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ORIGINAL ARTICLE

Atheroprotective and hepatoprotective effects of trans-chalcone through modification of *eNOS/AMPK/KLF-2* pathway and regulation of *COX-2*, *Ang-II*, and *PDGF* mRNA expression in NMRI mice fed HCD

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Abstract

Background The effects of *trans*-chalcone on atherosclerosis and NAFLD have been investigated. However, the underlying molecular mechanisms of these effects are not completely understood. This study aimed to deduce the impacts of *trans*-chalcone on the *eNOS/AMPK/KLF-2* pathway in the heart tissues and the expression of *Ang-II*, *PDFG*, and *COX-2* genes in liver sections of NMRI mice fed HCD.

Methods and results Thirty-two male mice were divided into four groups (n = 8): control group; fed normal diet. HCD group; fed HCD (consisted of 2% cholesterol) (12 weeks). TCh groups; received HCD (12 weeks) besides co-treated with *trans*-chalcone (20 mg/kg and 40 mg/kg b.w. dosages respectively) for 4 weeks. Finally, the blood samples were collected to evaluate the biochemical parameters. Histopathological observations of aorta and liver sections were performed by H&E staining. The real-time PCR method was used for assessing the expression of the aforementioned genes. Histopathological examination demonstrated atheroma plaque formation and fatty liver in mice fed HCD which were accomplished with alteration in biochemical factors and Real-time PCR outcomes. Administration of *trans*-chalcone significantly modulated the serum of biochemical parameters. These effects were accompanied by significant increasing the expression of *eNOS*, *AMPK*, *KLF*-2 genes in heart sections and significant decrease in *COX-2*, *Ang-II*, and *PDGF* mRNA expression in liver sections.

Conclusion Our findings propose that the atheroprotective and hepatoprotective effects of *trans*-chalcone may be attributed to the activation of the *eNOS/AMPK/KLF-2* pathway and down-regulation of *Ang-II*, *PDFG*, and *COX-2* genes, respectively.

Keywords *Trans*-chalcone · Atherosclerosis · NAFLD · *eNOS/AMPK/KLF-2* pathway · *COX-2* · *Ang-II* · *PDGF*

Introduction

Hypercholesterolemia is a notorious risk factor occurring in a plethora of metabolic disorders, including fatty liver, obesity, type-2 diabetes (T2D), and cardiovascular diseases (CVD) [1]. Atherosclerosis, the main contributing factor in cardiovascular disease, with a high worldwide mortality rate specified by continuous lipid disposition, smooth muscle cell proliferation, the divergence of monocytes to the endothelium, and production of macrophage-derived foam. It is consequently ensued by endothelial cell dysfunction associated with oxidative stress [2].

Reduced endothelial nitric oxide synthetase (*eNOS*)-derived nitric oxide (NO), and thereby the impairment of endothelium-dependent vascular dysfunction, is an essential indicator of arterial pathogenesis, which is a crucial characteristic of endothelial dysfunction [3]. *eNOS*

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dysfunction pathophysiology has been of great significance in many cardiovascular diseases; atherosclerosis is a notable example [4]. It has been shown that *AMP*-activated protein kinase (*AMPK*) phosphorylates *eNOS* at the Ser-1177/1179 site; hence, the bioavailability of *eNOS*-derived *NO* is enhanced. *AMPK* is also paramount in maintaining endothelial cell function and NO activity [5]. *AMPK*, a serine/threonine kinase, acts as an integral regulator of metabolic energy homeostasis [6]. In addition, the atheroprotective effects of *AMPK* have been demonstrated by modulating macrophage polarization, monocyte differentiation, and the decline of macrophages in plaques [7].

Another integral factor in atheroma plaque formation is further inhibited by Kruppel-like factor 2 (*KLF-2*) [8]. *KLF-2*, a transcription factor is in the family of *KLF* zinc-finger transcription factors, acts as vital regulators of cell development and differentiation in atherogenesis [9]. Following previous reports, *KLF-2* mediates the anti-inflammatory property of statin 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase inhibitors. Drugs in this classification not only decrease cholesterol but also provide many benefits to the endothelium directly [10]. Through the process of atherosclerosis, *KLF-2* expression is induced, which engenders the down-regulation of genes controlling inflammatory responses; for instance, vascular cell adhesion molecule (VCAM) and E-selectins in the endothelium and monocytes [11].

Non-alcoholic fatty liver disease (NAFLD) is known as one of the utmost pervasive diseases worldwide, prevalent to metabolic syndrome disorders, categorized by hepatic steatosis in the absenteeism of alcohol usage [12]. NAFLD is defined by the imbalance between synthesis, oxidation, transportation, and absorption of fatty acids [13]. Hepatic steatosis likely contributes to the pathogenesis of atherosclerosis. Comprehensive examinations have depicted that NAFLD is linked to increased intima-media density, which is a primary marker of atherosclerosis [14, 15]. As the understanding of the mechanism and treatment options for NAFLD remains insubstantial, thorough research has been conducted on NAFLD to elucidate the mechanism and developing treatment.

A variety of genes have been identified to be susceptive to progress from steatosis to nonalcoholic steatohepatitis (*NASH*). Cyclooxygenase-2 (*COX-2*) is a significant enzyme that incites arachidonic acid metabolism and acts as an indispensable mediator in cancer and inflammation [16]. The levels of *COX-2* in hepatic cells are eminently low. Nonetheless, in patients with NASH, the *COX-2* level is elevated due to heightened cytokine expression, including tumor necrosis factor-α (*TNF-α*) and interleukin-6 (IL-6). Both oxidative stress and lipid peroxidase also increase, additionally [17]. On the contrary, NAFLD progression inhibition was seen in diet-induced obesity rats when treated with a selective *COX-2* suppressor [18].

