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Research Article

Casein or soy protein supplementation protects against hepatotoxicity in hypercholesterolemic rats treated with rosuvastatin

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Abstract: Rosuvastatin (Rvs) is used for hypercholesterolemia therapeutic but it induces adverse effects including hepatotoxicity. This study aimed to evaluate the hepatoprotective role of casein and soy protein in hypercholesterolemic rats received Rvs. Seven groups of male Wistar rats were treated for 8 weeks including; control group, high-cholesterol diet (HCD) group, the groups treated orally with casein or soy protein (300 mg/kg bw) suspended in distilled water, the group received HCD for 4 weeks and treated orally with Rvs (20 mg/kg bw) for another 4 weeks, and the groups received HCD for 4 weeks and treated orally with casein or soy protein for another 4 weeks. Blood and tissue samples were collected for different assays. The results indicated that Rvs improved lipid profile in hypercholesterolemic rats, but it disturbed the liver and kidney indices, oxidative stress markers, the hepatic mRNA expression of *SREBP-1c*, *SREBP-2*, *FAS*, and *ACC-1* and the histopathological picture in hypercholesterolemic rats. Casein and soy protein improved the all the tested parameters, the histological picture, and mRNA expression of the tested genes, and soy protein was more effective than casein. Casein and soy protein supplementation can protect against Rvs-induced hepatotoxicity under hypercholesterolemic conditions.

Keywords: rosuvastatin; hypercholesterolemia; casein; soy protein; hepatoprotective

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1 Introduction

The liver damage induced by dietary supplements, herbal products or drug is an unfavorable response that is mostly unpredictable and is linked with several frequently used medications that cause potential chronic and acute liver damage[1]. The likelihood of an individual drug causing liver injury is relatively low (i.e., only 1 per 10,000 to 100,000 treated patients); however, the incidence may reach 100 per 100,000 for some drugs (e.g. isoniazid and chlorpromazine)[2]. Drug-induced liver injury (DILI) has a global estimated annual incidence of 1.3–19.1 per 100,000 people exposed[2]. The prevalence and cause of DILI vary geographically[3], and it accounts for about 10% of all cases of acute hepatitis[4]. It is the cause of acute jaundice in 50% of patients with new jaundice and accounts for up to half of the cases of acute liver failure in Western countries[5]. DILI is accountable for liver failure worldwide e.g. 3% in Spain[6], 5.9% in Denmark[7], 4.2% in New Zealand[8], 1.4% in India[9], 11.4% in France[10], and 11% in the USA[11]. The use of warnings and the removal of drugs from the markets are critical reasons for adverse hepatic reactions[12].

Statins are common drugs widely used for the therapeutic of hypercholesterolemia and the prevention of cardiovascular diseases[13]. However, it induces various roles such as cerebrovascular disease[14], decreasing the risk of heart failure[13], depression[15], chronic subdural hematoma[16], pancreatic cancer[17], rheumatoid arthritis[18], and contrast-induced acute kidney injury[19]. Rosuvastatin (Rvs) is used to lower blood cholesterol (Chol) levels and is considered safe at therapeutic doses ranging from 10 mg/day to 80 mg/day[20]; however, it can pose a considerable risk symptoms to different organs that are not adequately recognized or reported[21]. These adverse effects include arthralgia, diarrhea, dyspepsia, nausea, insomnia, nasopharyngitis, extremities, and infection of the urinary tract[22]. According to LiverTox[23], Rvs therapy is associated with mild, asymptomatic, and usually transient serum aminotransferase elevations in 1%–3% of patients. Moreover, Rvs may cause myalgia[21], hearing loss[24], hepatotoxicity, diabetes mellitus, neurological and neurocognitive effects[25] and elevation in liver enzyme[26], although the incidence of paramount liver injury is very low[27]. The United States Food and Drug Administration (USFDA) recommended continuous monitoring of liver function enzymes during statin therapy[28]. In the United States, 300,000–3,000,000 of those treated with statins have been denied due to unwarranted concern[29] and also because of the cost of the semiannual liver monitoring which

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was estimated to be $3 billion a year[30].

Soy foods are associated with several health benefits, particularly the reduction of Cardiovascular disease (CVD) risk through the low-density lipoprotein cholesterol (LDL-C) lowering effect and the hypocholesterolemic properties[31]. The bioactive peptides in soy protein primarily exert their effects through mechanisms involving the LDL-C receptor (LDLR) and bile acid regulation[32, 33]. It was reported that 25 g of soy protein reduced the risk of coronary heart disease (CHD) in Canada[34], and the United States[35]. Moreover, other components in soybeans have been shown to exert several health benefits, such as the reduction of the risk of cardiovascular disease. The phytochemicals constituted in soybeans such as isoflavones, lecithins, phytosterols, polysaccharides, soluble fibers, and saponins may act collectively or through different independent mechanisms to exert unparalleled health benefits[36, 37]. It is well documented that saponins and lecithins in soy play a role in the metabolism of lipids; linoleic acid and phytosterols induce hypocholesterolemic activities[36], in addition to the soy fibers that promote weight loss[36]. Moreover, the LDL-C lowering effect of soy protein offers protection against oxidative stress[38] and renal dysfunction[39] through the improvement of endothelial function markers[40].

Milk-derived bioactive peptides can be regarded as potential functional food ingredients[41, 42]. Casein is the major protein component in milk rich in hydrophobic or hydrophilic amino acid residues (amyloid proteins)[43, 44]. β-Lactogenesis and lactostatin derived from milk lactoglobulin were identified as hypocholesterolemic peptides[45, 46]. Several mechanisms for hypolipidemic peptides have been proposed for cholesterol-lowering activity including binding to bile acid[47], inhibition of Chol absorption *in vivo* and micellar solubility of Chol *in vitro*[48], inhibiting the activity of 3-hydroxy-3-methylglutaryl CoA reductase[49], and targeting the expression of proteins involved in the metabolism of cholesterol[50]. Moreover, casein is poor in aromatic amino acids and is rich in branched amino acids which makes it very proper to treat liver disorders[51]. The current study aimed to evaluate the hepatotoxicity of Rvs and the possible protective role of casein and/or soybeans supplementation, and to assess whether these proteins can affect the action of Rvs on Chol homeostasis.

