum of mice fed with standard diet, CSAA or CDAA diet. It was observed that the amount of TG was significantly elevated in the serum of the mice fed with CDAA diet compared to that in serum of mice fed with standard or CSAA diet. The maximum increase in serum TG levels was observed after 54 weeks of CDAA diet (Fig. 6A) ($n = 6$; $*p \leq 0.05$ and $\#p \leq 0.01$).

The expression of miR-22 was quantified in the liver tissues isolated from these mice. A significant increase in the expression of miR-22 was observed in the liver of CDAA fed mice compared with liver of either standard diet or CSAA diet fed mice (Fig. 6B) ($n = 6$; $*p \leq 0.05$ and $\#p \leq 0.01$). There was a time-dependent increase in the miR-22 levels found in the liver of CDAA diet-fed mice.

### 3.6. CDAA diet inhibits gluconeogenic enzymes in mice liver

The expression of PGC1α, SIRT-1, PEPCK, and G6P was significantly decreased in the liver of mice fed with CDAA diet compared to those fed with standard or CSAA diet (Fig. 7A, C, E and G). The Western blot quantification showed that there was a significant inhibition in the expression of the gluconeogenic enzymes in CDAA-fed mice but not in standard or CSAA-fed mice (Figs. 7B, D, F and H) ($n = 6$; $*p \leq 0.05$, $\#p \leq 0.01$ and $\#\#p \leq 0.001$).

## 4. Discussion

Several attempts have been made to understand the mechanisms regulating NAFLD and NASH although the complete mechanism is not yet understood [33,34]. Previous studies have shown that there was a direct involvement of FFAs in lipid accumulation, thereby contributing to the development of NAFLD, which might be due to improper metabolism in the liver and accumulation of triglycerides in the liver [35–38]. PGC-1α is shown to regulate mitochondrial biogenesis, fatty acid oxidation, adaptive thermogenesis, and gluconeogenesis [39,40]. PGC-1α is also involved in the detoxification of reactive oxygen species in mitochondria which are generated during mitochondrial respiration, thereby increasing the efficiency of mitochondrial functions [41–43]. There are some physiological conditions, such as, exercise, fasting, and low temperature, that stimulate the gene expression of PGC-1α [39]. High-fat diet was shown to inhibit PGC-1α expression in mice [44]. The same study also showed that LA inhibited PGC-1α expression in Hep G2 cells [44].

In this study, three FFAs were selected based on their saturation levels. PA is a saturated fatty acid; OA is a monounsaturated fatty acid, while LA is a polyunsaturated fatty acid. Preliminary experiments were performed in Huh7 cells to check the cytotoxicity of different concentrations of FFAs