Studies have suggested that oxidative stress and inflammation induced by obesity and hypercholesterolemia play fundamental roles in *NAFLD* pathogenesis [19]. *Angiotensin-II* (*Ang-II*), a primary constituent of the renin-angiotensin system, is a potential mediator of hypertension as its plasma level is heightened with liver cirrhosis. *Ang-II* elevation is related to inflammatory factors and reactive oxygen species (ROS) [20]. This was demonstrated by inhibiting *Ang-II,* which has proven to debilitate the accumulation of triglyceride (TG) in the liver of Zucker obese rats [21]. Based on these data, *Ang-II* is of great significance in the appearance and subsequent progression of NAFLD.

Small-scale studies have taken place on the effects of platelet-driver growth factor (*PDGF*) on fatty liver development. *PDGF* is a biologically active peptide with a manifold of functions. The prior substance is essentially expressed in platelet alpha-granules under physiological conditions [22]. In the case of liver damage, *PDGF* is likely to be expressed in macrophages in great quantities due to activated endothelial cells and hepatic stellate cells (HSC) [23].

Chalcones function as the precursor for flavonoids and are phenolic flavonoids compounds with anti-cancer, antioxidant, anti-inflammatory, and atheroprotective properties [24]. The core structure of chalcones is *trans*-chalcone; proven to have anti-diabetic and hypoglycemic properties in mice with T2D [25]. A report on healthy rats has indicated that *trans*-chalcone, via modifying microRNA-451 (*miR-451*) reduces interleukin-8 (*IL-8*) in liver cells [26]. Moreover, by modulating *miR-34a* and *miR-451* regulation in the livers of rats suffering from NAFLD, the positive effects of *trans*-chalcone were demonstrated on liver inflammation and insulin resistance [27]. Also, it has been shown the cardioprotective effect of *trans*-chalcone by up-regulation of collagen type I, which was modified through inhibiting transforming growth factor-beta 1 (*TGF-β1*)-connective tissue growth factor (*CTGF*) pathway in HFD-fed rats [28]. We previously indicated that *trans*-chalcone has potential impacts on liver fibrosis and atherosclerosis through increasing adiponectin gene expression in the adipose tissues and modulation of plasma antioxidant enzymes in mice species that were fed high cholesterol diet (HCD) [20]. To develop a deep understanding of whether *trans*-chalcone ameliorates atherosclerosis and reduce lipid accumulation and inflammation in the liver, this study initially aims to assess the impact of *trans*-chalcone supplementation on the expression of *AMPK*, *KLF-2,* and *eNOS* genes in the heart tissues and *COX-2*, *Ang-II,* and *PDGF* genes in the liver sections of NMRI mice feeding HCD.

Material and methods

Animals

Thirty-two male mice weighing in the vicinity of (18–22 g) were utilized in this study obtained via the Razi Vaccine and Serum Institute, Karaj, Iran. The animals were maintained under standard laboratory conditions with air-conditioned quarters and at a controlled temperature of (24 ± 1 °C) with a 12 h light–dark cycle and 55 ± 5% relative humidity with easy access to a standard pellet and water. In conformance with the Guide for the Care and Use of Laboratory Animals, the welfare of the animals was maintained, and experimental procedures were carried out accurately; In addition, the animal protocol was authorized by the Science and Research Branch of the Islamic Azad University, Animal Ethics Committee (approval code: 176947). This study was carried out in the laboratory complex of the Science and Research Branch, Islamic Azad University, Tehran, Iran.

Experimental design

Ensuring a week of accommodation, the animals were distributed into four groups (n = 8) at random, as follows:

- Control group: fed a regular diet (pellet) with sunflower oil vehicle orally (gavage); 0.5 ml once a day for 12 consecutive weeks.
- HCD group: fed a high-cholesterol diet (comprised of 1% cholesterol combined with regular diet) and received 1% cholesterol (3.5 g/kg b.w.) dissolved in 0.5 ml sunflower oil vehicle orally (gavage) once daily for 12 consecutive weeks [20].
- TCh-20 group (*trans*-chalcone-20): fed with HCD and received 1% cholesterol (3.5 g/kg b.w.) dissolved in 0.5 ml sunflower oil orally once daily for 12 weeks. After 8 weeks of feeding HCD, the animals were treated with *trans*-chalcone (20 mg/kg b.w.) once a day for 4 weeks via gavage simultaneously [29].
- TCh-40 group (*tans*-chalcone-40): fed with HCD and received 1% cholesterol (3.5 g/kg b.w.) dissolved in 0.5 ml sunflower oil orally once daily for 12 weeks. After 8 weeks of feeding HCD, the animals were treated with *trans*-chalcone (40 mg/kg b.w.) once a day for 4 weeks via gavage simultaneously.

Each dose of *trans*-chalcone (20 my/kg and 40 my/kg) was resolved in 0.5 cc sunflower oil by using a sonicator homogenizer operation (Basic T-10, IKA, Germany). Then animals were fed *trans*-chalcone through gavage. The utilized *trans*-chalcone and cholesterol were obtained from Sigma–Aldrich, St. Louis, MO, USA. All other chemicals were provided of analytical grade and good quality.

Biochemical measurements

After a 14-h fasting period at the culmination of the experimental period, ketamine (90 mg/kg b.w.) and xylazine (10 mg/kg b.w.) were utilized intraperitoneally to anesthetize the animals; additionally, terminal blood samples were obtained from the cardiac ventricles. Ensuing centrifugation at 2500 rpm at 37 °C for 10 min, the blood samples were stored at room temperature for 1 h. Then the collected serum was measured to establish the levels of low-density lipoprotein-cholesterol (LDL-C), high-density lipoprotein-cholesterol (HDL-C), triglycerides (TG), total cholesterol (TCh), alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), amylase and glucose by using the enzymatic method from commercially available kits (Pars Azmoon, Tehran, Iran).

