

## Original Research

**Choline and Vitamin E Combination Alleviates Biochemical, Molecular and Histopathological Effects of Non-alcoholic Fatty Liver Disease in Rats**

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E-mail address: Omniamostafa6694@gmail.com**Abstract**

A sedentary lifestyle with a poor diet is associated with non-alcoholic fatty liver disease (NAFLD) occurrence. An increase in the NAFLD prevalence is associated with an increase in obesity in the world. Choline as a lipotropic factor and vitamin E as an antioxidant would possess beneficial effects in NAFLD treatment. This study aimed to investigate the synergistic effect of choline and vitamin E on lipid profile, liver function enzymes, antioxidant status and hepatic lipid metabolism-related genes. Albino rats (n=50) were randomly divided into 5 groups. G1: Control group (n= 10), G2: Rats were induced by NAFLD (n=10), G3: Rats were induced by NAFLD and received low dose of choline (25 mg/kg BW orally) and vitamin E (50 mg/kg BW orally), G4: Rats were induced by NAFLD and received medium dose of choline (50 mg/kg BW orally) and vitamin E (100 mg/kg BW orally), G5: Rats were induced by NAFLD and received high dose of choline (100 mg/kg BW orally) and vitamin E (200 mg/kg BW orally). The results demonstrated that treatment of rats with different doses of choline and vitamin E significantly decreased AST, ALT, ALP, GGT, TC, TG, LDL, VLDL, plasma glucose, hepatic lipid peroxidation (MDA), hepatic mRNA expression of inflammatory cytokines (IL-1 $\beta$  and TNF $\alpha$ ) and mRNA expression of hepatic SREBP-1c in compared to NAFLD group. Moreover, significant increase of total protein and albumin, HDL, insulin, antioxidant enzymes (SOD and catalase), mRNA expression of fatty acid oxidation genes (PPAR- $\alpha$  and CPT-1), mRNA expression of hepatic PEMT was detected in treated groups in compared to NAFLD group. In conclusion, choline and vitamin E combination alleviates the biochemical, molecular, and histopathological effects of NAFLD in rats with a potential effect to the highest dose of this combination.

## KEYWORDS

NAFLD, Choline, High fat diet, Vitamin E.

**INTRODUCTION**

Fatty liver is an abnormal accumulation of cholesterol and triglycerides within hepatocytes that exceeds 5% of the weight of the liver (Bellentani *et al.*, 2010). Excess buildup of lipids in the liver due to a deficit in hepatic lipid intake, synthesis, degradation, and secretion (Tessari *et al.*, 2009). Recently, the occurrence of non-alcoholic fatty liver disease (NAFLD) has significantly risen and affects approximately one-fourth (25%) of the global population (Younossi *et al.*, 2018). Moreover, NAFLD is one of the most common liver disorders worldwide (Sharma *et al.*, 2015, Younossi *et al.*, 2018). A sharp increase in the prevalence of NAFLD is associated with an increase in obesity in the world. Unhealthy dietary habits are one of the main factors contributing to the onset and progression of NAFLD (Vancells Lujan *et al.*, 2021).

NAFLD is associated with metabolic syndrome, diabetes mellitus, insulin resistance, hyperlipidemia, obesity, visceral adiposity, and hypertension (Chalasanani *et al.*, 2012). Additionally, it causes the development of multiple diseases like osteoporosis, kidney impairment, and cardiovascular disease (Araujo *et al.*, 2018; Schiavo *et al.*, 2018; Younossi *et al.*, 2018). The multi-hit hypothesis describes the pathophysiology of NAFLD as hepatic process include decrease in hepatic oxidation of free fatty acids, an increase in de novo hepatic lipogenesis, and decrease in lipid export from the liver. Non-hepatic processes include oxidative

stress, apoptosis, and increased proinflammatory cytokines. Additionally, adipocytes release cytokines such as interleukin-6 and tumor necrosis factor alpha (TNF- $\alpha$ ) (Kim and Younossi, 2008; Buzzetti *et al.*, 2016).