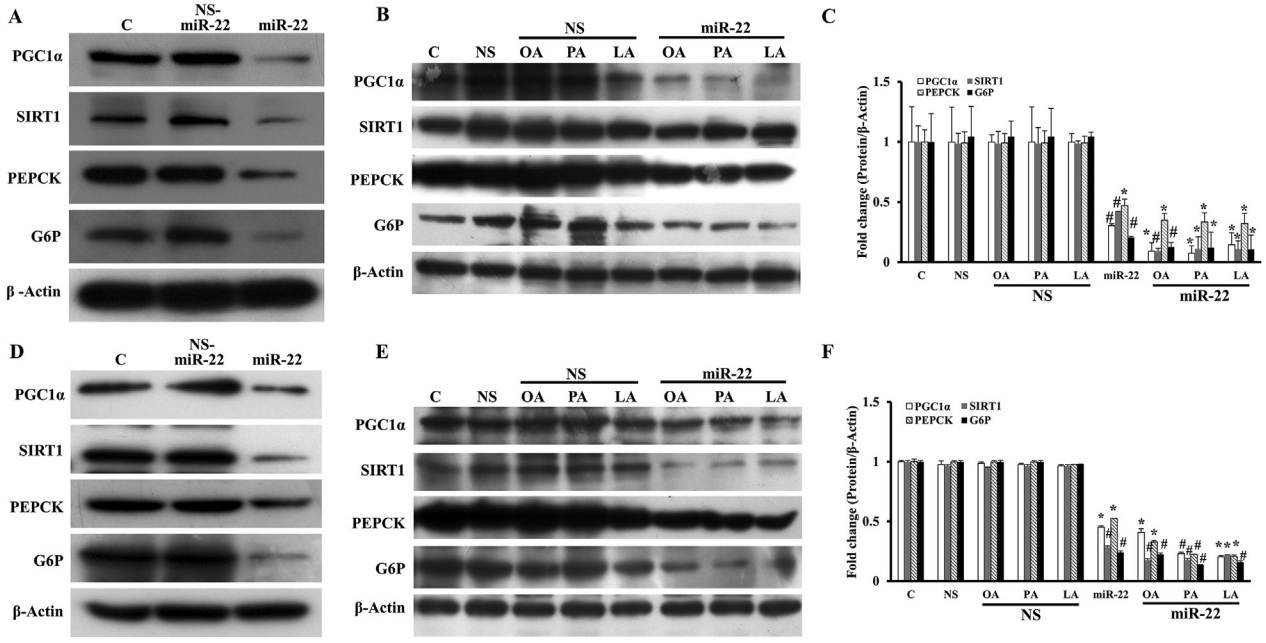


**Fig. 2. Effect of overexpression of miR-22 on the expression of gluconeogenic enzymes in FFA-treated cells**. Huh7 cells (A–C) and WRL-68 cells (D–F) were transfected with miR-22 premiRs, followed by treatment with 300 μM FFAs (OA or PA or LA) and the cells were collected after 48 h or 72 h after transfection for the isolation of total RNA or total protein respectively. (A and D) The representative images of the Western blot results for the expression of PGC1α, SIRT1, PEPCK, G6P, and β-actin ($n = 3$) in miR-22-transfected cells. Lane 1, control cells; lane 2, NS-miR-transfected cells; and lane 3, miR-22-transfected cells. (B and E) The representative images of the Western blot results for the expression of PGC1α, SIRT1, PEPCK, G6P, and β-actin ($n = 3$) in FFA-treated with miR-22-transfected cells. (C and F) Densitometric analysis of the band intensities of the relative expression of PGC1α, SIRT1, PEPCK, and G6P compared to β-actin ($n = 3$; $*p \leq 0.05$; $\#p \leq 0.01$; $\#\#p \leq 0.001$). FFAs, free fatty acids; G6P, glucose-6-phosphatase; LA, linoleic acid; OA, oleic acid; PA, palmitic acid; PEPCK, phosphoenolpyruvate carboxykinase; PGC1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; RT-PCR, real-time polymerase chain reaction; SIRT-1, sirtuin 1.