Histopathological examination

To evaluate plaque formation in the aorta arteries, the proximal aorta sections were segregated. The rest of the heart tissues obtained were reserved at a temperature of -70° C for future molecular analysis. In addition, the liver tissues of all treated animals were divided into two equal sections to determine the molecular examination (stored at – 70 °C) and histological changes. For further observation of tissue alterations, the separated aorta and liver sections were fixated in 10% buffered formalin solution for a 24-h period, enclosed in paraffin, and cross-sectioned into 5 µm thick cells stained with Haematoxylin and Eosin (H&E) complying with routine protocols. The samples were analyzed using light microscopy (Olympus SZX10 microscope, Tokyo, Japan).

Real-time quantitative polymerase chain reaction (PCR)

In order to evaluate the expression levels of *AMPK*, *KLF-2*, and *eNOS* genes, total *RNA* was extracted from heart tissues. For assessing the expression levels of *COX-2*, *Ang-II*, and *PDGF*, liver sections were used to isolate the total RNA. Total RNA extraction of all samples was carried out using RNA isolation kits (Fermentase, Cinagen Co Iran) as per the manufacturer's guidelines. Moreover, complementary DNA (cDNA) synthesis was executed by the producer, as previously described [20]. Real-time PCR reactions were used to quantify the expression of mentioned genes in addition to glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), which is a housekeeping gene, by amplifying *SYBR* Green PCR Master Mix (Fermentase, Germany) with *ABI*-step 1

system. By using the $2^{-\Delta\Delta Ct}$ method, the relative quantitative expression of the genes mentioned prior was calculated. The primer sequences of all genes and *GAPDH* were taken from the National Center for Biotechnology Information (NCBI) website, as listed in Table 1.

Statistical analysis

All data was defined as mean ± SEM. SPSS 22.0 software (SPSS Inc., IL, USA.) was used for data analysis. Group means were set side by side via one-way analysis of variance (ANOVA) followed by Tukey's Post hoc test; According to what was mentioned, $P < 0.05$ was considered statistically significant.

Results

Trans-chalcone improved lipid profiles, liver indexes as well as amylase, and glucose levels in mice on fed HCD

According to the biochemical results, Table 2, the serum levels of cholesterol, TG, and LDL-C in mice fed high cholesterol diet for 12 weeks had significantly risen ($P < 0.001$) in comparison to the control group, while the HDL-C level was markedly decreased ($P < 0.001$). Administration of *trans*-chalcone mitigated these changed parameters. As indicated in Table 2, compared with the HCD group, serum cholesterol was significantly decreased in TCh groups ($P < 0.001$). In addition, serum TG has demonstrated a significant reduction ($P < 0.001$) in the TCh-40 group compared to HCD induced animals. Regarding LDL-C, no statistically significant alteration was recorded between TCh and HCD groups. On the other hand, *trans*-chalcone supplementation significantly increased the serum level of HDL-C in both

Table 1 The primers sequences of genes which were used in real-time PCR analysis

GENES	F 5 > 3	R 5 > 3	Length
eNOS	CGCGGCTGGTACATGAGTTC	CCGGGTGTCTAGATCCATGC	103 bp
AMPK	TAGTGAGAGCCGAGGTCCA	CTTGCGGTGGTCATTCGGT	150 bp
KLF-2	TCAGCGAGCCTATCTTGCC	GTTTAGGTCCTCATCCGTGCT	116 bp
COX-2	TATCAGGTCATCGGTGGAGAG	ACTCTGTTGTGCTCCCGAAGG	176 bp
Ang-II	CTGGCTGTGGCTGACTTACTT	CACTTTGCACATCACAGGTC	102 bp
PDGF	GGCTCGAAGTCAGATCCACA	ATGGGCTCTCAGGTTTGTCT	100 bp
GAPDH	GAAGCTGGTCATCAACGGGA	GAAGGGGCGGAGATGATGAC	180 bp

eNOS: endothelial nitric oxide synthetase, AMPK: AMP-activated protein kinase, KLF-2: Kruppel-like factor 2, COX-2: Cyclooxygenase-2, Ang-II: Angiotensin-II, PDFG: platelet-driver growth factor

Table 2 Effect of trans-chalcone on biochemical parameters in mice fed with HCD

Groups	Control	HFD	TCh-20 (20 mg/kg)	TCh-40 (40gm/kg)
Parameters				
TCh (mg/dl)	203.66 ± 8.09	289.50 ± 8.01^{+++}	212.66 ± 8.88***	189 ± 8.46***
TG (mg/dl)	140.33 ± 8.63	238.50 ± 10.32^{+++}	206.66 ± 9.75	117.83 ± 7.40***
LDL-C (mg/dl)	26.44 ± 1.40	41 ± 1.52^{+++}	39.83 ± 1.97	37.83 ± 1.35
HDL-C (mg/dl)	45.33 ± 1.97	34.16 ± 1.88^{+++}	41.16 ± 1.07*	45.66 ± 1.66***
ALT (U/L)	82 ± 5.07	120.33 ± 2.92^{+++}	112.16 ± 4.36	79.50 ± 4.03***
AST (U/L)	467 ± 13.09	666.66 ± 17.12^{+++}	608.16 ± 8.25*	539.16 ± 11.0***
ALP (U/L)	227.66 ± 10.18	256.66 ± 9.67	224.16 ± 6.72	190.33 ± 10.38***
Glucose (mg/dl)	69.33 ± 2.21	125.4 ± 4.66^{+++}	104 ± 3.56**	82.16 ± 3.66***
Amylase (U/L)	2482 ± 76.16	3491.16 ± 98.55^{+++}	3354.16 ± 54.67	2971.18 ± 51.35***

TCh Total cholesterol, *TG* Triglyceride, *LDL-C* Low-density lipoprotein cholesterol, *HDL-C* High-density lipoprotein-cholesterol, *AST* Aspartate aminotransferase, *ALT* alanine aminotransferase, *ALP* alkaline phosphatase, *TCh-20* trans-chalcone with 20 mg/kg dose, *TCh-40* Trans-chalcone with 40 mg/kg dose

$^{+++}P < 0.001$ compared with control group (n = 8)

***P < 0.001, **P < 0.01 and *P < 0.05 compared with HCD group (n = 8)

the TCh-20 ($P < 0.05$) and the TCh-40 ($P < 0.001$) groups versus the HCD group.

