

# Excess fructose and fatty acids trigger a model of non-alcoholic fatty liver disease progression *in vitro*: Protective effect of the flavonoid silybin

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Received February 19, 2019; Accepted May 7, 2019

DOI: 10.3892/ijmm.2019.4234

**Abstract.** Overconsumption of fats and sugars is a major cause of development of non-alcoholic fatty liver disease (NAFLD). The main objectives of the present study were to explore the pathways sustaining the interfering metabolic effects of excess fructose and fatty acids in hepatocytes, and to clarify the mechanisms through which the nutraceutical silybin rescues the functional and metabolic alterations that are associated with the NAFLD progression. Cultured hepatocytes were exposed to fructose and fatty acids, alone or in combination, to induce different grades of steatosis *in vitro*. Cell viability, apoptosis, free radical production, lipid content, lipid peroxidation and activity of lipogenic enzymes were assessed by spectrophotometric assays. Oxygen consumption and mitochondrial respiration parameters were measured using a Seahorse analyzer. Expression of markers for liver steatosis and dysfunction were also evaluated by reverse transcription-quantitative polymerase chain reaction. The data revealed that fructose and fatty acid combination *in vitro* had a positive interference on lipogenic pathways, leading to more severe steatosis and liver dysfunction, reduced cell viability, increased apoptosis, oxidative stress and mitochondrial respiration. Hepatic cell abnormalities were almost completely alleviated by silybin treatment. These findings suggest that silybin may serve as a novel and cost-effective dietary supplement for treatment and/or prevention of hepatosteatosis associated with NAFLD progression.

## Introduction

Overnutrition plays a pivotal role in obesity and comorbidities including nonalcoholic fatty liver disease (NAFLD), type 2 diabetes mellitus and cardiovascular disease (CVD) (1). NAFLD is characterized by hepatic accumulation of fat, particularly triglycerides (TGs), and may range from simple steatosis to nonalcoholic steatohepatitis (NASH), cirrhosis and hepatocellular carcinoma (2). In liver cells, excess TGs are stored in lipid droplets (LDs), and LD-associated proteins, such as the adipose differentiation-related protein (ADRP), regulate lipid packing and traffic (3). TG synthesis is a beneficial response against excess of potentially toxic fatty acids (FAs), leading to inflammation and reactive oxygen species (ROS) formation, particularly in mitochondria (4), which trigger lipid peroxidation of membranes acting in NAFLD progression (5).

Fructose-enriched food may contribute to the development of NAFLD (6). Fructose can enter *de novo* FA synthesis in liver cells through the action of fatty acid synthase (FAS). However, the extent to which fructose contributes to the metabolic disorders remains unclear, as only a limited number of data reporting its direct effects on hepatocyte during NAFLD progression are available (7).

In liver cells, NAFLD is associated with alterations in lipogenic and lipolytic pathways, which are controlled by a number of transcription factors, such as peroxisome proliferator-activated receptor (PPAR) (8), and by microRNAs (miRNAs/miRs), including miR-122, which is the most abundant hepatic miRNA (9). Dysregulation of miRNA expression has been reported in rodent models of NAFLD, and in certain cases aligned with the changes observed in obese patients with steatosis (10).

A deeper understanding of the mechanisms underlying NAFLD progression would help identifying novel cost-effective therapeutic strategies. It has been reported that plant polyphenols are promising molecules for the management of NAFLD (11). Silybin, the most relevant flavonolignan extract from the seeds of milk thistle (*Silybum marianum*) (12),

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**Key words:** non-alcoholic fatty liver disease, dietary fructose, fatty acids, lipid metabolism, oxidative stress, silybin

exhibited certain beneficial effects in a preliminary study on NAFLD patients (13).

In the present study, an *in vitro* model of NAFLD progression was established to identify the pathways sustaining the interference between excess fructose and fatty acids on dysregulating lipid and radical metabolism in hepatocytes, and to verify the ability of silybin to reverse these alterations. The results may have an important translational value for possible therapy of hepatic steatosis associated with NAFLD.

## Materials and methods

**Cell treatments.** Rat hepatoma FaO cells (European Collection of Authenticated Cell Cultures, Salisbury, UK; cat. no. 89042701) were supplied as mycoplasma-free and cultured in Coon's modified Ham's F12 with 10% fetal bovine serum (South American origin, EU-approved; Euroclone, Milan, Italy). When 80% confluence was reached, the cells were incubated in starvation medium containing 0.25% bovine serum albumin (BSA). Subsequently, cells were treated with an oleate/palmitate mixture (2:1 molar ratio; final concentration, 0.75 mM) for 3 h (referred to as the FA treatment group), with 5.5 mM fructose for 72 h (Fru group), or with sequential combination of fructose for 72 h and FAs for 3 h (Fru/FA group). Cells in the Fru/FA group were then treated for 24 h with 50  $\mu$ M silybin (stock solution, 10 mM in dimethyl sulfoxide). Silybin treatment was also performed on untreated FaO cells, which served as the control group.