2 Materials and methods

2.1 Materials, chemicals, and kits

Chol powder (≥99%; CAS# 57-88-5) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Rvs was purchased from Chemipharm Pharmaceutical Industries (Cairo, Egypt). Casein soluble light white was purchased from BDH Chemicals Ltd. (Poole, England). Soy protein was provided by the Agriculture Research Centre, Soybean Unit, (Giza, Egypt). Kits for Transaminases (alanine transaminase (ALT) and aspartate aminotransferase (AST)), creatinine, urea, Chol, triglycerides (TriG), low- and high-density lipoprotein (LDL and HDL), and total protein (TP) were purchased from Randox Co., (Antrim, UK). A kit for nitric oxide (NO) was purchased from Eagle Diagnostics (Dallas, TX, USA). Malondialdehyde (MDA) was purchased from Elabscience (TX, USA), catalase (CAT) was purchased from BioTrend (Köln-Germany), and glutathione peroxidase (GPx) was purchased from BioVision (MA, USA). Kit for first-strand cDNA synthesis was purchased from iNtRON Biotechnology (Seoul, Korea). SYBRR Premix Ex *Taq*™ kit was purchased from TaKaRa Biotech. Co., Ltd. (Shiga, Japan). TRIzol reagent and RNAse-free DNAse kit were purchased from Canvas (Valladolid, Spain).

2.2 Experimental animals

Male albino Wistar rats (3 months old, (150 ± 15) g) were supplied by the National Organization of Drug Control and Research (NODCR). Animals were maintained on a basal diet: a balanced basal diet consists of 30% (*m*/*m*, the same below) sucrose, 16% whey protein, 35% starch, 10% groundnut oil, 5% fiber, 3.5% salt mixture (AIN 76), 1% vitamin mixture (AIN 76), and 0.2% choline chloride. The standard diet was ground to a fine powder and Chol was added at a ratio of 3% to prepare the high-cholesterol diet (HCD). All animals were housed in filter-top polycarbonate cages in a room free from any source of chemical contamination, artificially illuminated (12 h dark/light cycle), and thermally controlled ((25 ± 1) °C) and humidity ((50 ± 5)%) at the Animal House Lab, NODCR, and Giza, Egypt. All animal experiments comply with the ARRIVE guidelines and were carried out in accordance with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978), and the protocol was permitted and approved by the General Division Committee for Biological Control and Research. National Organization for Drug Control and Research (Approval No. 234/6/11/2019)

2.3 Experimental design

All animals were left for 1 week as an acclimatization period before starting the experiment. Then the rats were divided into 7 groups (8 rats per group) and treated daily for 8 weeks and including group (1) the untreated control animals received 0.5 mL distilled water, group (2) animals that fed HCD (3%) for 8 weeks, group (3) animals that fed the basal diet and treated by gavage with casein (300 mg/kg bw) for 8 weeks suspended in 0.5 mL distilled water, group (4) animals that fed basal diet and treated by gavage with soy protein (300 mg/kg bw) for 8 weeks suspended in 0.5 mL distilled water, group (5) animals that fed HCD for the first 4 weeks then treated by gavage with a daily dose of Rvs (20 mg/kg bw) dissolved in 0.5 mL distilled water along with HCD diet for another 4 weeks, group (6) animals that fed HCD for the first 4 weeks then treated by gavage with casein and Rvs for another 4 weeks along with HCD diet, and group (7) animals that fed HCD for the first 4 weeks then treated by gavage with soy protein and Rvs for another 4 weeks along with HCD diet. At the termination of the experimental period, all animals fasted for 12 h, and then blood samples were withdrawn from the retro-orbital venous plexus under isoflurane anesthesia. Sera were separated by centrifugation (1,700 r/min and 4 °C) and kept at −20 °C until analysis. The sera were used for the assay of liver and kidney indices, and lipid profile, according to the kit's instructions using a spectrophotometer. After the collection of blood samples, all animals were euthanized and samples were collected. Liver and kidney samples were weighed, homogenized in a phosphate buffer (pH 7.4), and centrifuged (1,700 r/min and 4 °C for 10 min). The supernatant was used for NO, MDA, CAT, and GPx assays[52]. Other liver samples from each animal were fixed in formal saline (10%), dehydrated by a graded series of alcohol, cleaned in xylene, embedded in paraffin wax, and sliced at 5 μm thickness. The sections were stained with hematoxylin and eosin stains for histopathological investigation using a light microscope[53]. However, other samples of the liver were kept in liquid nitrogen

−80 °C for genetic analysis.

2.4 Gene expression analysis

TRIzol® reagent was used to isolate the total genomic RNA from frozen liver tissues (−80 °C) of all treated animals following the manufacturer's protocol. The concentration and purity of RNA were determined by NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific, USA). Reverse transcription-cDNA synthesis was performed with the cDNA using a PreMix cDNA Kit (iNtRON Biotechnology, Korea). SYBR® Premix Ex *Taq*™ kit was utilized to assay the quantitative reverse transcription polymerase chain reaction (qRT-PCR) analyses as previously described[54]. The gene-specific primer sequences for *GAPDH*, sterol-regulatory-element-binding protein-1c (*SREBP-1c*), sterol regulatory element-binding protein-2 (*SREBP-2*), fatty acid synthesis (*FAS*), and acetyl-CoA carboxylase 1 (*ACC-1*) are presented in Table 1.

RT-qPCR was carried out on Stratagene Mx3005P Real-Time PCR System (Agilent Technologies) in a 20-μL reaction volume using, 1 μL cDNA, 10 μmol/L of forward and reverse primers, 10 μL TOPreal™ qPCR 2× PreMIX (SYBR Green with low ROX) (Enzynomics) and DNAse-free water. All samples were amplified in a minimum of triplicates. Amplification was performed as follows: 2 min at 50 °C and then 10 min at 95 °C, followed by two-step PCR for 40 cycles at 95 °C for 15 s followed by 60 °C for 1 min. The expression level was calculated from the PCR cycle number (Ct) where the increased fluorescence curve passes across a threshold value. The relative expression of target genes was obtained using the comparative Ct ($2^{-\Delta\Delta Ct}$) method. The ΔCt was calculated by subtracting *GAPDH* Ct from that of the target gene whereas ΔΔCt was obtained by subtracting the ΔCt of the calibrator from that of the test sample. Relative expression levels of transcription factor of *SREBP-1c*, *SREBP-2*, *FAS* and *ACC-1* genes in the liver were assessed and normalized to *GAPDH* and *β-actin*. RT-qPCR data were obtained as a threshold cycle (Ct) value and were used to calculate ΔCt values for each sample[55].