AST, ALT, and ALP levels in serum are conventionally inferred as markers of liver damage. As illustrated in Table 2, the serum levels of both AST and ALT had ascended in the HCD group when compared with the control group ($P < 0.001$); However, only ALP increase levels were insignificant ($P > 0.05$). Co-treatment of HCD induced animals with *trans*-chalcone doses (20 mg/kg or 40 mg/kg) for 30 days significantly diminished the AST levels in $P < 0.05$ and $P < 0.001$. Furthermore, *trans*-chalcone attenuated the levels of ALT and ALP in a dose-dependent manner ($P < 0.001$).

A high-cholesterol diet also elevated serum amylase and glucose levels in comparison to the control group. However, the serum glucose levels had significantly declined when co-treatment with *trans*-chalcone in TCh-20 and TCh-40 groups ($P < 0.01$ and $P < 0.001$ respectively), only the serum amylase levels experienced a significant reduction in the TCh-40 group ($P < 0.001$) in comparison to the HCD one.

Trans-chalcone significantly ameliorated the histopathological alterations in aorta and liver tissues in mice with atheroma plaque and NAFLD

As shown in Fig. 1, histopathological evaluation of the aortic arteries exhibited a typical and average structure with intact intima in the control group (Fig. 1A). At the same time, a high-cholesterol diet led to the proliferation and migration of smooth muscle cells, the thickening of the intima, and the accumulation of cholesterol plaques in the HCD group (Fig. 1B). The evaluation of the HCD aortic section group with ×400 magnification illustrated the significant presence of numerous cholesterol plaques and macrophages (Fig. 1C) and hyperplasia of inner layers (Fig. 1D). These changes could ascertain the formation of atheroma plaque. The aortic arteries figures of the TCh-20 group showed limited cholesterol plaques with middle hyperplasia in the medial layer (Fig. 1E, F). The presence of fat displacement and thickened intima in the TCh-40 group was lowered by *trans*-chalcone supplementation (Fig. 1G). No typical alterations were identified in the development of atheroma plaque in both TCh-20 and especially in the TCh-40 groups.

Staining the liver sections by H&E (Fig. 2A) revealed a normal hepatocyte structure without fat deposition in the control group. On the contrary, histopathological observations of the HCD group indicated microvesicular and macrovesicular fatty droplets (Fig. 2B) with severe penetration of mononuclear inflammatory cells (Fig. 2C), which can confirm the appearance of NAFLD. Administration of *trans*-chalcone in both TCh groups modified the degree of fat accumulation around the central vein and weakened the infiltration of mononuclear cells as inflammation indicators (Fig. 2D, E).

Trans-chalcone up-regulated the mRNA level of KLF2, AMPK, and eNOS in heart tissues from mice under a high-cholesterol diet

To determine whether *trans*-chalcone affected the *KLF-2/AMPK/eNOS* pathway, we measured the expression of these genes in heart tissues by the real-time PCR method. Our results exhibited that the mRNA level of *KLF-2* and *AMPK* were highly down-regulated by fed a high-cholesterol diet in the HCD group ($P < 0.001$) when compared with the control group. HCD also experienced a substantial decline in *eNOS* gene expression in $P < 0.01$ versus the control group. However, supplemented *HCD* with *trans*-chalcone for 30 days could reverse these changes. As seen in Fig. 3, *trans*-chalcone supplementation significantly up-regulated *KLF-2* gene expression in both doses (20 mg/kg or 40 mg/kg) similarly ($P < 0.001$). Additionally, *AMPK* gene expression was also significantly up-regulated by *trans*-chalcone in TCh-20 ($P < 0.01$) and TCh-40 ($P < 0.001$) groups compared to the HCD group. Furthermore, *eNOS* gene expression significantly enhanced in the TCh-20 group ($P < 0.001$) more than the TCh-40 group ($P < 0.05$) in comparison to the HCD one (Fig. 3).

Trans-chalcone down-regulated the expression of PDGF, COX-2, and Ang-II genes in liver tissues from mice under a high-cholesterol diet.

To investigate how *trans*-chalcone impacts fatty liver functionality, the expression of *PDGF*, *Ang-II*, and *COX-2* genes was assessed in liver tissues of all animals (Fig. 4). The current research exemplified that HCD substantially reinforced the expression of the mentioned genes in the HCD group ($P < 0.001$). Interestingly, co-treatment with *trans*-chalcone considerably declined *PDGF, Ang-II,* and *COX-2* gene expression in the TCh-20 group in $P < 0.05$ compared to the HCD group. Moreover, administration of *trans*-chalcone with 40 mg/kg dosage significantly down-regulated the mRNA expression of *PDGF, Ang-II,* and *COX-2* in a dose-dependent manner ($P < 0.01$ and $P < 0.001$) when evaluated with that of the HCD group (Fig. 4).