Various treatments can be applied to treat NAFLD, such as lifestyle changes to promote weight loss, medications to lower lipid levels, antioxidants and cytoprotective agents to protect liver cells, anti-TNF agents to reduce inflammation, and other treatments. Lifestyle and diet are the first and most important step in treating NAFLD (Younossi *et al.*, 2019). Choline is a lipotropic factor which is an essential nutrient, a major methyl donor, and has a role in fat transport and metabolism (Li and Vance, 2008). Additionally, it is necessary for the synthesis of phospholipids such sphingolipid and phosphatidylcholine (PC) (Zeisel *et al.*, 1991). PC can be synthesized through the cytidine diphosphocholine (CDP-choline) pathway or de novo choline synthesis, which involves three consecutive methylation reactions using SAM as the methyl donor and phosphatidylethanolamine N-methyltransferase (PEMT) as the key enzyme (Gibellini and Smith, 2010). PC is required for the structural integrity of cell membranes, it is the major phospholipid in plasma lipoproteins and is required for lipoproteins structure and cholesterol solubilization in bile (Glier *et al.*, 2014), as well as playing an important role in very low density lipoproteins (VLDL) secretion. In the liver, choline plays important role in maintaining normal membrane integrity and

managing the normal metabolism of cholesterol including low density lipoproteins (LDL) and VLDL (Mehedint and Zeisel, 2013).

Choline deficiency impairs phosphatidylcholine synthesis from both pathways, resulting in intrahepatic triglyceride accumulation, as PC are required for the formation and secretion of VLDL particles containing an abundance of triglycerides (Noga et al., 2002), so a deficiency in choline can significantly reduce VLDL secretion. Furthermore, choline deficiency increases release of reactive oxygen species (ROS) from mitochondria due to change in the mitochondrial membrane, reduced the potential of the mitochondrial membrane and resulting in a decrease in fatty acid oxidation. This increase in ROS can trigger lipid peroxidation (Corbin and Zeisel, 2012). Therefore, whereas choline deficiency causes hepatic steatosis, PE deficiency promotes de novo hepatic lipogenesis and is associated with a higher risk of NAFLD (Bale et al., 2019).

Fat accumulation in hepatocytes is caused by a disruption in lipid metabolism, leading to the production of ROS by intracellular organelles like mitochondria, endoplasmic reticulum, and NADPH oxidase. Oxidative stress has been associated with the onset and progression of NAFLD (Chen et al., 2020). Moreover in NAFLD, oxidative stress can activate the proinflammatory cytokines such as tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) and interleukins leading to liver inflammation, liver fibrosis, and cause cell death (Guo et al., 2020).

Antioxidants include vitamin E. It is classified as a fat-soluble antioxidant. Its main role is scavenging free radicals and preventing oxidative damage in the cell (Polimeni et al., 2015). This damage can cause cellular senescence and apoptosis (El Hadi et al., 2018).  $\alpha$ -Tocopherol regulates the expression of genes involved in cholesterol homeostasis, inflammatory pathways, cellular trafficking, and lipid uptake (El Hadi et al., 2018). It is absorbed through the lymphatic system and transported along with chylomicrons in the body.  $\alpha$ -Tocopherol is found in all fractions of lipoprotein in plasma, but commonly associated with lipoproteins containing apo B. When secreted from the liver, it is associated with VLDL. It inhibits oxidation of polyunsaturated fatty acids (PUFA) and LDL so lowering the risk of atherosclerosis. Moreover, vitamin E enhances the activity of antioxidant enzymes such as SOD, catalase, and glutathione peroxidase (Tabei et al., 2015; Debbabi et al., 2016; Nor Azman et al., 2018). Vitamin E can reduce the inflammatory response in NAFLD by suppressing the expression of the cytokines such as interleukins and TNF- $\alpha$  (Ahsan et al., 2014).

This study aims to investigate the biochemical and molecular effects of choline and Vitamin E combination on HFD-induced NAFLD in rats. We postulated that choline and vitamin E have synergistic ameliorative effect on lipid profile, liver function enzymes, antioxidant status, inflammatory cytokines genes (IL-1 $\beta$  and TNF $\alpha$ ) and hepatic lipid metabolism-related genes in HFD-induced NAFLD in rats.