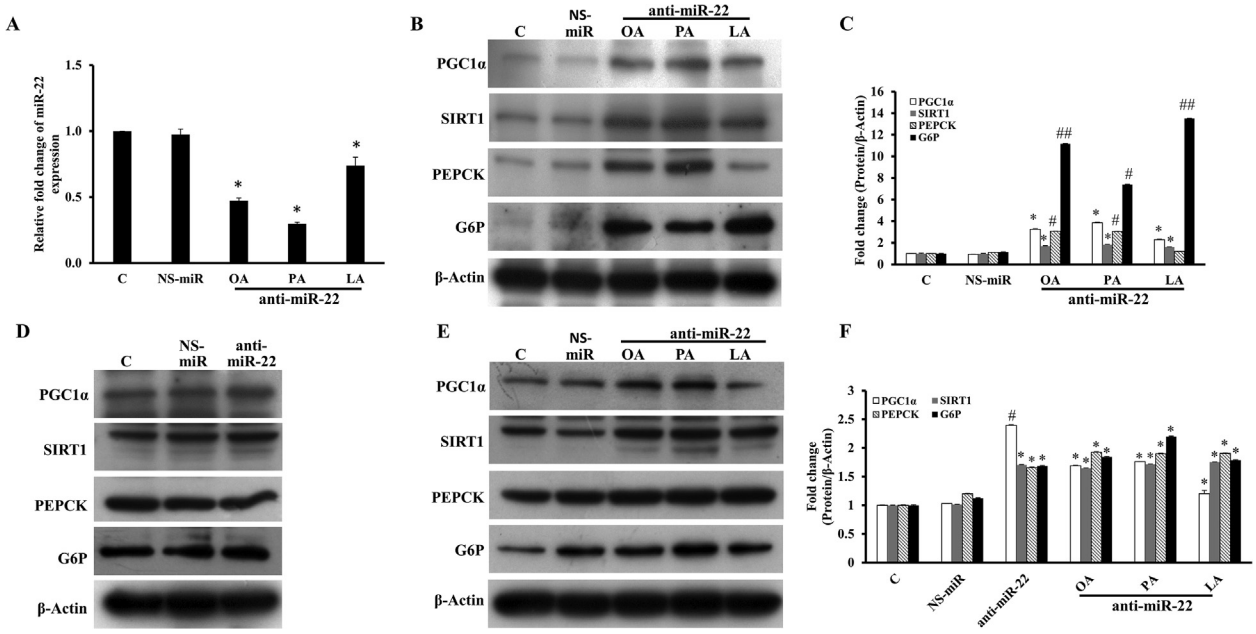


**Fig. 3. Effect of inhibition of miR-22 on the expression of gluconeogenic enzymes in FFA-treated cells**. Huh7 cells (A–C) and WRL-68 cells (D–F) were transfected with anti-miR-22 oligos, followed by treatment with 300 μM FFAs (OA or PA or LA) and the cells were collected after 48 h or 72 h after transfection. (A) Relative expression of miR-22 in OA, PA, or LA-treated miR-22 transfected cells ($n = 3$; $^*p \leq 0.05$). (B and E) The representative images of the Western blot results for the expression of PGC1α, SIRT1, PEPCK, G6P and β-actin ($n = 3$) in FFA-treated with anti-miR-22-transfected cells. (D) The representative images of the Western blot results for the expression of PGC1α, SIRT1, PEPCK, G6P and β-actin ($n = 3$) in anti-miR-22-transfected cells. (C and F) Densitometric analysis of the band intensities of the relative expression of PGC1α, SIRT1, PEPCK, G6P compared to β-actin ($n = 3$; $^*p \leq 0.05$; $\#p \leq 0.01$; $\#\#p \leq 0.001$). FFAs, free fatty acids; G6P, glucose-6-phosphatase; LA, linoleic acid; OA, oleic acid; PA, palmitic acid; PEPCK, phosphoenolpyruvate carboxykinase; PGC1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; RT-PCR, real-time polymerase chain reaction; SIRT-1, sirtuin 1.

ranging from 100 to 500 μM for 24–72 h. Cells incubated with upto 300 μM concentration for 72 h did not show any cytotoxic effect; hence, this concentration and time point were chosen for all the experiments (data not shown). Several researchers have treated hepatic cells with 1 mM FFAs for upto 24 h to study their effects [45,46]. The lower dose and longer duration of FFAs treatment used in our study mimicked the long-term effects of accumulated fatty acids in hepatic cells.

Previously, it was shown that when primary mouse hepatocytes, isolated from the overnight starved mice, were incubated with FFAs for up to 4 h, the mRNA levels of PEPCK and G6P were increased via activation of