Discussion

The beneficial effects of chalcone compounds on different liver diseases and atherosclerosis have been shown by various molecular mechanisms [20, 24, 28–30]. Initially, the objective of this study was to determine whether or not oral administration of *trans*-chalcone exhibits its effects on atheroma plaque and fatty liver by evaluating *AMPK*,

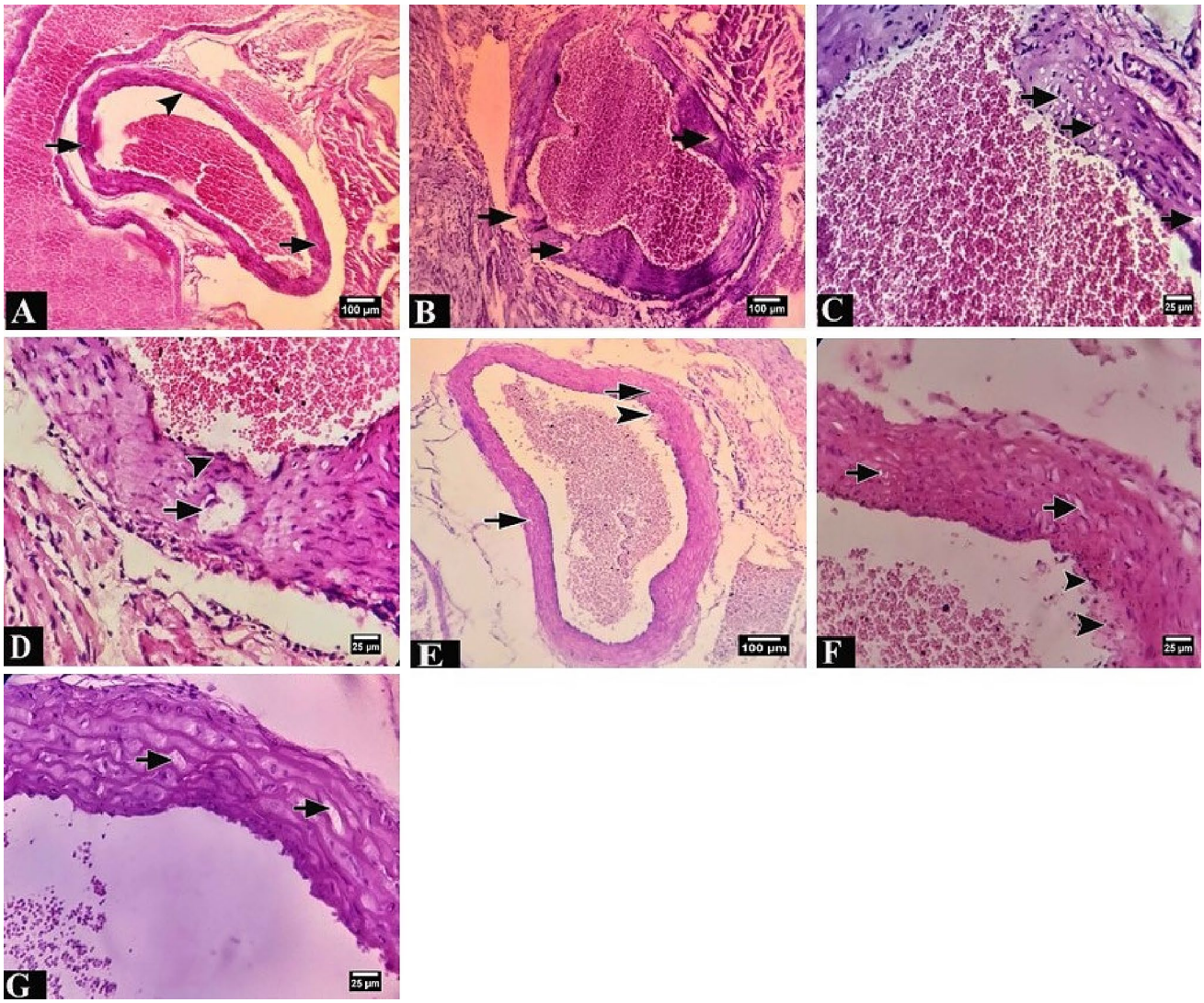

Fig. 1 The effect of trans-chalcone on aortic tissues of mice fed with HCD. The aortic sections were stained by H&E. **A** Control group (×100); the arrows and arrow icons showed the normal structure of the inner layer and endothelium of the aorta. **B** HCD group aortic section (×100); the arrow icons indicated thickened intima layer and hyperplasia. **C** and **D** HCD group aortic sections (×400); the existence of cholesterol plaques and hyperplasia in the inner and the outer layer of the aorta were recognized by arrows and arrow icons. **E** TCh-20 (trans-chalcone with 20 mg/kg dose) group (×100). **F** TCh-20 group (×400); the arrows and arrow icons indicated the moderate presence of fat deposition and hyperplasia in both pictures. **G** TCh-40 (trans-chalcone with 40 mg/kg dose) group (×400); the arrow icons exhibited the smooth vessels of the aorta without hyperplasia and atheroma plaque

KLF2, *eNOS* genes in heart samples as well as evaluating the expression of *COX-2*, *Ang-II,* and *PDGF* genes in liver sections of mice underfeeding HCD.

Hypercholesterolemia-induced dyslipidemia, a prominent risk factor in NAFLD and CVD identifies by abnormalities in lipid profile and obesity [31]. Some studies have documented that a diet rich in cholesterol results in the development of fatty liver in animals [31–33] in addition to atheroma plaque formation [1]. This study illustrated that mice fed HCD for 12 weeks had abnormalities in their plasma lipids. Prior investigations have implied that *trans*-chalcone modulates lipid profile alteration besides increasing fatty acid oxidation [20, 29]. It also decreases hepatic lipogenesis by down-regulation of sterol regulatory element-binding protein *(SREBP)-1c*, *SREBP-2*, hepatic fatty acid synthase (FAS) enzyme, peroxisome proliferator-activated receptor (*PPAR*)-*γ2* levels (30), and malondialdehyde (MDA) [20]. As depicted in Table 2, *trans*-chalcone ameliorated the serum level of lipids in TCh groups compared to the HCD group. These results verified the earlier explorations. In this regard, according to our histopathological observations, in comparison to the control group, the HCD group demonstrated infiltration of the inflammatory cells between hepatocytes resulting in hepatic steatosis. Additionally,