**Cell viability and apoptosis.** The sulforhodamine B (SRB) assay, relying on the property of SRB to bind stoichiometrically to proteins, is used to determine cell density. Briefly,  $1.5 \times 10^4$  cells/well were seeded in 96-well culture plates and treated. Next, the cells were fixed and incubated with 0.5% SRB in 1% acetic acid for 1 h at 37°C. The dye bound to proteins was extracted with 10 mM Tris-HCl (pH 10), and quantified in a Varian Cary-50 Bio spectrophotometer (Agilent Technologies, Inc., Milan, Italy) (14). Caspase 3-like activity is a marker of apoptosis as it initiates DNA fragmentation (15). Caspase activity was measured in cell extracts containing 25  $\mu$ g proteins determined by the bicinchoninic acid method (16). Following resuspension in 20 mM HEPES/NaOH (pH 7.5), 250 mM sucrose, 10 mM KCl, 2 mM  $MgCl_2$ , 1 mM EDTA, 2 mM dithiothreitol (DTT) and 100  $\mu$ M phenylmethylsulfonyl fluoride, the cell extracts were incubated for 1 h at 37°C in 25 mM HEPES (pH 7.5), 10% sucrose, 10 mM DTT, 0.1% CHAPS and 100  $\mu$ M caspase substrate Ac-DEVD-pNA. The released pNA was measured spectrophotometrically, and the results are expressed as nmol of pNA released per  $\mu$ g of protein (17).

**Lipid quantification and imaging.** TGs were extracted from the different cell groups and spectrophotometrically quantified as previously described (18). Data are expressed as the percent TG content relative to the control group. For LD visualization, cells growing on coverslips were treated as aforementioned, rinsed with PBS, fixed with 4% paraformaldehyde, stained by Oil Red O (19) and then examined with a Leica DMRB light microscope equipped with a Leica CCD camera DFC420C (Leica, Wetzlar, Germany).

**FAS activity.** FAS activity in the different cell groups was measured according to Goodridge (20). Briefly, cell lysate was obtained by mixing cells with 0.1 M KPi (pH 7.0), 3 mM EDTA and 1 mM DTT via a syringe needle. Then 20  $\mu$ g of lysate were mixed to 0.1 M KPi (pH 7.0), 0.025 mM acetyl coenzyme A (CoA), 0.2 mM NADPH, 3 mM EDTA, 1 mM DTT, 25 mg/ml BSA and 0.1 mM malonyl-CoA. NADPH disappearance was followed by spectrophotometric examination. FAS activity (nmol NADPH/min/mg protein) was expressed as the percentage relative to the control group.

**ROS production and lipid peroxidation.** ROS production was quantified through the oxidation of 2',7'-dichlorofluorescein diacetate (DCF-DA; Fluka, Germany) to 2',7'-dichlorofluorescein (DCF), which was measured using a LS50B fluorimeter (PerkinElmer, Inc., Waltham, MA, USA). Briefly, suspended cells were loaded with 10  $\mu$ M DCF-DA at 37°C in the dark, centrifuged (800 x g for 10 min at 4°C) and resuspended in PBS (21). The fluorescent intensity was normalized to the protein content. Lipid peroxidation was then evaluated through the thiobarbituric acid reactive substance assay, as previously described (22). Cells were incubated for 45 min at 95°C with 2 vol thiobarbituric acid (TBA) solution, containing 0.375% TBA, 15% trichloroacetic acid and 0.25 N HCl. Subsequently, 1 vol N-butanol was added, and the absorbance of the organic phase was measured. Values [pmol of malondialdehyde (MDA) per ml/mg protein] were expressed as the percentage relative to the controls.

**RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Total RNA was extracted using TRIzol reagent (Thermo Fisher Scientific, Inc., Milan, Italy) and quantified spectrophotometrically. Then, cDNA was synthesized by using RevertAid H Minus transcriptase according to manufacturer's instructions (Thermo Fisher Scientific, Inc.); qPCR was performed in quadruplicate using 1X IQ™ SYBR® Green SuperMix and a Chromo4™ system (Bio-Rad Laboratories, Inc., Milan, Italy) (23). Primer pairs for the assessed genes were designed *ad hoc* starting from the coding sequences of *Rattus norvegicus* (<http://www.ncbi.nlm.nih.gov/Genbank/GenbankSearch.html>) and listed in Table I. The amplification conditions were as follows: 3 min at 95°C, followed by 40 cycles consisting of 5 sec at 95°C, 30 sec of annealing (temperatures listed in Table I), and 40 sec of extension at 72°C. At the end, a melting curve ranging between 55 and 95°C was measured. The relative quantity of target mRNA was calculated by using the comparative Cq method and was normalized for the expression of GAPDH gene (24).