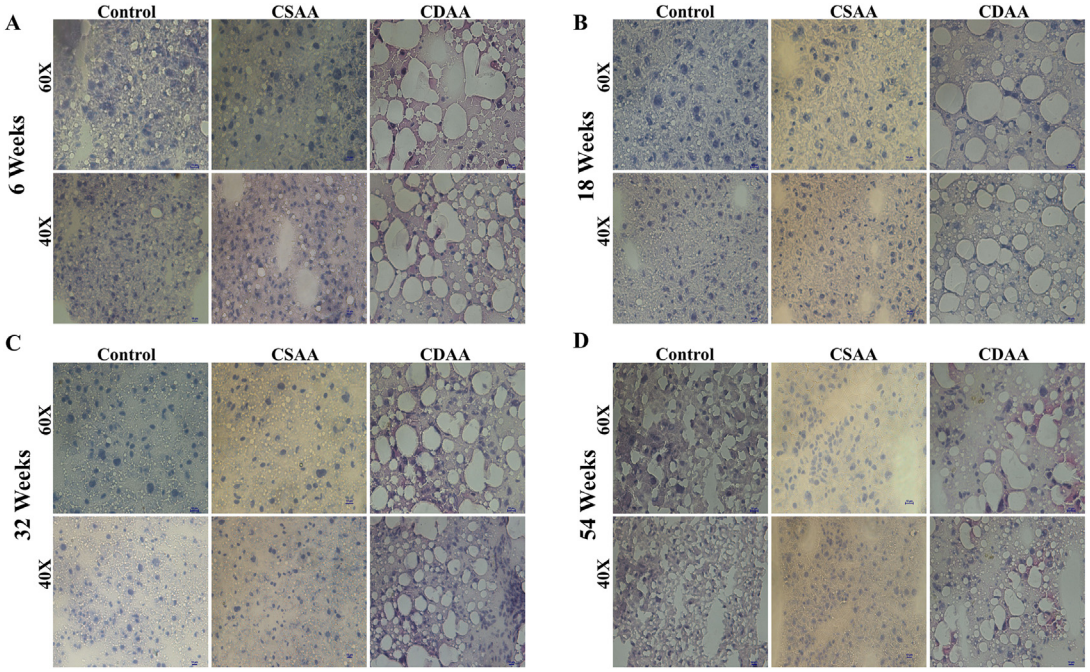


**Fig. 4. Hematoxylin and eosin (H&E) staining of mice liver tissue**. The mice liver tissues were embedded into OCT and sectioned into slices of 5 μm thickness. The sectioned tissues were fixed with 4% paraformaldehyde and then stained with H&E as per the standard procedures mentioned in materials and methods.