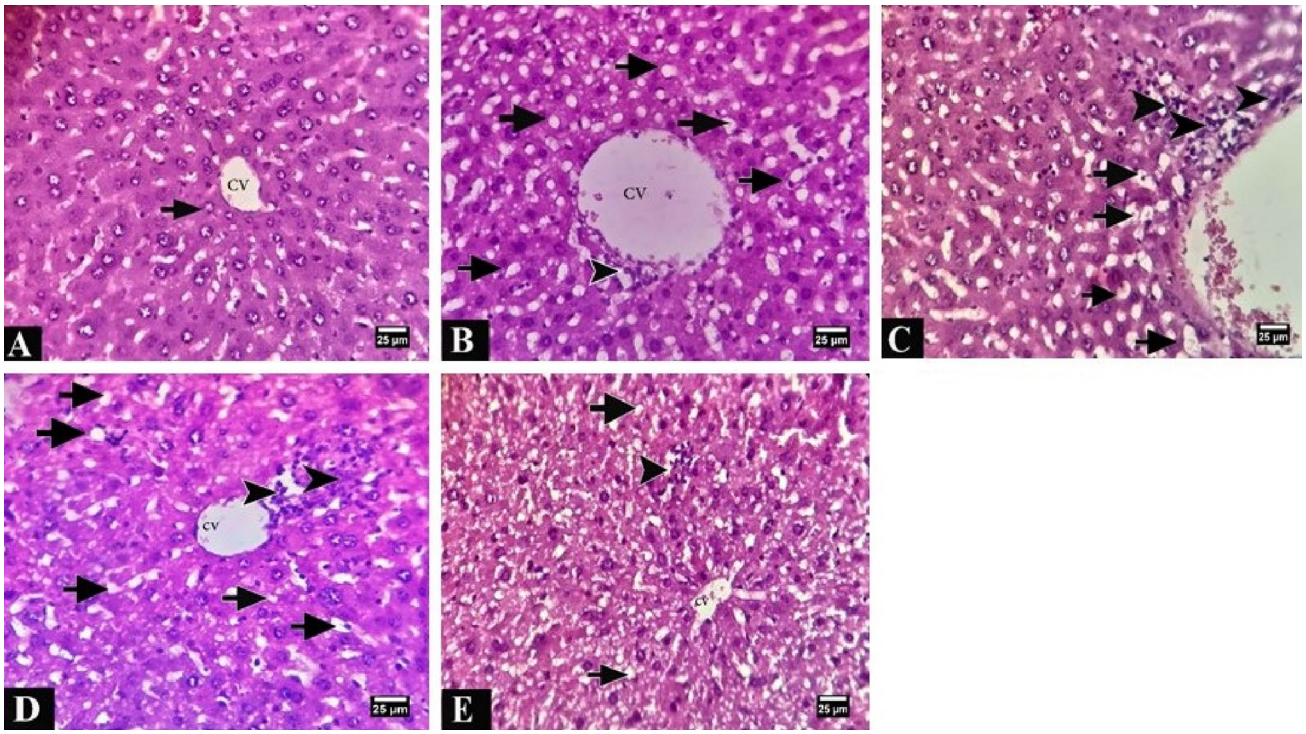

Fig. 2 The effects of trans-chalcone on liver tissues in mice fed HCD. The liver sections were stained by H&E (×100). **A** control group. **B** and **C** HCD group, the existence of micro-vesicular and macro-vesicular fatty droplets was specified by arrows and arrow icons between hepatocytes. In **C** the arrows illustrated the fat droplets, and the arrow icons indicated the infiltration of mononuclear inflammatory cells. **D** TCh-20 group, the arrow, and arrow icons indicated the fatty droplets, and the penetration of mononuclear cells around the cervical vein was decreased by trans-chalcone (20 mg/kg) in comparison to the HCD group. **E** TCh-40 group, the arrow and arrow icons showed that the fat deposition and mononuclear cells were significantly diminished by oral administration of trans-chalcone (40 mg/kg) compared with the HCD group

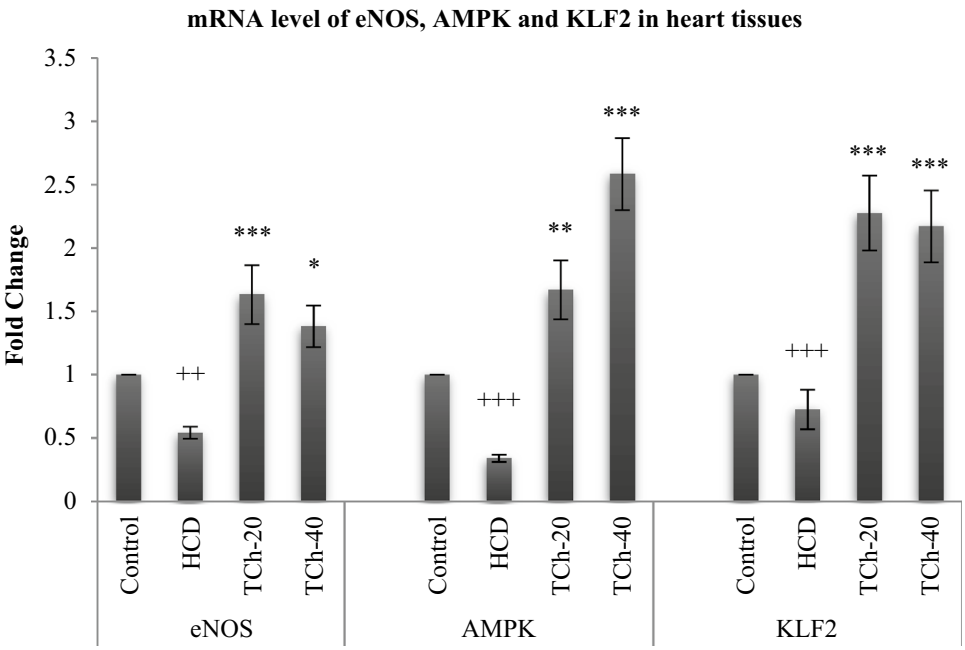

Fig. 3 Effect of trans-chalcone on mRNA level of *eNOS*, *AMPK,* and *KLF-2* in heart tissues of mice fed HCD. *eNOS* Endothelial nitric oxide synthase, *AMPK* adenosine monophosphate-activated protein kinase, *KLF-2* Kruppel-like factor-2, *HCD* high-cholesterol diet, *TCh-20* trans-chalcone with 20 mg/kg dose, *TCh-40* trans-chalcone with 40 mg/kg dose. Data were expressed as means ± SEM deviation. $^{+++}P < 0.001$ and $^{++}P < 0.01$ versus control group (n = 8). ***P < 0.001, **P < 0.01 and *P < 0.05 versus HCD group (n = 8)