In order to measure miR-122 expression, the High-Capacity cDNA RT kit and the miRNA-specific primers provided with the TaqMan MicroRNA Assay kit (Thermo Fisher Scientific, Inc.) were used. Amplification was performed using the StepOnePlus Real-Time PCR system (Thermo Fisher Scientific, Inc.). Probe and primers for miR-122-5p (4427975-002245) and miRNA U6 (4427975-001973) were provided by Thermo Fisher Scientific, Inc. The relative expression of miR-122 and mRNAs was calculated by the comparative Cq method using miRNA U6 and GAPDH as housekeeping genes (24).

**Oxygen consumption.** Oxygen consumption was measured using the Seahorse XFe96 Extracellular Flux analyzer

Table I. Primer sequences table.

Gene	Forward primer	Reverse primer	Annealing temperature (°C)	Accession ID
ADRP	CCGAGCGTGGTGACGAGGG	GAGGTCACGGTCCTCACTCCC	64	AAH85861
GAPDH	GACCCCTTCATTGACCTCAAC	CGCTCCTGGGAAGATGGTGATGGG	60	DQ403053
IKBIP	CAGAACAGTGAGCAGGCAAG	ACGGCATTCTCTATGGTTGG	60	NM_001009430.2
PPAR $\alpha$	CCCCACTTGAAGCAGATGACC	CCCTAAGTACTGGTAGTCCGC	60	NM_013196
PPAR $\gamma$	CGGAGTCCTCCAGCTGTTCCGC	GGCTCATATCTGTCTCCGTCTTC	60	Y12882
PPAR $\delta$	AATGCCTACCTGAAAACTTCAAC	TGCCTGCCACAGCGTCTCAAT	60	AJ306400.1
CPT1	CCGCTCATGGTCAACAGCA	CAGCAGTATGGCGTGGATGG	60	NM_031559
UCP2	CGTCGGACCTAGCCGTCTGCA	CGGAGTCGGGAGGGTGCTTTG	56	BC062230

ADRP, adipose differentiation-related protein; IKBIP, inhibitor of nuclear factor- $\kappa$ B kinase subunit  $\beta$ -interacting protein; PPAR, peroxisome proliferator-activated receptor; CPT1, carnitine palmitoyltransferase 1; UCP2, uncoupling protein 2.

(Agilent Technologies, Inc., Santa Clara, CA, USA) as previously described (25). Briefly, approximately  $2 \times 10^4$  cells/well were seeded into 96-well plates. A final concentration of  $3 \mu\text{M}$  oligomycin,  $1 \mu\text{M}$  FCCP, and a mixture of  $1 \mu\text{M}$  rotenone and  $1 \mu\text{M}$  antimycin were added sequentially to cells. The sensor cartridge and the calibration plate were used for calibration. Three baseline rate measurements of oxygen consumption rate (OCR) were made using a 3-min mixing and 3-min measure cycle. The compounds were injected pneumatically by the Seahorse XFe96 analyzer into each well and mixed, following which the OCR measurements were conducted using the 3-min mixing and 3-min measure cycle (26).

**Statistical analysis.** Data are expressed as the mean  $\pm$  standard deviation of at least three independent biological experiments performed as technical triplicates. Statistical analysis was performed using analysis of variance with Tukey's post-test (GraphPad Software, Inc., San Diego, CA, USA). Differences with  $P \leq 0.05$  values were considered as statistically significant.

## Results

**Excess FAs and fructose alter lipid metabolism and cell function.** The extent of steatosis was assessed by Oil Red O staining and TG quantification. The steatosis features were assessed in terms of the accumulation of cytosolic LDs, whose number and size markedly increased in all steatotic cells compared with the control cells (Fig. 1A). As shown in Fig. 1B, quantification of intracellular TGs revealed that fructose and fatty acids alone increased the TG content by 57% ( $P \leq 0.01$ ) and 87% ( $P \leq 0.001$ ), respectively, compared with the control group, while their combination (Fru/FA) led to a larger increase of 277% vs. the control group ( $P \leq 0.001$ ). As markers for LD accumulation and hepatic cell dysfunction, the mRNA expression levels of ADRP and inhibitor of nuclear factor- $\kappa$ B kinase subunit  $\beta$ -interacting protein (IKBIP), respectively, were then assessed (Fig. 1C). ADRP expression was upregulated by FAs alone (1.85-fold induction vs. control;  $P \leq 0.001$ ), with even greater upregulation induced by the Fru/FA combination (2.08-fold induction vs. control;  $P \leq 0.001$ ). However, IKBIP expression was significantly upregulated only by

the Fru/FA combination (1.61-fold induction vs. control;  $P \leq 0.001$ ). Furthermore, it was observed that Fru alone, but not FAs, stimulated the FAS activity by 125% as compared with the control group ( $P \leq 0.05$ ), whereas the Fru/FA combination markedly reduced this Fru-induced activity by 59% (vs. Fru group;  $P \leq 0.05$ ; Fig. 1D). On the other hand, the expression of miR-122 showed a significant increase only in Fru/FA-treated cells (1.52-fold induction vs. control;  $P \leq 0.05$ ; Fig. 1E).