The $2^{-\Delta\Delta Ct}$ method was applied for the determination of the quantitative values of the specific genes to (*GAPDH*) and *β-actin*. The relative quantification of the target gene to the reference was calculated using the following $2^{-\Delta\Delta Ct}$ equations:

$$\Delta Ct_{(test)} = Ct_{(target,test)} - Ct_{(reference,test)} \quad (1)$$

$$\Delta Ct_{(calibrator)} = Ct_{(target,calibrator)} - Ct_{(reference,calibrator)} \quad (2)$$

$$\Delta\Delta Ct = \Delta Ct_{(test)} - \Delta Ct_{(calibrator)} \quad (3)$$

2.5 Statistical analyses

Statistical analyses were carried out using SPSS 16. Data were expressed as mean ± SE. Variables were compared using one-way ANOVA; post hoc Duncan's test and the significance of differences among means were determined at $P \leq 0.05$.

3 Results

The effect of different treatments on serum lipid (Table 2) revealed that animals that received Chol alone showed a significant increase in serum Chol, TriG, and LDL-Chol (1.4, 1.3, and 1.9 folds compared to the control group) and a significant decrease in HDL-Chol (1.5-fold compared to the control). Chol, TriG, and LDL-Chol did not significantly change in the groups that received soy protein or casein; however, animals that were treated with Rvs plus Chol showed a significant improvement in all serum lipid profiles compared to the group that received Chol alone. This improvement reached 13.7%, 8.4%, 22.7%, and 21.1% for Chol, TriG, LDL-Chol, and HDL-Chol, respectively. The combined treatment with soy protein or casein plus Rvs and Chol induced a significant improvement in the lipid profile toward the control levels and soy protein was more effective although it did not normalize any of these lipid parameters except LDL-Chol which was lower than the control level. The improvement in Chol,

Table 1 Details giving primer sequences for the genes amplified

cDNA	Sequence from (5' to 3')	Accession number	RT-PCR product size (bp)
SREBP-1c	F: GGAGCCATGGATTGCACATT R: AGGAAGGCTTCCAGAGAGGA	NM_001276708.1	191
SREBP 2	F: GTGCAGACAGTCGCTACACC R: AATCTGAGGCTGAACCAGGA	NM_001033694.2	60
FAS	F: AAGATCCCGGAAAGCAAGAT R: TGATACCAGCACTGGAGCAG	NM_139194.3	92
ACC1	F: CCCAACAGAATAAAGCTACTCTGG R: TCCTTTTGTGCAACTAGGAACGT	>NM_022193.1	70
GAPDH	F: AAGGTCATCCATGACAACTTTG R: GTCCACCACCCTGTTGCTGTAG	NM_017008.4	496
β-actin	R: CCACCATGTACCCAGGCATT F: CGGACTCATCGTACTCCTGC	NM_031144	189

Table 2 Effect of casein and soy protein on lipid profile parameters (mg/dL) in Rvs-treated hypercholesterolemic rats

Groups	Chol	TriG	LDL-Chol	HDL-Chol
Control	120.85 ± 1.13^{a}	284.12 ± 2.52^{a}	42.57 ± 0.91^{a}	46.41 ± 1.43^{a}
Chol	167.02 ± 2.94^{b}	358.85 ± 4.16^{b}	79.76 ± 5.03^{b}	29.98 ± 0.27^{b}
Casein	115.97 ± 5.54^{ac}	281.19 ± 2.05^{a}	40.68 ± 0.33^{a}	49.69 ± 0.32^{c}
Soy	109.34 ± 2.53^{c}	$280.78 \pm .4.19^{a}$	41.07 ± 0.64^{a}	47.54 ± 1.47^{a}
Chol + Rvs	144.02 ± 4.17^{d}	328.82 ± 1.67^{c}	61.69 ± 0.93^{c}	37.98 ± 0.93^{d}
Chol + Rvs + Casein	139.17 ± 1.33^{e}	313.08 ± 4.46^{e}	58.19 ± 1.14^{cd}	39.42 ± 1.52^{de}
Chol + Rvs + Soy	129.88 ± 3.22^{f}	298.06 ± 1.42^{f}	47.75 ± 0.59^{e}	40.50 ± 0.44^{e}

Note: Within each column, means superscripts with different letters are significantly different ($P < 0.05$).

TriG, LDL-Chol, and HDL-Chol in these groups reached 16.7%, 12.8%, 27.0%, and 23.9%, respectively for the group that received Chol plus Rvs and Casein and it reached 22.0%, 16.9%, 40.4% and 25.9%, respectively for the group received Chol plus Rvs and soy protein.

The results showed that Chol administration elevated the serum levels of AST, ALT, urea, creatinine, and a significantly decreased serum TP (Table 3). Insignificant changes were observed in these parameters in the groups that received soy or casein. Animals that received Rvs plus Chol showed a significant increase in ALT, AST, urea, and creatinine (1.45, 1.12, 1.42, and 2.68 folds, respectively) accompanied by a significant decrease in TP (1.37-fold). The elevation of ALT, AST, urea, and creatinine in the group that received Chol plus Rvs reached 67.5%, 3.0%, 22.9%, and 40.3%, respectively; however, the decrease in TP in this group reached 9.0%. Co-administration of Rvs and Chol plus soy protein or casein resulted in a significant improvement in these biochemical parameters and soy protein was more effective compared to casein. The improvement in ALT, AST, TP, urea, and creatinine in the group the was received Chol and Rvs plus casein reached 20.2%, 20.4%, 37.5%, 21.7%, and 19.2%, respectively; however, it reached 48.8%, 22.9%, 36.9%, 42.2% and 39.6%, respectively in the group that was received Chol and Rvs plus soy protein.