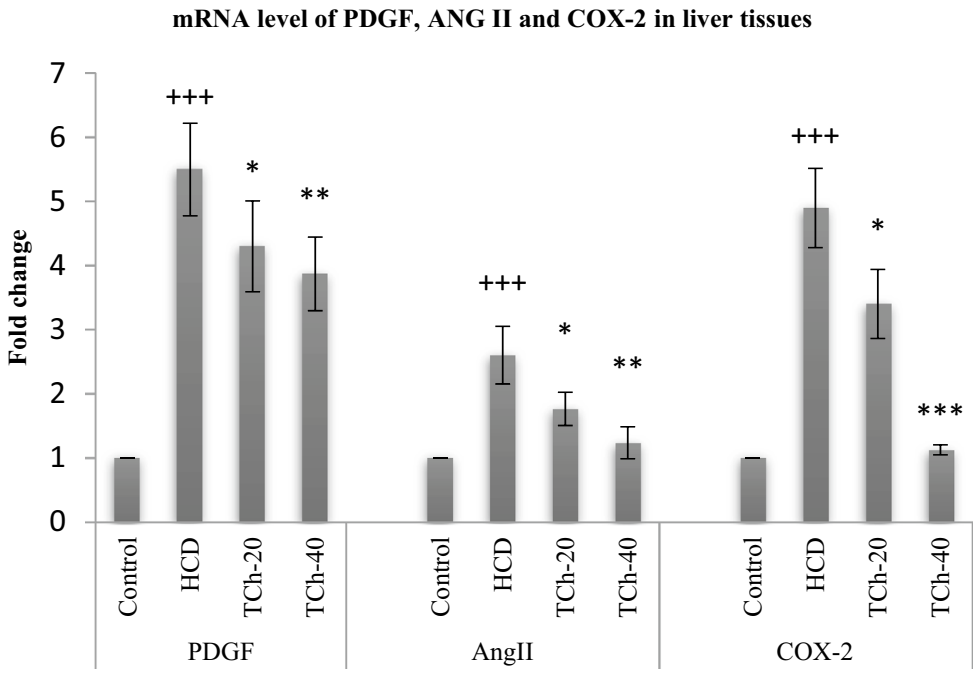

Fig. 4 Effect of trans-chalcone on mRNA level of *PDGF*, *Ang-II,* and *COX-2* in liver tissues of mice fed HCD. *PDGF*: Platelet-derived growth factor, *Ang-II* angiotensin-II, *COX-2* Cyclooxygenase-2, *HCD* high-cholesterol diet, *TCh-20* trans-chalcone with 20 mg/kg dose, *TCh-40* trans-chalcone with 40 mg/kg dose. Data were expressed as means ± SEM deviation. $^{+++}P < 0.001$ versus control group (n = 8). ***P < 0.001, **P < 0.01 and *P < 0.05 versus HCD group (n = 8)

histopathological examination of aorta sections showcased vessel media layer hyperplasia and cholesterol plaques. However, atheroma plaque and steatohepatitis were inhibited by administering *trans*-chalcone in the TCh groups, which ascertain the former investigations [20, 27, 28].

During hepatocyte injury, liver biomarkers in the plasma such as AST, ALP, and ALT are enhanced. This implies that cytosolic enzymes had leaked into the bloodstream, which could be an indication of CVD and liver malfunction due to underfeeding with HFD [31, 34, 35]. Based on our results, while treating animals with *trans*-chalcone, steatosis and liver function were ameliorated by restoring hepatic enzyme levels to an average degree in TCh groups compared with the HCD group. It has been shown that *trans*-chalcone elevated glutathione (GSH) and superoxide dismutase (SOD) as antioxidant indexes [20] in addition to inhibiting *IL-8* in mice fed HCD, resulting in hepatic injury improvement [26]. In parallel, Signh et al. indicated that *trans*-chalcone reduced *TNF-α* level and hepatic markers in CCl4 and PCM-induced hepatic damage in rats [36]. The current biochemical evaluations were amended by the administration of *trans*-chalcone, consistent with other documents.