Lipid peroxidation was also assessed as a marker of oxidative stress. The MDA level increased by 89 and 67% in response to FAs alone and Fru/FA combination, respectively, as compared with the control group ( $P \leq 0.001$ ; Fig. 1F). This oxidative imbalance was paralleled by changes in caspase 3-like activity, which increased in cells exposed to FAs alone or Fru/FA combination (+142 and +145% vs. control, respectively;  $P \leq 0.001$ ; Fig. 1G). By contrast, cell viability did not change in FA cells, but it was significantly reduced by 13% in cells exposed to Fru alone as compared with the control cells ( $P \leq 0.01$ ), and further reduced by 23% in the Fru/FA combination group vs. the control ( $P \leq 0.01$ ; Fig. 1H).

**Silybin counteracts the steatogenic effects of fructose and FAs.** Exposure of Fru/FA cells to  $50 \mu\text{M}$  silybin for 24 h markedly reduced the steatosis grade by 35% of TG content ( $P \leq 0.01$ ), and the IKBIP upregulation by 32% ( $P \leq 0.001$ ) compared with Fru/FA cells (Fig. 2A and B). However, silybin did not change the ADRP mRNA level (Fig. 2C). Moreover, silybin treatment led to changes in the lipogenic transcription factor PPAR $\gamma$ , whose expression was upregulated in Fru/FA cells (1.55-fold induction vs. control;  $P \leq 0.01$ ) and reduced by 67% upon silybin treatment with respect to Fru/FA ( $P \leq 0.001$ ); by contrast, PPAR $\alpha$  and PPAR $\delta$  levels were not significantly altered by silybin (Fig. 2D). Exposure to silybin further increased miR-122 expression (1.84-fold induction vs. control;  $P \leq 0.001$ ) in Fru/FA cells, in which this miRNA was already overexpressed (Fig. 2E). Treatment of control cells with silybin had no effects on the expression of these genes (data not shown).

**Silybin counteracts apoptosis and mitochondrial dysfunction.** Silybin treatment did not rescue the reduction in cell viability