The data presented in Table 4 showed the effect of Rvs on oxidative stress markers in the liver and kidney during hypercholesterolemia and the role of soy protein and casein. Animals that received Chol showed a significant increase in NO levels in both the liver (1.19-fold) and kidney (1.13-fold). Casein or soy protein alone induced a significant decrease in NO compared to the control animals. However, animals treated with Chol plus Rvs showed a greater elevation in NO in the hepatic and renal tissues reaching 11.49% in the liver and 15.86% in the kidney. Chol plus Rvs and casein induced a significant decrease in NO in the liver (1.45%) and the kidney (23.72%); however, Chol plus Rvs and soy protein showed a marked decrease in NO in the liver (22.29%) and kidney (29.19%) compared to hypercholesterolemic animals that received the Rvs alone, although none of these treatments could normalize NO in both tissues. The data also revealed that MDA level was significantly high in the liver (1.14-fold) and kidney (1.73-fold) of the animals that were treated with Chol alone compared to the control group. Casein alone induced a significant decrease in MDA level in the liver and did not affect its level in the kidney; however, soy protein alone induced a significant decrease in the liver and kidney compared to the control animals. Interestingly, hypercholesterolemic animals that received Rvs showed a significant increase in MDA levels in the liver (16.92%) and kidney (3.52%) compared to the hypercholesterolemic group. Administration of casein plus Rvs to the hypercholesterolemic animals induced a significant decrease in MDA levels in both liver (48.21%) and kidney (41.37%). Whereas, administration of soy protein plus Rvs to the hypercholesterolemic group showed a more reduction in MDA level reaching 55.7% in the liver and 42.96% in the kidney.

The antioxidant enzymes (GPx and CAT) in different treatment groups (Table 5) showed a significant decrease in both tissues in the hypercholesterolemic animals reaching 41.49% in the liver and 49.80% in the kidney. Casein or soy protein alone induced a significant increase in these markers in both organs except the hepatic CAT which was at the normal level of the control. Hypercholesterolemic animals that received Rvs showed a significant decrease in GPx and CAT in the hepatic (46.85%) and renal (66.28%) tissues compared to the control group. However, co-administration of Chol plus Rvs and casein induced a significant improvement in the hepatic and renal GPx reaching 21.42% and 27.55%, and hepatic and renal CAT reached 14.67% and 38.13%, respectively compared to the group that received Chol plus Rvs alone. On the other hand, the group that received Chol plus Rvs and soy protein showed an improvement in both enzymes reached 34.47% and 23.45% for GPx and reached 33.34% and 34.66% for CAT in both tissues, respectively compared to the group that received Chol plus Rvs alone.

The results of the relative concentration of the transcription

Table 3 Effect of soy protein or casein on serum biochemical parameters in Rvs-treated hypercholesterolemic rats

Groups	ALT (U/L)	AST (U/L)	TP (g/dL)	Urea (mg/dL)	Creatinine (mg/dL)
Control	17.67 ± 0.33^{a}	56.33 ± 0.88^{a}	6.48 ± 0.21^{a}	26.53 ± 0.75^{a}	0.98 ± 0.15^{a}
Chol	25.67 ± 1.20^{b}	63.33 ± 0.88^{b}	4.73 ± 0.23^{b}	37.77 ± 1.76^{b}	2.63 ± 0.18^{b}
Casein	18.00 ± 0.58^{a}	57.67 ± 0.88^{a}	6.27 ± 0.35^{a}	28.30 ± 0.84^{a}	0.84 ± 0.03^{c}
Soy	18.33 ± 1.20^{a}	55.33 ± 1.45^{a}	6.85 ± 0.29^{c}	28.47 ± 1.39^{a}	0.92 ± 0.03^{ad}
Chol + Rvs	43.00 ± 1.15^{c}	65.33 ± 1.20^{b}	4.30 ± 0.06^{bd}	46.43 ± 0.84^{c}	3.69 ± 0.10^{e}
Chol + Rvs + Casein	34.33 ± 0.88^{d}	52.00 ± 0.58^{c}	6.88 ± 0.21^{c}	36.37 ± 0.80^{b}	2.98 ± 0.08^{bf}
Chol + Rvs + Soy	22.00 ± 1.15^{e}	50.33 ± 1.20^{cd}	6.79 ± 0.29^{ce}	26.90 ± 1.49^{a}	2.23 ± 0.04^{g}

Note: Within each column, means superscripts with different letters are significantly different ($P < 0.05$).

Table 4 Effect casein and soy protein on hepatic and renal No and MDA in Rvs-treated hypercholesterolemic rats

Groups	NO (μmol/g tissue)		MDA (nmol/g tissue)	
	Liver	Kidney	Liver	Kidney
Control	117.68 ± 2.30^{a}	116.43 ± 0.64^{a}	132.69 ± 1.48^{a}	70.85 ± 1.93^{a}
Chol	139.70 ± 0.91^{b}	131.30 ± 1.11^{b}	151.28 ± 9.34^{b}	122.40 ± 5.92^{b}
Casein	103.07 ± 5.61^{c}	109.46 ± 0.69^{c}	126.58 ± 9.93^{c}	71.05 ± 1.62^{a}
Soy	111.63 ± 1.86^{d}	105.65 ± 0.90^{d}	96.28 ± 0.98^{d}	65.47 ± 2.15^{c}
Chol + Rvs	155.75 ± 1.80^{e}	152.12 ± 4.04^{e}	176.88 ± 6.69^{e}	126.71 ± 1.58^{d}
Chol + Rvs + Casein	136.36 ± 1.20^{f}	116.04 ± 0.82^{a}	91.61 ± 1.38^{f}	74.28 ± 1.93^{e}
Chol + Rvs + Soy	121.04 ± 0.85^{g}	107.71 ± 0.90^{cd}	80.10 ± 1.13^{g}	72.27 ± 4.46^{a}

Note: Within each column for each organ, means superscripts with different letters are significantly different ($P < 0.05$).