In the previous research, *trans*-chalcone increased the *adiponectin* gene expression in obese mice with atherosclerosis [20]. *Adiponectin* up-regulates the *AMPK/eNOS* pathway [37]. It has been demonstrated that *AMPK* inhibits VSMC proliferation and attenuates myocardial ischemia. Also, *AMPK* improves vascular endothelial function through attenuating free radicals and improving metabolic profiles [5, 7]. The activation of *AMPK* can ameliorate atherosclerosis by preventing monocyte differentiation to macrophages and reducing macrophage accumulation in plaques. However, *AMPK* mRNA expression was decreased in obesity and atherosclerosis in diet-induced insulin resistance rats and HCD-fed mice that experienced the formation of atheroma plaque [38] which the current investigation showed the same result. The present outcomes revealed that *trans*-chalcone supplementation significantly promotes *AMPK* gene expression in mice treated with *HCD*. It is noteworthy that *AMPK* can act as an *eNOS* regulator [5]. NO has potential effects on suppressing leukocyte-endothelial adhesion VSMC migration and proliferation [4, 39]. However, the enzymatic activity of *eNOS* is inhibited under hyperlipidemia conditions and atherosclerosis which contributes to producing superoxide and endothelial dysfunction [40]. In this study, *eNOS* gene expression had highly diminished in the heart tissues of mice fed HCD; However, it was reverted to normal by utilizing *trans*-chalcone. Furthermore, *eNOS* was proven to be a gene that *KLF-2* targeted [11]. Based on several experimental studies, *KLF-2* can potentially induce *eNOS* expression besides preventing cytokine-mediated induction of VCAM-1 and E-selectin in endothelial cells [11, 41]. *KLF-2* exerts endothelial cell protection against endothelial activation through the statin-activated expression of Thrombomodulin and *eNOS* [10]. In addition, *KLF-2* can also prevent atherosclerosis by reducing inflammation in the vascular endothelium and inhibiting blood clotting and platelet aggregation. The anti-inflammatory properties of *KLF-2* are mediated by inhibiting nuclear factor kappa B (NF-ƙB) signaling and thus suppressing the cellular response to inflammatory cytokines *IL-1β* and *TNF-α* [42]. In the current research, it seems that the anti-inflammatory effect of *trans*-chalcone

on atherosclerosis was illustrated by increasing the *KLF-2* gene expression, which may induce the *eNOS* expression. Furthermore, *AMPK* functions as an upstream kinase regulating *KLF-2* expression, and the *AMPK/KLF-2* signaling pathway can mediate the differentiation of the endothelial cells [43]. These findings might suggest that *trans*-chalcone positively ameliorates atheroma plaque formation through up-regulation of *Adiponectin/AMPK/KLF-2/eNOS* signaling pathway in the aorta vessels of mice on HCD.

Multiple previous studies have proposed that *trans*-chalcone has indicated hepatoprotective and anti-inflammatory properties against HFD-induced liver injury by different molecular mechanisms [20, 29, 36]. Oral administration of *trans*-chalcone has been shown to attenuate *COX-2* expression in hepatic tissue of mice on HCD in the current research. Following the results of our study, preliminary examinations depicted that *COX-2* expression was higher in patients afflicted with NASH and experimental NAFLD animals [16, 44]. Nevertheless, the hepatoprotective properties of *trans*-chalcone have been revealed through reducing the transforming growth factor-beta 1 (*TGF-β1*) and *TNF-α* in the liver damage of rats induced by CCl-4 [36] in addition to down-regulation of the *SREBP-1c, SREBP-2, and PPAR-γ2* as well as up-regulation of *PPAR-α* mRNA expression in obese mice [29]. Moreover, inflammation caused by *COX-2* is in parallel with the increase of plasma leptin levels, oxidative stress, and insulin resistance [44]. Previous studies exhibited that insulin resistance and serum leptin were lowered by administrating *trans*-chalcone in obese animals suffering from a fatty liver [20, 26]. Thus, it can be assumed that the anti-inflammatory properties of *trans*-chalcone can be mediated by reducing *COX-2* gene expression in hepatocytes which ultimately leads to modification the insulin resistance and leptin in diet-related liver abnormalities.

Ang-II is of substantial importance in NAFLD pathogenesis [45]. It has been reported that infusion of *Ang-II* into normal mice induced hepatic steatosis and fibrosis [46]. Furthermore, an increased level of *Ang-II* has a direct relationship with insulin resistance and hepatic *TG* accumulation in animal models with fatty liver disease [45, 46]. In agreement with the previous researches, in this study, the *Ang-II* gene expression had increased substantially in the *HCD* classification. At the same time, it was decreased by oral administration of *trans*-chalcone in *TCh* groups. *Ang-II* is well known as a regulator of adipocytokine productions which reduces adiponectin secretion via down-regulation of *adiponectin* gene expression in adipose tissues [47]. According to the previous results, which exhibited the positive effects of *trans*-chalcone on *adiponectin* gene expression [20], the benefits of *trans*-chalcone against steatohepatitis may be modulated by suppressing the *Ang-II* expression results in the up-regulation of adiponectin in the liver of mice fed HCD.

Another segment of the general study depicted that dose-dependent administration of *trans*-chalcone significantly inhibited *PDGF* mRNA levels in the TCh-40 group. Similarly, following Deng et al. (2017), *PDGF* expression level was incremented in NAFLD mice liver samples that were fed HFD [23]. It has been signified that *PDGF* has a predominant role in hepatic satellite cell proliferation and hepatic fibrosis promotion [22]. *Trans*-chalcone likely inhibits hepatocyte activation and related signal transduction via *PGDF* pathway suppression in hepatocytes. However, further molecular and cellular investigations besides human studies are required to confirm the hepatoprotective and atheroprotective effects of trans-chalcone.

Conclusion

In conclusion, the present study proposed that *trans*-chalcone exerted its atheroprotective effects via up-regulation of *AMPK*, *KLF2*, and *eNOS* genes expression as a possible pathway in the heart tissues of mice fed HCD. Moreover, *trans*-chalcone indicated positive influences on hepatic steatosis and liver function by down-regulating of *COX-2*, *Ang-II*, and *PDGF* gene expression. Overall, the histopathological observations of aorta and liver sections indicated the cardioprotective and hepatoprotective effects of *trans*-chalcone, which was ascertained by regulating assessed genes expression and evaluated biochemical markers.

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Data availability The datasets used and analyzed during the current study are available from the corresponding author on reasonable request. (https://doi.org/10.6084/m9.figshare.14748543).

Declarations

Conflicts of interest No potential conflict of interest relevant to this article was reported.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. The animal protocol was authorized by the Science and Research Branch of the Islamic Azad University, Animal Ethics Committee (approval code: 176947). This study was carried out in the laboratory complex of the Science and Research Branch, Islamic Azad University, Tehran, Iran.

Informed consent Not applicable.

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