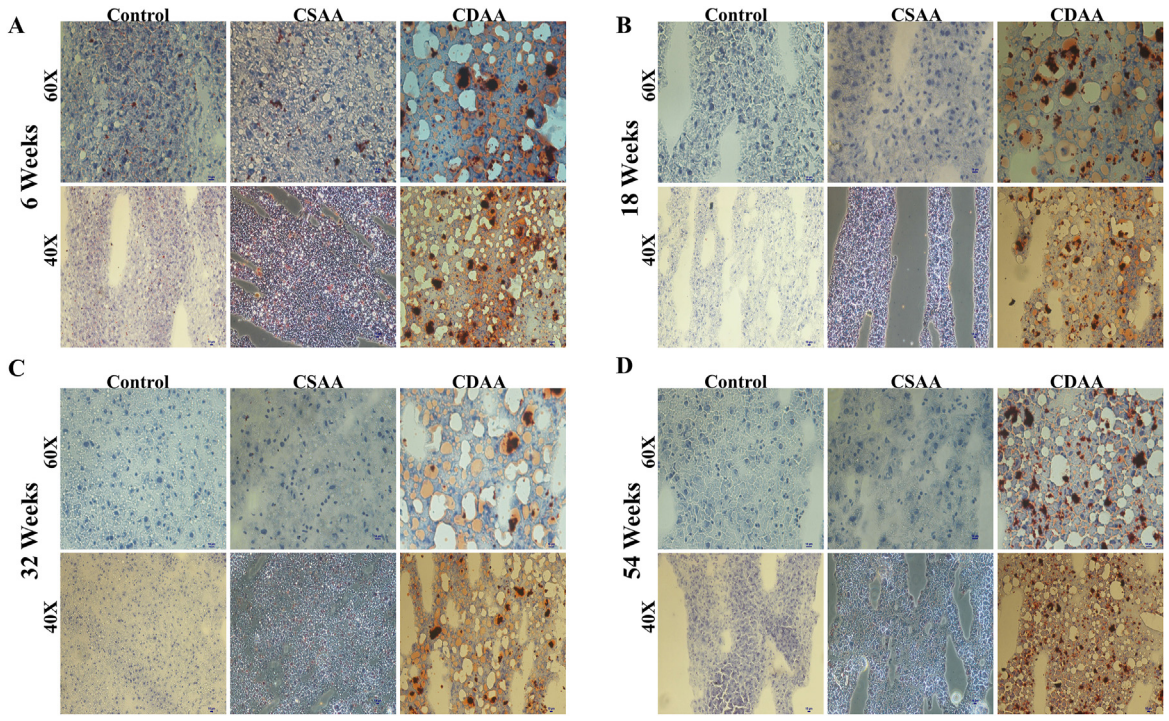


**Fig. 5. Accumulation of lipid in hepatocytes**. The mice liver tissues were embedded into OCT and sectioned into slices of 5 μm thickness. The tissue sections were fixed using 4% paraformaldehyde for 10 min and after washing with PBS, the tissue sections were incubated with Oil Red O stain for 30 min, followed by 1 min of counterstaining with hematoxylin.

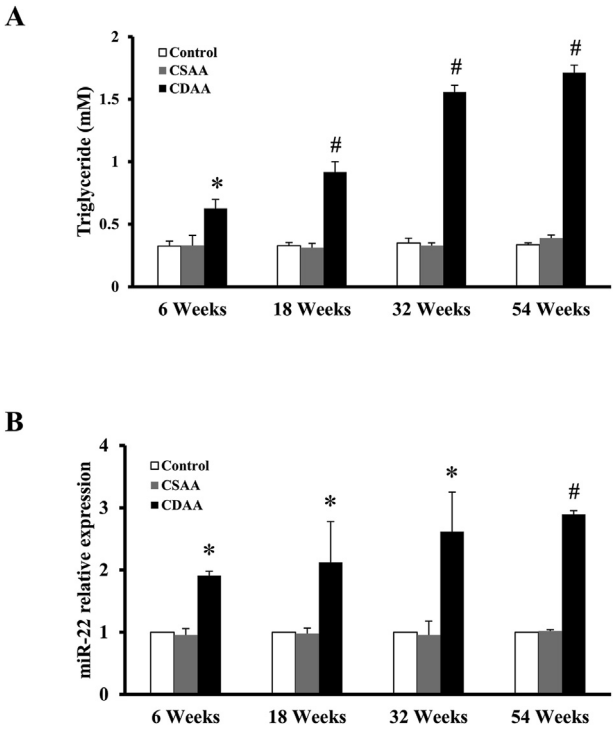


**Fig. 6. Triglyceride (TG) content and miR-22 levels in the CDAA-induced NAFLD mice model system**. (A) TG levels in the serum of C57BL/6 mice fed with standard-, CSAA- or CDAA-diet for the duration of 6, 18, 32 and 54 weeks ($n = 6$; $*p \leq 0.05$; $\#p \leq 0.01$). (B) Expression of miR-22 in the livers of C57BL/6 mice fed with control, CSAA or CDAA diet for the duration of 6, 18, 32 and 54 weeks ($n = 6$; $*p \leq 0.05$ and $\#p \leq 0.01$). CDAA, choline-deficient l-amino acid diet; CSAA, choline-sufficient l-amino acid diet; NAFLD, nonalcoholic fatty liver disease.

p38 mitogen-activated protein kinase [47]. However, other studies have shown that when the hepatic cells are treated with FFAs for longer duration, gluconeogenesis gets inhibited [48,49]. In the present study, the hepatic cells incubated with FFAs for up to 72 h showed an inhibition of the expression of gluconeogenic enzymes. These data suggest that the fatty acids can induce gluconeogenesis under starved conditions, and they might inhibit gluconeogenesis under pathological conditions or fed state.