Table 5 Effect of casein and soy protein on hepatic and renal antioxidant enzymes in Rvs-treated hypercholesterolemic rats

Groups	GPX (U/mg tissue)		CAT (U/g tissue)	
	Liver	Kidney	Liver	Kidney
Control	322.54 ± 3.49^{a}	232.09 ± 3.84^{a}	91.50 ±2.80^{a}	86.12 ± 0.25^{a}
Chol	188.73 ± 5.70^{b}	116.75 ± 2.63^{b}	73.15 ± 0.59^{b}	62.37 ± 0.65^{b}
Casein	351.16 ± 4.25^{c}	241.01 ± 0.86^{c}	91.30 ± 4.45^{a}	114.15 ± 0.64^{c}
Soy	342.61 ± 5.48^{d}	241.72 ± 1.72^{c}	91.90 ± 1.22^{a}	122.13 ± 1.32^{d}
Chol + Rvs	171.42 ± 3.18^{e}	108.93 ± 1.11^{d}	69.33 ± 1.18^{bc}	53.28 ± 1.74^{e}
Chol + Rvs + Casein	218.15 ± 2.29^{f}	150.35 ± 1.03^{e}	81.25 ± 1.30^{d}	69.65 ± 1.03^{f}
Chol + Rvs + Soy	261.60 ± 1.07^{g}	160.99 ± 0.61^{f}	90.57 ± 1.51^{a}	81.54 ± 1.12^{g}

Note: Within each column for each organ, means superscripts with different letters are significantly different ($P < 0.05$).

factor of *SREBP-1c* in the liver (Fig. 1A) showed a significant increase (6-folds, higher) in mRNA content in the animals that received Chol alone compared to the control. Animals that received casein showed a moderate increase in mRNA expression of *SREBP-1c* (2-folds); however, soy protein did not affect the mRNA expression of this gene. Co-administration of Chol plus Rvs induced a significant decrease in mRNA expression of *SREBP-1c* reaching 33.3% compared to Chol alone group. Administration of casein or soy protein plus Rvs in the hypercholesterolemic animals induced a significant decrease in mRNA expression of *SREBP-1c*. This decrease reached 50% in the case of casein and 70% in the case of soy protein compared to the Chol alone-treated group. Thus, soy protein was more effective than casein but none of these treatments normalized the mRNA expression of this gene. The mRNA expression of *SREBP-2* showed an insignificant increase in Chol-treated animals (Fig. 1B). Casein alone decreased the expression of *SREBP-2* mRNA by 20% compared to the control; however, soy protein did not affect the mRNA expression of this gene. On the other hand, Rvs, Rvs plus casein, or Rvs plus soy protein induced a significant decrease in the mRNA expression of *SREBP-2* but casein was more effective than the other treatments since the decrease reached 10%, 30% and 20% in hypercholesterolemic animals that received Rvs, Rvs plus casein and Rvs plus soy protein, respectively. The mRNA expression of the *FAS* gene (Fig. 1C) or the *ACC-1* gene (Fig. 1D) showed the same pattern as *SREBP-1c*. The Chol group showed a 1.8-fold increase in the *FAS* mRNA expression, however, casein or soy protein alone showed a 0.8- and 0.5-fold decrease compared to the control. The increase in *FAS* mRNA expression in the hypercholesterolemic animals that received Rvs was increased by 1.5-fold and the co-administration of casein or soy protein plus Rvs to the hypercholesterolemic animals induced a reduction in *FAS* mRNA expression by 1.4- and 1.1-fold, respectively. Additionally, Chol alone induced a 1.91-fold increase in *ACC-1* mRNA compared to the control group. Casein and soy protein induced a reduction in *ACC-1* mRNA expression which reached 0.8- and 0.6-fold compared to the control group. Hypercholesterolemic animals that received Rvs showed a high expression in *ACC-1* mRNA reaching 2.2-fold compared to the control animals. Co-administration of Rvs plus casein or soy protein to the hypercholesterolemic animals resulted in a decrease in *ACC-1* mRNA expression reached 4.5% and 13.6% of the hypercholesterolemic group that received Rvs alone.

The histopathological examination of the liver sections of the control animals showed normal histology of hepatic cells with a preserved nucleus, cytoplasm, and hepatocytes which were arrayed in well-formed nucleus cords around the central vein (Fig. 2A). The liver sections of rats treated with Chol showed fibrous tissues around the portal tract, and hepatocytes have marked fatty, hydropic degeneration and necrosis (Fig. 2B). The liver sections of

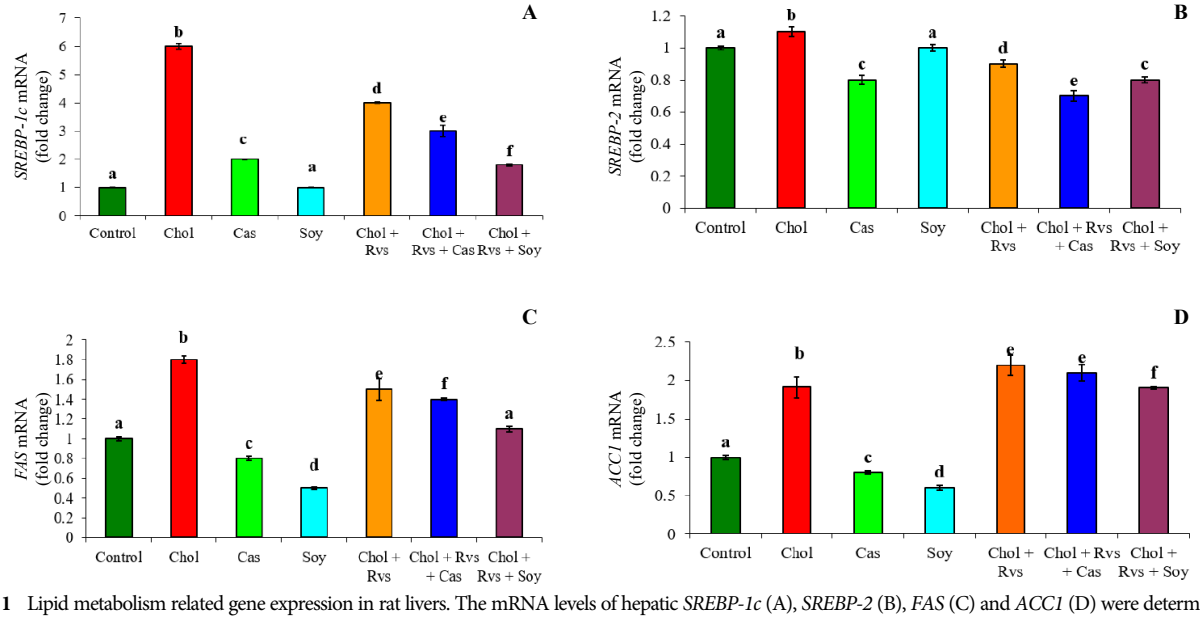

Figure 1 Lipid metabolism related gene expression in rat livers. The mRNA levels of hepatic *SREBP-1c* (A), *SREBP-2* (B), *FAS* (C) and *ACC1* (D) were determined by quantitative real time PCR analysis. Results were normalized to *GAPDH* and *β-actin* expression and then expressed as fold changes relative to gene expression. Data are expressed as mean ± SE. Means with different letters are significantly different at $P \leq 0.05$.