## MATERIALS AND METHODS

### Experimental animals

Fifty male Sprague-Dawley rats, weighing around 100-120 g, were purchased from the farm of laboratory animals of Faculty of Veterinary Medicine, Zagazig University, Egypt. The rats were accommodated to the laboratory conditions for two weeks before the experiment began. They were kept in a carefully regulated environment to ensure that certain factors, such as temperature, humidity, and lighting, placed in their stainless-steel cages, with a temperature range of 21-25°C, a relative humidity

of 50-60%, and a 12-hour light-dark cycle. The rats were handled in accordance with the National Institutes of Health (NIH) guidelines for the care and use of laboratory animals. The Ethics of Animal Use in Research Committee of Zagazig University, Egypt, approved the current protocol. The approval number is (ZU\_IA-CUC/2/F/54/2022).

### Chemical compounds

Choline chloride (ChCl) (70% Liquid) and Vitamin E (DL- $\alpha$ -Tocopherol 94% purity) were obtained from Phytex Pharma Co., 6th October, Giza, Egypt.

### Experimental design

Fifty rats were divided into five experimental groups, each group contained ten rats. The first group (G1) was designated as control group and was fed a standard diet. G2: Rats were induced by NAFLD (n=10), G3: Rats were induced by NAFLD and received low dose of choline (25 mg/kg BW orally) and vitamin E (50 mg/kg BW orally), G4: Rats were induced by NAFLD and received medium dose of choline (50 mg/kg BW orally) and vitamin E (100 mg/kg BW orally), G5: Rats were induced by NAFLD and received high dose of choline (100 mg/kg BW orally) and vitamin E (200 mg/kg BW orally), rats received daily orally doses for 8 weeks.

### Sampling

The rats were euthanized using cervical decapitation at the end of the experiment. Blood samples were then taken both with and without an anticoagulant. Serum and plasma were separated for use in various biochemical tests. The liver sample was divided into three sections. The 1<sup>st</sup> part was collected in 1 ml Trizol and stored at -80°C until used for gene expression. The 2<sup>nd</sup> part was collected for measurement of antioxidant enzymes (SOD and catalase) and MDA. The 3<sup>rd</sup> part was fixed in 10% neutral buffered formalin for histopathological examination.

### Liver function measurements

Serum ALT was assessed according to the instructions of the kit (NADH. Kinetic UV. IFCC rec., SPINREACT, Ctra. Santa, Coloma, SPAIN) (Murray and Kaplan, 1984b). Serum AST was assessed using kit (NADH. Kinetic UV. IFCC rec. Liquid, SPINREACT, Ctra. Santa, Coloma, SPAIN) (Murray and Kaplan, 1984a). Moreover, alkaline phosphatase was assayed using Kit (p-Nitrophenyl phosphate, kinetic. Liquid. DGKC, SPINREACT, Ctra. Santa, Coloma, SPAIN) (Wenger et al., 1984; Rosalki et al., 1993). Total protein was assessed using kit (Biuret. Colorimetric, SPINREACT, Ctra. Santa, Coloma, SPAIN) (Koller and Kaplan, 1984; Burtis, 1999). Albumin was assayed using kit (Bromocresol green. Colorimetric, SPINREACT, Ctra. Santa, Coloma, SPAIN) (Rodkey, 1965; Doumas et al., 1971; Webster, 1974; Gendler, 1984).

### Lipid profile measurements

TC was assessed according to the instructions of the kit (CHOD-POD. Liquid, SPINREACT, Ctra. Santa, Coloma, SPAIN) (Meiattini et al., 1978; Naito and Kaplan, 1984). Triglycerides (TG) was assayed using kit (GPO-POD. Enzymatic colorimetric, SPINREACT, Ctra. Santa, Coloma, SPAIN) (Buccolo, 1973; Fossati and Prencipe, 1982; Kaplan et al., 1984). HDL Cholesterol was assayed using kit (Direct. Enzymatic colorimetric) (SPINREACT, Ctra. Santa Coloma, SPAIN) (Jacobs et al., 1990; Shih et al., 2000). LDL Choles-

terol was assayed using kit (Direct. Enzymatic Colorimetric) (Friedewald *et al.*, 1972; Wieland and Seidel, 1983). and the VLDL-c was calculated using the Friedewald formula (Friedewald *et al.*, 1972).