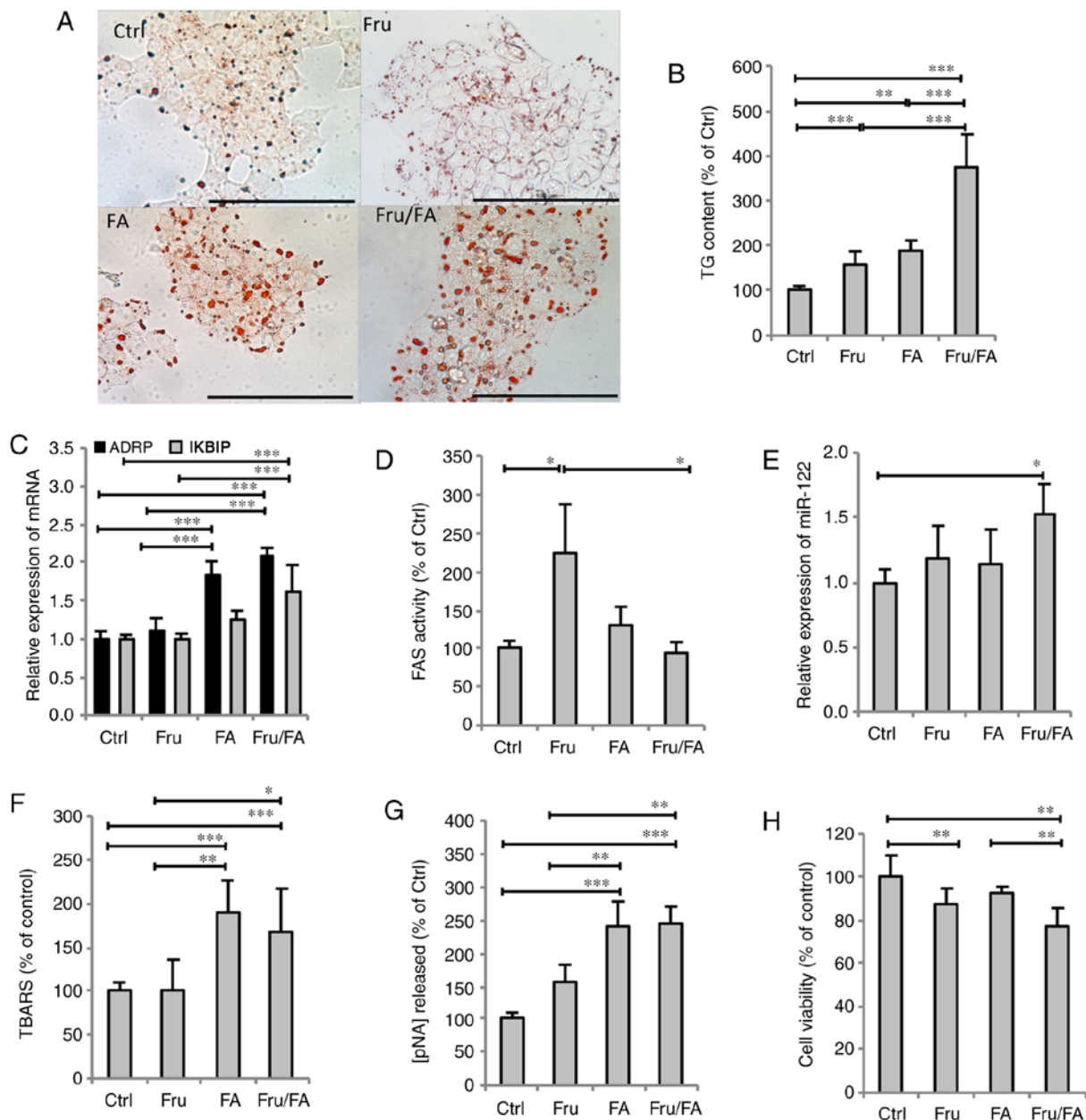


Figure 1. Steatogenic effects of Fru, FAs and their combination (Fru/FA) in FaO cells. (A) Lipid droplet accumulation was visualized by Oil Red O staining (magnification,  $\times 40$ ; bar, 100  $\mu\text{m}$ ). (B) TG content was quantified spectrophotometrically and normalized to total protein level. (C) Expression levels of ADRP and IKBIP mRNA were evaluated by RT-qPCR and expressed as the fold induction relative to the control. (D) FAS activity (nmol NADPH/min/mg protein) was quantified spectrophotometrically. (E) miR-122 expression was evaluated by RT-qPCR using U6 as the internal control and is expressed as the fold induction relative to the control. (F) Malondialdehyde level (pmol MDA/ml/mg protein) was quantified by TBARS assay. (G) Activity of caspase 3 (nmol of pNA released/ $\mu\text{g}$  protein) was measured spectrophotometrically. (H) Metabolic activity was measured by sulforhodamine B assay (percentage relative to the controls). All values are expressed as the mean  $\pm$  standard deviation from at least three independent experiments. \* $P \leq 0.05$ , \*\* $P \leq 0.01$  and \*\*\* $P \leq 0.001$ . Fru, fructose; FA, fatty acid; Ctrl, control; TG, triglyceride; ADRP, adipose differentiation-related protein; IKBIP, inhibitor of nuclear factor- $\kappa\text{B}$  kinase subunit  $\beta$ -interacting protein; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; FAS, fatty acid synthase; TBARS, thiobarbituric acid reactive substance.

caused by Fru/FA, but it had anti-apoptotic effects as indicated by the decrease in the caspase 3-like activity by 38% compared with the Fru/FA cells ( $P \leq 0.001$ ; Fig. 3A and B). Silybin was also able to counteract the lipid peroxidation (reduction by 30% as compared with Fru/FA;  $P \leq 0.05$ ) and the ROS levels (reduction by 50% as compared with Fru/FA;  $P \leq 0.001$ ; Fig. 3C and D) associated with excess fat in Fru/FA cells.

Steatotic hepatocytes typically stimulate respiration and ATP production in an attempt to counteract the excess TGs (21). Silybin reduced basal respiration by 56% ( $P \leq 0.01$ ), maximal

respiration by 62% ( $P \leq 0.001$ ) and ATP production by 60% ( $P \leq 0.001$ ) in Fru/FA cells, without significant effects on proton leak (Fig. 4A-D). Moreover, the expression of the mitochondrial proteins carnitine palmitoyltransferase 1 (CPT1) and uncoupling protein 2 (UCP2), which is the main regulatory step of mitochondrial FA oxidation, was increased in Fru/FA cells (2.29- and 1.74-fold induction, respectively, vs. control;  $P \leq 0.001$  and  $P \leq 0.01$ ). Further upregulation to the CPT1 and UCP2 levels by 68 and 50%, respectively, was observed upon exposure to silybin ( $P \leq 0.001$  for both; Fig. 4E). Treatment of control cells