rats that received casein showed the hepatocytes around the central vein are nearly normal with minimal focal necrosis with fibrosis around the portal tract (Fig. 2C). However, the liver sections of the rats that received soy protein showed nearly normal hepatic architecture around blood vessels (Fig. 2D). The liver sections of the animals treated with Chol plus Rvs (Fig. 2E), Chol plus Rvs and casein (Fig. 2F), or Chol plus Rvs and soy protein (Fig. 2G) showed marked improvement in hepatic cells and blood vessels.

The microscopic examination of the kidney sections of control rats (Fig. 3A), rats treated with Chol (Fig. 3B), rats treated with casein (Fig. 3C), and rats treated with soy protein (Fig. 3D) showed no histological changes in kidney tubules and glomeruli. The kidney sections of rats treated with Chol plus Rvs (Fig. 3E), Chol plus Rvs and casein (Fig. 3F), or Chol plus Rvs and soy protein (Fig. 3G) showed few renal histological changes where glomerular mesangial cells are expanded and renal tubules are dilated.

4 Discussion

Rvs is among the statins used globally in patients suffering from hypercholesterolemia worldwide[25]. Despite the effective role of Rvs in lowering serum Chol, several reports showed that it induced liver injury and elevation of transaminases[56]. In the current study, the selective dose of Chol was selected based on the previous work[57]. However, the selected dose of Rvs was calculated as 1/100 of lethal dose (LD_{50}) as described previously[58]. On the other hand, the doses selected for casein and soy were based on the work of Cohn *et al.*[59] The results revealed that animals fed HCD (3%) for 8 weeks disturbed lipid profile parameters. These results indicated the induction of hypercholesterolemia in these animals[60]. The marked elevation in serum Chol and TriG is probably due to the enhancement of Chol biosynthesis during the high intake of Chol[61], and/or the increase of exogenous Chol uptake[62] in addition to the collapse of the oxidative defense systems and the increase in inflammation under hypercholesterolemic conditions[63].

The liver function enzymes (ALT and AST) were significantly high in hypercholesterolemic animals. The activity of ALT and AST are well-known parameters for the assessment of a healthy liver[64, 65]. ALT is considered the most specific hepatic damage maker which is found mainly in the hepatic tissue[66, 67]. Increased levels of serum ALT are associated with several risk factors for different diseases such as diabetes, metabolic syndrome, cardiovascular diseases, increased blood pressure, hyperglycemia, obesity, and dyslipidemia, and are correlated with non-alcoholic fatty liver disease (NAFLD)[68]. A similar increase in the hepatic enzymes was reported in the previous reports in hypercholesterolemic rats[69]. These studies showed that the extremist lipid accumulation in hepatocytes as a result of the imbalance between the formation and the degradation of lipids induced a decrease in enzyme leakage from the injured hepatic cells[66]. Hence, we can assume that the high level of lipid fractions Chol, TriG, and LDL-Chol and the decrease in HDL-Chol are correlated to NAFLD and dyslipidemia as major factors in the development of fatty liver disease. Thus, the elevated liver markers are indicative of liver injury due to the excess fat deposition in the hepatic tissue. Additionally, the accumulation of such lipids in the hepatic tissue induces the inflammation of the hepatic tissue and enhances the generation of free radicals[70] leading to cell death or fibrosis and the leakage of liver enzymes from the damaged