*Glycemic index*

Glucose was assessed according to the instructions of the kit (GOD-POD. Liquid, SPINREACT, Ctra. Santa, Coloma, SPAIN) (Trinder, 1969; Kaplan, 1984). Serum insulin level was determined using an ELISA kit.

*Hepatic oxidative assay*

The concentrations of superoxide dismutase (SOD) (Nishikimi *et al.*, 1972), catalase (Fossati *et al.*, 1980), and MDA (lipid peroxidation biomarker) (Kei, 1978; Ohkawa *et al.*, 1979) were estimated in the liver by colorimetric method following the instructions of the kits (Biodiagnostic, Giza, Egypt).

*Gene expression*

To extract total RNA from the tissue, Trizol was utilized. The quality of the RNA was evaluated by analyzing the A260/A280 ratio using the NanoDrop® ND-1000 Spectrophotometer. A High-Capacity cDNA Reverse Transcription Kit cDNA Kit (Applied Biosystems™, USA) was used for cDNA synthesis. Primers were prepared in accordance with the manufacturer’s instructions. The real-time RT-PCR was performed in a Mx3005P Real-Time PCR System using TOPreal™ qPCR 2X PreMIX (SYBR Green with low ROX) following the manufacturer’s instructions (Khamis *et al.*, 2020; 2021). Oligonucleotide primers specific to the target genes were synthesized by Sangon Biotech (Beijing, China), and their sequences are presented in Table 1. Following PCR amplification, a melting curve analysis was done. The mRNA expression of a known housekeeping gene, GAPDH, was used to normalize the target gene expression levels. The results were presented as fold-changes in comparison to the control group, using the 2<sup>-ΔΔCT</sup> method (Livak and Schmittgen, 2001).

*Histopathological technique*

Liver samples were fixed in 10% neutral formalin buffered

solution for 48 h, dehydrated in a series of ethyl alcohol solutions of increasing concentrations and cleared in xylol before being covered with paraffin. Microtome was used to obtain paraffin sections with thicknesses 5 micron. For histopathology examinations, the sections were regularly stained with hematoxylin and eosin (H&E) (Suvarna *et al.*, 2018). All of the section images were captured using a Leica® microscope equipped with an AmScope® microscope digital camera. The lesions were scored using a semiquantitative method as follows: 0 = no detectable histopathological changes, 1 = minimal or focal changes that were rare, 2 = multifocal changes, and 3 = patchy or diffuse changes (Gibson-Corley *et al.*, 2013) (Table 2).

*Statistical analysis*

The data were presented as mean ± SEM. One-way ANOVA was used to compare the means of various parameters across groups. Following significant ANOVA results, Duncan’s multiple range test was employed as a post hoc test to figure out differences among groups. SPSS version 28 was used for statistical data analysis. A significance level of P < 0.05 was considered statistically significant.

**RESULTS**

*Effect of Choline and vitamin E combination on the liver function tests in rats induced by NAFLD*

Significant (P < 0.01) increase in serum AST, ALT, ALP and GGT concentrations as well as significant (P < 0.01) decrease of total protein and albumin concentrations in NAFLD group in comparison to the control group. Moreover, significant (P < 0.01) decline in the concentration of serum ALT, AST, ALP and GGT as well as significant (P < 0.01) increase total protein and albumin concentration were detected in choline and vitamin E combination treated groups in compare to NAFLD group (Figure 1). Increasing doses of choline and vitamin E improved the liver function tests in NAFLD rat model.

*Effect of choline and vitamin E combination on the lipid profile parameters in rats induced by NAFLD*

The levels of TC, TG, LDL-c, and VLDL-c were significantly (P < 0.01) increase while HDL-c level were significantly (P < 0.01)

Table 1. Oligonucleotide primer sequences for real-time PCR.