In our study, treating Huh7 cells with FFAs significantly inhibited the expression of PGC-1α. It is known that PGC-1α is necessary to activate the gluconeogenic genes, such as PEPCK and G6P. As expected, our results showed that the expression of these two genes was also down-regulated significantly in hepatic cells treated with FFAs. The upstream regulator of PGC-1α activity is SIRT-1, and interestingly, our data showed that incubation with FFAs reduced SIRT-1 expression as well.

Previously, we had shown that miR-22 inhibited SIRT-1 expression and Akt phosphorylation in hepatic cells [25]. Hence, we hypothesized that FFAs might induce miR-22, which in turn might inhibit SIRT-1, ultimately leading to decreased PGC-1α activation. Indeed, incubation of Huh7 cells with FFAs resulted in increased expression of miR-22, suggesting that inhibition of hepatic gluconeogenesis by FFAs is mediated by miR-22 and SIRT-1. Further, transfection of two different hepatic cell lines, Huh7 and WRL-68, with miR-22 premiRs or anti-miR-22 confirmed the mediatory role played by miR-22 and SIRT-1 in regulating gluconeogenesis in hepatocytes. WRL-68 cells show morphological and functional characteristics of a human hepatic cell line and can be used as an in vitro hepatic model [50]. These cells were previously taken as a model of normal hepatic cells to show the effects of FFAs on apoptosis [51].

CDAA diet-fed mice model is one of the accepted models for the development of NASH/NAFLD in mice. Feeding mice with CDAA diet triggers insulin resistance, which is directly associated with pathological conditions of the liver such as NAFLD, NASH, and HCC. Hence, CDAA diet-induced NAFLD mice model was utilized in this study to verify whether expression of miR-22 and gluconeogenetic enzymes is modulated during progression of NAFLD, and we did not observe HCC in mice fed with CDAA diet for 54 weeks. The histological analysis of the liver