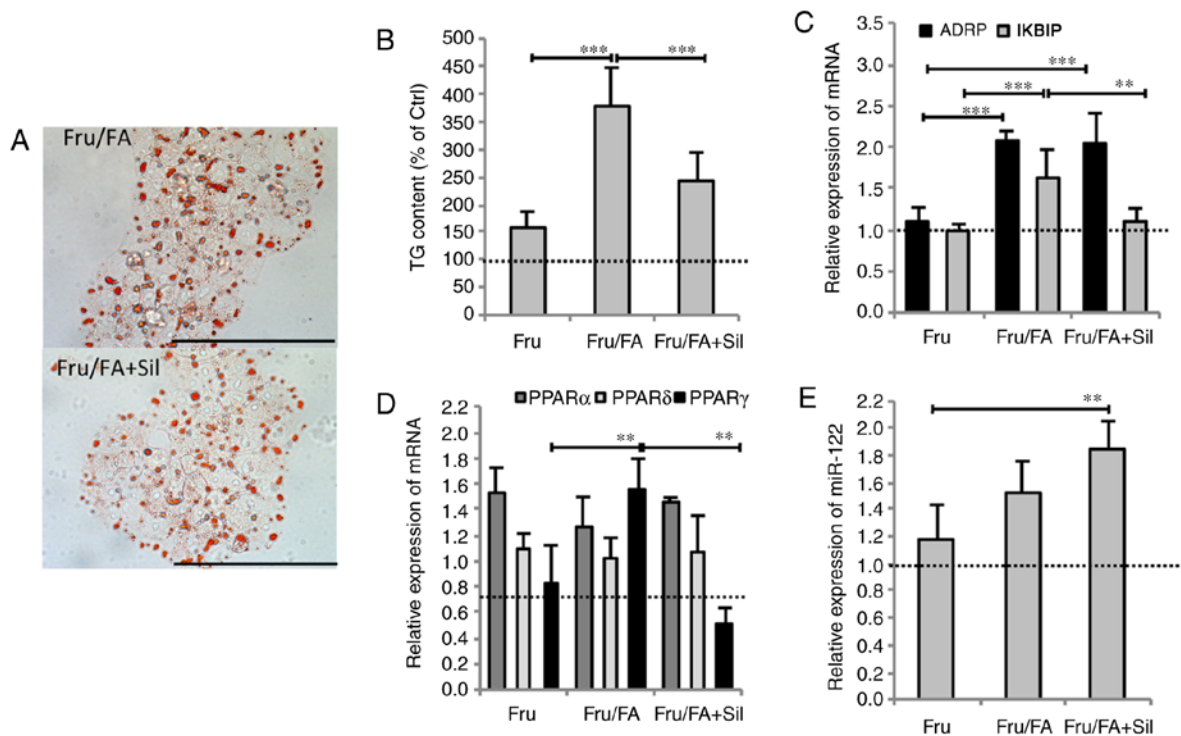


Figure 2. Silybin counteracts lipid metabolism dysregulation. Cells incubated with Fru/FA were then treated for 24 h with 50  $\mu$ M silybin. (A) Microphotographs of Oil Red O-stained cells at a magnification of x40 (bar, 100  $\mu$ m), and (B) histogram of TG content. (C) mRNA expression levels of ADPR and IKBP, (D) mRNA expression levels of PPAR $\alpha$ ,  $\gamma$  and  $\delta$ , and (E) miR-122 expression. All values are expressed as the mean  $\pm$  standard deviation from at least three independent experiments. \*\* $P \leq 0.01$  and \*\*\* $P \leq 0.001$ . Fru, fructose; FA, fatty acid; TG, triglyceride; ADPR, adipose differentiation-related protein; IKBP, inhibitor of nuclear factor- $\kappa$ B kinase subunit  $\beta$ -interacting protein.

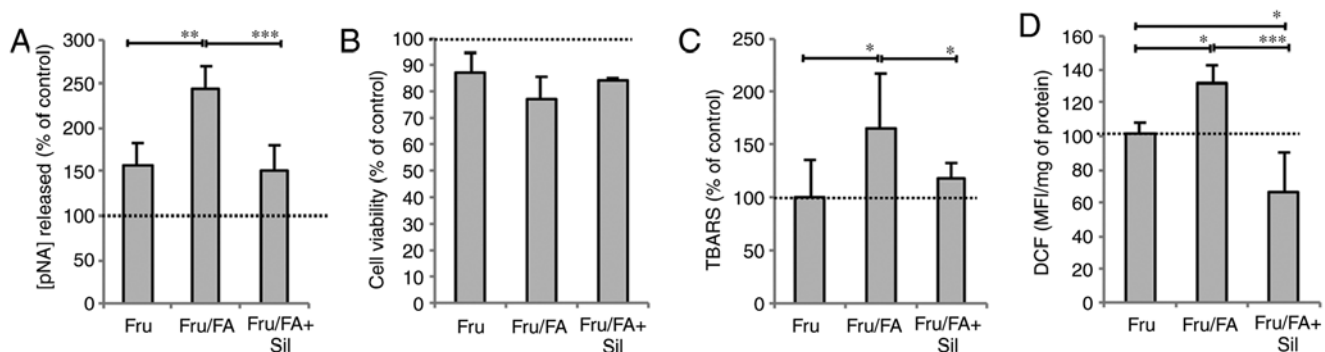


Figure 3. Silybin counteracts the cell viability and oxidative stress. Cells incubated with Fru/FA were then treated with silybin. (A) Activity of caspase 3. (B) Metabolic activity measured by sulforhodamine B assay, indicating the cell viability. (C) Malondialdehyde level was quantified by TBARS assay. (D) Intracellular level of reactive oxygen species was quantified fluorimetrically. All values are expressed as the mean  $\pm$  standard deviation from at least three independent experiments. \* $P \leq 0.05$ , \*\* $P \leq 0.01$  and \*\*\* $P \leq 0.001$ . Fru, fructose; FA, fatty acid; TBARS, thiobarbituric acid reactive substance.

with silybin had no effects on apoptosis, lipid peroxidation and mitochondrial respiration (data not shown).