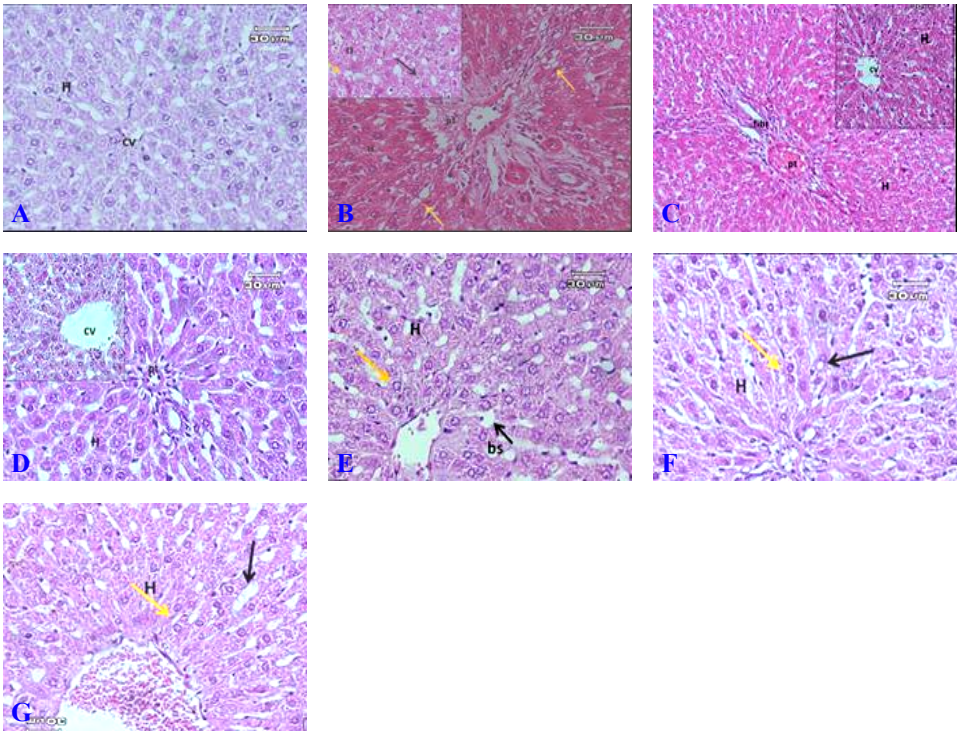

Figure 2 Photomicrographs of the liver sections of (A) control rats showing normal histology of hepatic cells (H) with preserved nucleus, cytoplasm and hepatocytes which were arrayed in well-formed nucleus cords around the central vein (CV), (B) rats treated with Chol showing the fibrous tissues around the portal tract, hepatocytes have marked fatty (black arrow), hydropic degeneration (yellow-arrow) and necrosis (n), (C) rats treated with casein showing the hepatocytes around the central vein are nearly normal (inset) and focal necrosis with fibrosis around portal tract (pt), (D) rats treated with soy protein showing nearly normal hepatic architecture around blood vessels, rats treated with Chol plus Rvs (E), Chol plus Rvs and casein (F) and Chol plus Rvs and soy protein (G) showing marked improvement in hepatic cells (H) and blood vessels (bs). Scale bar: ×400.

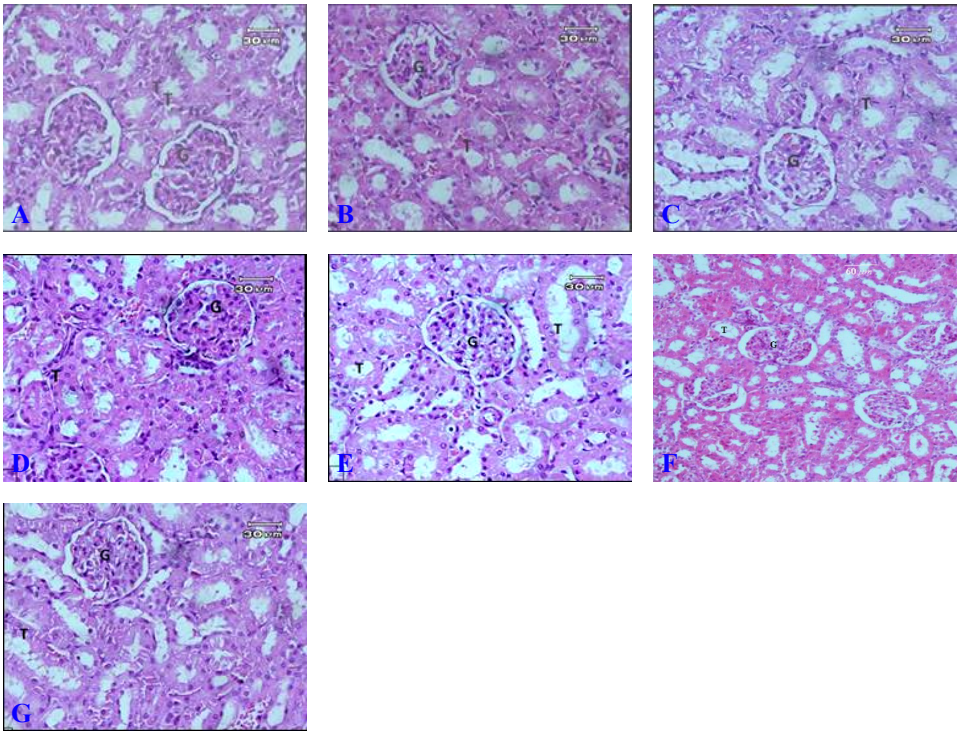

Figure 3 Photomicrographs of sections in the rat kidney from (A) control group, (B) Chol-treated group, (C) casein-treated group and (D) soy protein-treated group showing no histological changes in kidney tubules and glomeruli. The kidney sections of (E) Chol plus Rvs-treated group, (F) Chol plus Rvs and casein-treated group and (G) Chol plus Rvs and soy protein-treated group showing few renal histological changes where glomerular mesengial cells are expanded and renal tubules are dilated. Scale bar: ×400.

cells[71]. The elevation in serum creatinine and urea and the reduction in TP levels in hypercholesterolemic rats indicated the impairment of kidney function in this group. Previous reports suggested that a HCD induces impairment in the function of the kidney through the defect in permeability and integrity of the cell membranes[72]. Moreover, hypercholesterolemia induced the thickening of the glomerular basal membranes, mononuclear cell aggregation, and dilated glomerular capillaries[73].

Our results showed that HCD feeding elevates the oxidative markers (NO and MDA) and decreases the antioxidant enzyme activity (GPx and CAT) in the hepatic and renal tissue. It was reported that hypercholesterolemia is a metabolic disorder associated with the increase in free radical generation and the decrease in enzymatic and non-enzymatic antioxidants resulting in a case of oxidative stress[74,75]. The mechanism of hypercholesterolemia-induced oxidative stress was suggested to be through tissue damage[76]. Similar observations demonstrated that hyperlipidemia/obesity is an independent risk factor for the decline in cytoprotective defenses, increased lipid peroxidation, and cell death[77].

The results of gene expression analysis revealed that the transcription factors of *SREBP-1c*, *SREBP-2*, *FAS*, and *ACC-1* in the liver were increased in the hepatic tissue of rats fed HCD. *SREBP-1c* is the master transcriptional regulator of TriG and fatty acid synthesis and it is expressed at a high level upon feeding due to the stimulation of insulin[78,79]. The high expression of *SREBP-1c* in the liver induced the mRNA-encoding enzymes responsible for the synthesis of TriG and fatty acids such as *ACC-1* and *FAS*[80]. Thus, the current results are concordant with the previous reports which indicated that HCD increased the expression of these genes[81,82].

The histological examination of the liver and kidney tissue revealed that animals fed HCD showed severe changes in the hepatic tissue including fibrosis around the portal tract; hepatocytes have marked fatty, hydropic degeneration and necrosis. However, the kidney did not show any severe histological changes. Similar histological changes in the liver were reported in the literature[83,84]. However, the kidney sections of rats fed HCD appear normal which indicates that this treatment affects only the function of the kidney but not the tissue. Although some reports[85,86] indicated that Chol administration induced significant histological changes in the kidneys of rats, this dissimilarity may be due to the duration of Chol administration.