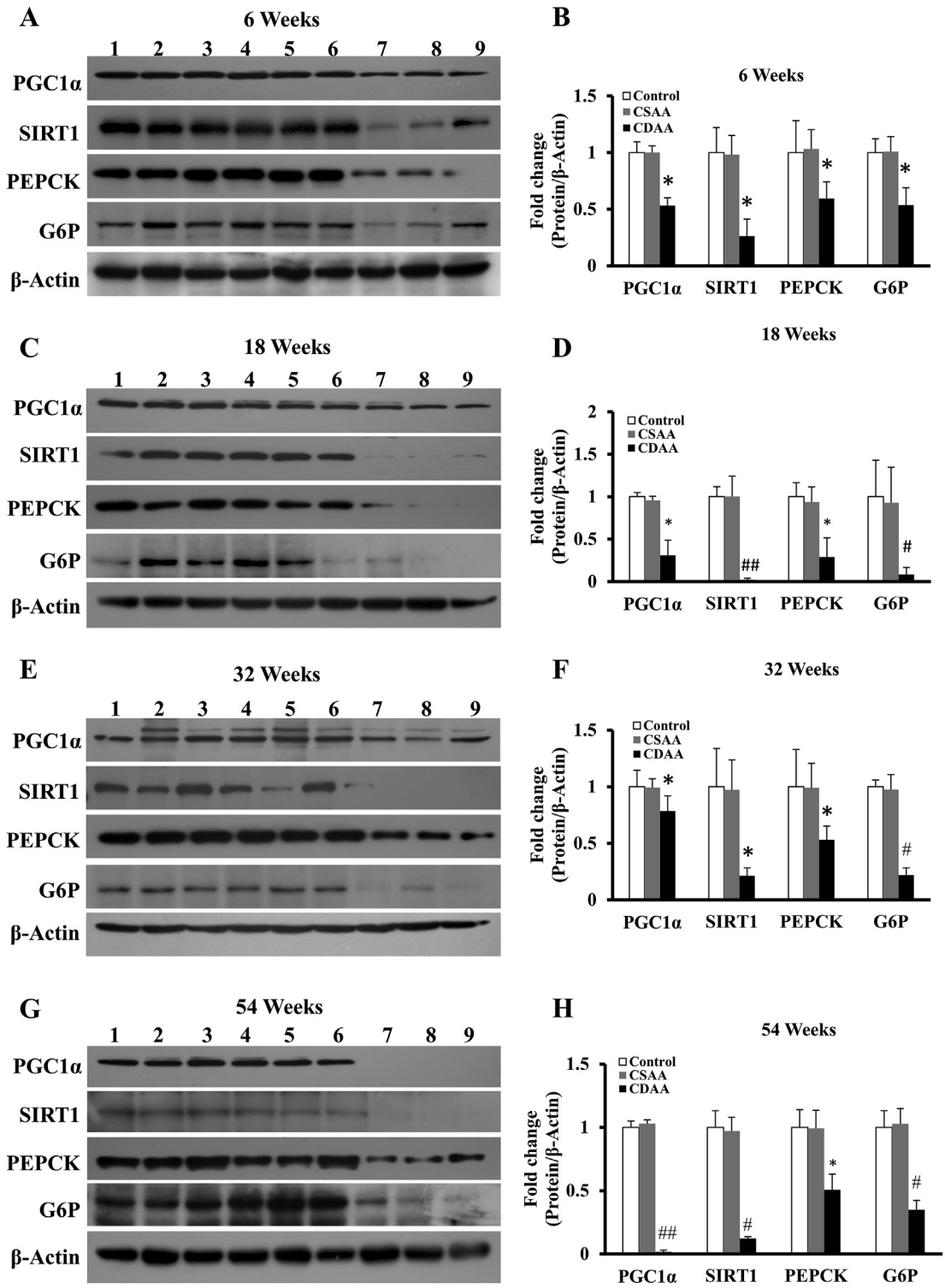


**Fig. 7. Effect of CDAA diet on gluconeogenesis in liver**. Expression of the gluconeogenic genes in the livers of C57BL/6 mice fed with control, CSAA or CDAA diet for the duration of 6, 18, 32 and 54 weeks. (A, C, E and G) The representative images show the Western blot results of the expression of PGC1α, SIRT1, PEPCK, G6P and β-actin (lanes 1–3, control; lanes 4–6, CSAA; and lane 7–9, CDAA). (B, D, F and H) Relative band intensities of PGC1α, SIRT1, PEPCK, G6P as compared to β-actin of the Western blots. (*$p \leq 0.05$, #$p \leq 0.01$ and ##$p \leq 0.001$). CDAA, choline-deficient l-amino acid diet; CSAA, choline-sufficient l-amino acid diet; FFAs, free fatty acids; G6P, glucose-6-phosphatase; PEPCK, phosphoenolpyruvate carboxykinase; PGC1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; SIRT-1, sirtuin 1.

tissues and measurement of TG in the serum samples confirmed that the NAFLD model had been established successfully. Previous studies have identified both HbA1C and TG as independent markers of NAFLD [52]. De Minicis et al. (2014) had shown that the liver of C57BL/6 mice fed with CDAA diet have reduced expression of gluconeogenic enzymes such as PEPCK and G6P [53]. This study also confirmed that the expression of SIRT-1, PGC-1α, PEPCK, and G6P was inhibited by CDAA diet.