## Discussion

The present study provided insights into the molecular mechanisms through which excess fructose impairs the lipogenic pathways in hepatocytes. In the past, fructose was considered as a beneficial dietary component since it does not stimulate insulin secretion; however, the harmful effects of fructose have recently gained mainstream attention. Studies have reported that high fructose intake stimulates *de novo* lipogenesis (27), and mice fed a diet of fats and high-fructose

corn syrup developed equally severe NAFLD (28). The findings of the present study revealed that exposure of FaO cells to a fructose/fatty acid combination led to larger TG synthesis and accumulation as compared with the single agents. In addition, it was observed that the more severe steatosis was associated with worsening of cell dysfunction parameters, including cell viability, oxidative stress and mitochondrial respiration.

In the cell model of the current study, the sequential exposure of hepatocytes to high fructose and fatty acids mimics the NAFLD progression *in vitro*. Excess fructose alone stimulates FAS activity resulting in TG overproduction, and Fru/FA combination led to more severe cell dysfunction compared with the single treatments, as confirmed by the

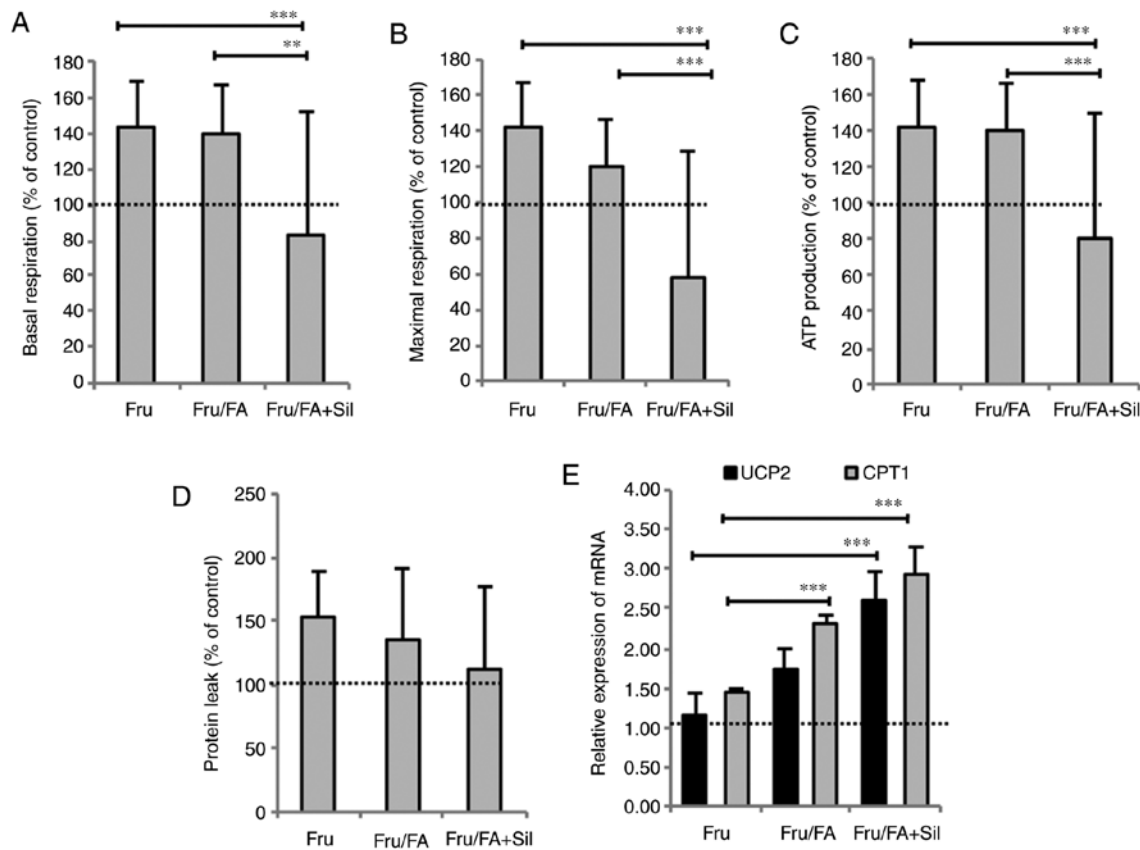


Figure 4. Silybin ameliorates mitochondrial dysfunction. Cells incubated with Fru/FA were then treated with silybin, and the following respiratory parameters were evaluated using the Seahorse XFe96 Extracellular Flux Analyzer: (A) Cell basal respiration, (B) cell maximal respiration, (C) ATP production and (D) proton leak (all presented as the OCR pmol/min/SRB labeling). Data are expressed as the mean  $\pm$  standard deviation of 14 separate experiments ( $n=16$ ). (E) mRNA expression levels of UCP2 and CPT1 were evaluated by reverse transcription-quantitative polymerase chain reaction. Values are expressed as the mean  $\pm$  standard deviation from at least three independent experiments ( $n=16$  for Seahorse experiments). \*\* $P \leq 0.01$  and \*\*\* $P \leq 0.001$ . Fru, fructose; FA, fatty acid; OCR, oxygen consumption rate; SRB, sulforhodamine B; CPT1, carnitine palmitoyltransferase 1; UCP2, uncoupling protein 2.