Although Rvs could improve the lipid profile, it induced a significant elevation in liver and kidney function. The mechanism by which Rvs-induced liver injury is still unknown. It was reported that less than 10% of Rvs is metabolized in the liver by cytochrome P450 family 2 subfamily C member 9 (CYP 2C9). The increase in liver enzymes in the groups that received Rvs may be due to the toxicity of an intermediate metabolite of the drug in addition to immune mechanisms[22]. Although Rvs is a poor substrate for CYP P450 isoenzymes, there is evidence that the drug is expected to interact with a few drug metabolites resulting from CYP 450-mediated metabolism[87]. In addition, the more efficient and more selective uptake of Rvs by the hepatocytes compared to other statin families may be another reasonable reason for the hepatotoxicity of Rvs[88]. Despite the feeding on HCD induced a significant elevation in the liver and kidney indices, co-administration of Rvs induced more elevation suggesting that this drug may act synergistically with Chol effects.

Administration of casein or soy protein did not affect the tested parameters in the control group, and they decreased the elevation of ALT and AST in Rvs-treated hypercholesterolemic rats indicating the hepatoprotective action of these agents. This protection was more noticeable in the soy protein-fed group. As a food source, casein supplies amino acids including histidine,

lysine, methionine, threonine, phenylalanine, tryptophan, isoleucine, leucine, valine, alanine, arginine, valine aspartic acid, cysteine/cysteine, proline, serine, glutamic acid, glycine, and tyrosine[89], carbohydrates including hexose, hexosamine, and sialic acid[90], and two inorganic elements, calcium and phosphorus in concentration reached 1,072 and 885 mg/kg, respectively[91]. Bioactive peptides from milk proteins and the two inorganic elements, calcium, and phosphorus have potency health benefits[92]. The small molecule peptides consisting of 2–20 amino acid residues are the most biologically active peptides in casein[93]; however, the other amino acids in casein can't be ignored as key factors in its bioactivity[94]. Additionally, casein was found to be effective in counteracting free radicals such as superoxide, hydroxyl, and oxygen radicals and Fe^{3+} reducing power[95], enhancing the activity of antioxidant enzymes in rats fed high-fat-diet[96], and thus protecting cells from oxidative damage[97]

On the other hand, soy protein is rich in saponins, and phytic acid isoflavones which exhibit strong antioxidant activity and show hypolipidemic effects[98]. The amino acid content plays a vital role in the bioactivity of soy protein[99]. It has a potent effect in reducing oxidative stress and prevents liver injury due to its high content of cysteine, glycine, and arginine[100]. Moreover, the high content of cysteine and arginine gives soy protein a higher advantage as an antiatherogenic and antioxidant than casein[101]. Previous studies indicated that soy protein reduced serum Chol, TriG, and LDL-Chol[102], and maintained the normal level of serum insulin concentrations compared with casein which in turn is accompanied by the low hepatic *SREBP-1c* mRNA expression and consequently decreased the expression of other lipogenic enzymes including FAS[103]. In this concern, we should notice that the protective role and the modulation of *SREBP-1c* mRNA of casein and soy protein are depending on the type of protein[103]. In addition to its content of amino acids, the isoflavones derivatives such as daidzein, genistein, glycerin, and their glycosides, glycoside acetates, and glycoside malonates, soy protein showed potent health benefits[104].

The current results indicated that the soy protein and casein act synergistically with Rvs in lowering Chol in hypercholesterolemic animals via different mechanisms. Rvs reduce Chol by blocking the actions of hydroxyméthylglutaryl-CoA (HMG-CoA) reductase, which is an early and rate-limiting step in the Chol synthesis process. Blocking the action of this enzyme leads to the inhibition of the conversion of HMG-CoA to mevalonic acid and a decrease in Chol production in the hepatocytes[105]. However, soy protein was more effective than casein in the lowering of cholesterol. Several mechanisms have been proposed to explain this phenomenon, including the metabolic effects brought about by differences in amino acid composition, changes in fecal steroid losses, changes in VLDL metabolism, changes in hepatic lipoprotein receptors, and endocrine changes[106]. Despite the mechanism of action by which casein exerts its hypocholesterolemic effect remains unclear, the level of plasma lipid is the net result of a dynamic process with lipoprotein production and removal. Hence, changes in the plasma lipid level must be due to changes in its production and/or removal rate[107]. Therefore, both soy protein and casein could reduce the elevation of lipid profile in the animals fed HCD, and protect the liver and kidney from the hazards of high Chol and Rvs. In general, the specific mechanism of hepatoprotection mediated by soy protein or casein may be attributed to increased glutathione synthesis as a result of their high sulfur-containing amino acid content. Higher levels of hepatic glutathione have been shown to protect against several types of liver injury, owing to glutathione's role as a key antioxidant and xenobiotic conjugation substrate[108]. However, soy protein was more effective than casein. These observations agree with the previous findings that soy protein can offer hepatoprotection in hypercholesterolemic rats[109,110] more than casein.

5 Conclusion

In the current results revealed that hypercholesterolemic rats administrated Rvs showed a marked increase in the liver and kidney function indices, and oxidative stress markers accompanied by a marked decrease in the antioxidant enzyme activity in both organs. Rvs also induced severe histological changes in the liver but not in the kidney and increased the mRNA expression of *SREBP-1c*, *SREBP-2*, *FAS*, and *ACC-1* in the liver. Casein or soy protein alone did not show any significant changes in the tested parameters or the histological picture of the liver and kidney. Supplementation with casein or soy protein to the HCD significantly improved all the tested parameters and the histological picture of the liver of hypercholesterolemic rats treated with Rvs suggesting the potential protective role of these agents. However, soy protein was more effective in the protection against Rvs suggesting that the type of protein is an important factor. This is considered a preliminary study to assess the hepatoprotective effects of soy protein and casein in hypercholesteremic rats given Rvs. However, more researches are needed to elucidate the pathways that may disclose the molecular mechanisms underpinning the advantages casein or soy protein in liver protection.

Conflicts of interest

The authors declare that there are no conflicts of interest in this work. Mosaad A. Abdel-Wahhab is an associate editor of Food & Medicine Homology, but was not involved in the processing, peer review or decision making of this article.

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