Several studies have shown that miRNAs could play a crucial role in lipid, carbohydrate, and glucose homeostasis. miR-122 was the first miRNA shown to play a role in the regulation of lipid metabolism [54,

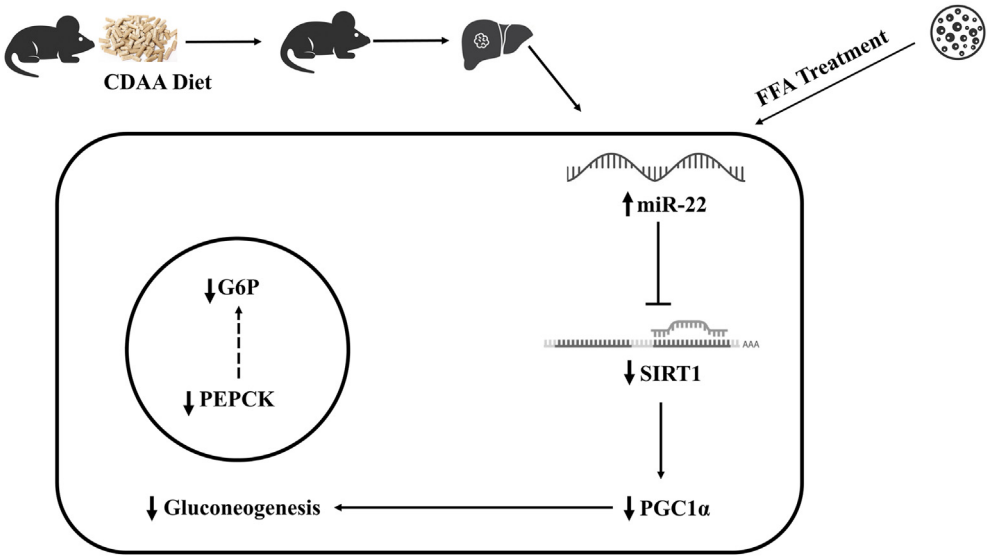


**Fig. 8.** Treatment of FFAs or CDAA diet in mice induced miR-22, which in turn inhibited the expression of SIRT-1, leading to decreased gluconeogenesis in hepatic cells. CDAA, choline-deficient l-amino acid diet; FFAs, free fatty acids; SIRT-1, sirtuin 1.

55]. Studies done in mice have shown that miR-34a is responsible for steatosis via SIRT1 suppression [56], while miR-132 and miR-30 are responsible for the regulation of lipid synthesis and lipoprotein secretion in the liver [57,58]. Another study done on ob/ob mice fed with high-fat diet has shown that miR-146a improves lipid and glucose metabolism by targeting MED1 [59]. The relationship between miR-22 and metabolic disorders has also been documented by several studies. One report showed that miRNA-22 played a negative role in human NAFLD and drug-induced steatosis as compared to healthy human [60]. Another study showed that inhibition of miR-22 in mice led to the prevention of high-fat diet-induced dyslipidemia [61]. Kaur et al. (2011) had shown that miR-22 and miRNA-34a were upregulated in the liver of db/db mice [62]. Our data showing the increased expression of miR-22 in the liver of CDAA diet-fed mice also supports these previous studies. However, we do not know the actual mechanisms by which FFAs or CDAA diet regulate miR-22, which needs to be explored in future studies.

In conclusion, the data from this study showed that treatment of hepatic cells with FFAs or lipid accumulation in liver induced miR-22 expression, which in turn inhibited the expression of SIRT-1, leading to decreased gluconeogenesis in hepatic cells (Fig. 8).

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## Author contributions

AKY has performed all transfection experiments, Western blots, TG assay, and other experiments. AKY also has analyzed data with the help of SKV and written the manuscript. DV, TNS, AKM, AKS, MAH, and KP helped in running Western blots and cell culture experiments. SKV designed the experiments, overseen the progress of work, and corrected the manuscript.

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## Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Senthil Kumar Venugopal reports was provided by India Ministry of Science & Technology Department of Science and Technology.

## Data available statement

Data and materials will be available from corresponding author upon reasonable upon request.

## Ethics statement

The study was approved by Institutional Animal Ethics Committee (Approval Number: SAU/IAEC/2018/01), South Asian University, New Delhi.

## Informed consent

Informed consent were waived for this study because no patients' data were reported.

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