following observations: i) Enhanced steatosis; ii) maximal upregulation of ADRP and IKBIP expression levels; and iii) enhanced lipid peroxidation, ROS production and caspase 3-like activity, which are indexes of oxidative stress and apoptosis. The extensive damaging effect of Fru/FA combination was also evident when looking at the expression of miR-122, which is reportedly involved in the onset/progression of NASH (10).

Mitochondria are the main site for FA degradation, and steatotic hepatocytes typically enhance mitochondrial  $\beta$ -oxidation to limit excess fat accumulation (8). Accordingly, the mitochondrial proteins CPT1 and UCP2 were found to be overexpressed in cells treated with Fru/FA combination in the present study. Furthermore, basal and maximal mitochondrial respiration, as well as ATP production, were stimulated by Fru/FA combination in an attempt to compensate for the increased FA oxidation. Of note, the increase in oxidative stress due to the overactive  $\beta$ -oxidation may trigger proinflammatory pathways sustaining NAFLD progression. The results of the present *in vitro* study are in agreement with previous findings described in patients and animals (29,30). While a 'high-fat' diet results in obesity, insulin resistance, and hepatic steatosis with minimal inflammation and no fibrosis, the 'Western' diet that is rich in fructose leads to steatosis associated with hepatic fibrosis, inflammation, oxidative stress and apoptosis (31).

The nutraceutical silybin is known as a general hepatoprotective, anti-steatotic agent (21,25), which has provided promising results in animal and cellular models of NAFLD (25,30), and in a number of clinical studies (32-34). Providing further insight into the effect of this agent, the present study demonstrated that silybin counteracted the metabolic dysfunctions caused by Fru/FA combination acting directly on hepatocytes. First, silybin reduced the large TG accumulation resulting from Fru/FA combination by down-regulating the expression of PPAR $\gamma$ , the main transcription factor for lipogenic genes. The hepatoprotective action of silybin was able to counteract *in vitro* the Fru/FA-dependent increase in terms of the following: i) IKBIP expression; ii) intracellular ROS production and lipid peroxidation; and iii) apoptosis rate. However, silybin was unable to alleviate the reduction in cell viability associated with exposure to Fru/FA combination. The action of silybin appears to be mainly dependent on its effects on mitochondria, with different mechanisms depending on the NAFLD grade (25). In our model mimicking a rather severe NAFLD, silybin exerts beneficial activity by inhibiting mitochondrial respiration, which is stimulated in steatosis progression as a consequence of an increased oxidative metabolism due to stimulation of anabolic pathways (35).

In conclusion, the cell model used in the present study, consisting of lipid-loaded hepatocytes mimicking the

progression of NAFLD *in vitro*, offered new insights into the harmful steatogenic effects of fructose on liver cells and supports the hepatoprotective activity of silybin. Further studies can translate these results into long-term beneficial effects in the hope that the onset, progression and deterioration of NAFLD/NASH will be prevented or delayed in patients by using nutraceutical approaches.

### Acknowledgements

The authors would like to thank Dr Moahamed Kalil, Dr Rita Fabbri and Dr Silvia Carestiatto (DISTAV, Genova, Italy) for their experimental assistance.

### Funding

The present study was supported by grants from the Compagnia di San Paolo (Torino, Italy) and the University of Genova, and funds from the Foundation for Science and Technology, Portugal (grant nos. PTDC/DTP-FTO/2433/2014, POCI-01-0145-FEDER-007440 and POCI-01-0145-FEDER-016659).

### Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

### Authors' contributions

All authors significantly contributed to this study. LV conceived and designed the study, analyzed and elaborated the data, and wrote the manuscript. EG carried out the qPCR analysis for miR-122 and spectrophotometric assay for FAS activity determination, and participated in writing the manuscript. FB performed cultures and treatments of FaO cells, fluorimetric and spectrophotometric assays, and qPCR measurements. GV carried out apoptosis determination and O<sub>2</sub> consumption evaluation. PJO supplied the Seahorse XFe96 Extracellular Flux Analyzer and supervised the experiments for mitochondria analyses. VAS participated in O<sub>2</sub> consumption analyses. AV participated in cell cultures and treatments, and critically revised the manuscript. PP participated in conceiving and designing the study, and critically revised the manuscript.

### Ethics approval and consent to participate

Not applicable.

### Patient consent for publication

Not applicable.

### Competing interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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