Gene	Forward primer (5' → 3')	Reverse primer (5' → 3')	Product size/bp	GenBank accession number
IL-1β	CACCTCTCAAGCAGAGCACA	ACGGGTTCCATGGTGAAGTC	81	NM_031512.2
TNF-α	GGCTTTCGGAACACTCACTGGA	GGGAACAGTCTGGGAAGCTC	164	NM_012675.3
PPAR-α	GTCCTCTGGTTGTCCCCTTG	GTCAGTTCACAGGGAAGGCA	176	NM_013196.2
CPT-1	TGCAGTCGACTCACCTTCC	TCAAAGAGCTCCACCTGCTG	93	NM_031559.2
SREBP-1c	GGAGCCATGGATTGCACATT	GCCTGTGTCTCTGTCTCAC	114	NM_001276708.1
PEMT	TTTCTTCTGGTTCTGGCCG	TAGGTAAGGGGACCCGAAGG	184	NM_013003.2
Gapdh	GCACTTCTTGTGCAGTGCC	TACGGCCAAATCCGTTACA	74	NM_017008.4

Table 2. Lesions score of the severity extent in the hepatic tissue.

lesions	Control group	NAFLD group	Low dose Ch & E group	Medium dose Ch & E group	High dose Ch & E group
MacroSteatosis	0	3	1	1	0
Microsteatosis	0	3	2	1	0
Albuminous degenerations	0	2	1	0	0
Round cells infiltrations	0	2	1	1	0

reduced in NAFLD group than the control group. Furthermore, significant ( $P < 0.01$ ) decrease in TC, TG, LDL-c, and VLDL-c levels was detected in choline and vitamin E combination treated groups in compared to NAFLD group (Figure 2). The lipid profile markers of a rat model of NAFLD were enhanced by administering higher doses of choline and vitamin E.

*Effect of choline and vitamin E combination on glucose homeostasis parameters in rats induced by NAFLD*

NAFLD group showed a significant higher ( $P < 0.01$ ) plasma glucose concentration and a significant ( $P < 0.01$ ) lower insulin level than the control group. Otherwise, choline and vitamin E combination treated groups showed significantly ( $P < 0.01$ ) decline in the plasma glucose concentration and a significantly ( $P < 0.01$ ) increase of insulin level in compared to the NAFLD group (Figure 3).

*Effect of choline and vitamin E combination on hepatic tissues antioxidant enzymes and lipid peroxidation in rats induced by NAFLD*

NAFLD group demonstrated a significant ( $P < 0.01$ ) reduction in SOD and catalase concentration of hepatic tissue compared to control group, while MDA level was significantly ( $P < 0.01$ ) higher in NAFLD group than control group. In choline and vitamin E combination treated groups, SOD and catalase concentrations in hepatic tissue significantly ( $P < 0.01$ ) increased and MDA lev-

el significantly ( $P < 0.01$ ) reduced in compared to NAFLD group (Figure 4). The Antioxidant status and lipid peroxidation in a rat model of NAFLD were enhanced by higher doses of choline and vitamin E.

*Effect of choline and vitamin E combination on hepatic mRNA expression of inflammatory cytokines (IL-1 $\beta$  and TNF $\alpha$ ) in rats induced by NAFLD*

mRNA expression levels of IL-1 $\beta$  and TNF $\alpha$  in hepatic tissue were significantly ( $P < 0.01$ ) higher in NAFLD group than control group. Furthermore, significant ( $P < 0.01$ ) reduction in mRNA expression level of IL-1 $\beta$  and TNF- $\alpha$  was detected in choline and vitamin E combination treated groups in compared to NAFLD group (Figure 5). Administering higher doses of choline and vitamin E resulted in an enhancement of hepatic mRNA expression of inflammatory cytokines in a rat model of NAFLD.

*Effect of choline and vitamin E combination on hepatic lipid metabolism related-gene expression in rats induced by NAFLD*

mRNA expression of fatty acid oxidation genes (PPAR- $\alpha$  and CPT-1)

Significant ( $P < 0.01$ ) lower level in hepatic mRNA expression of PPAR $\alpha$  and CPT-1 was detected in NAFLD group than control one. In choline and vitamin E combination treated groups, hepat-

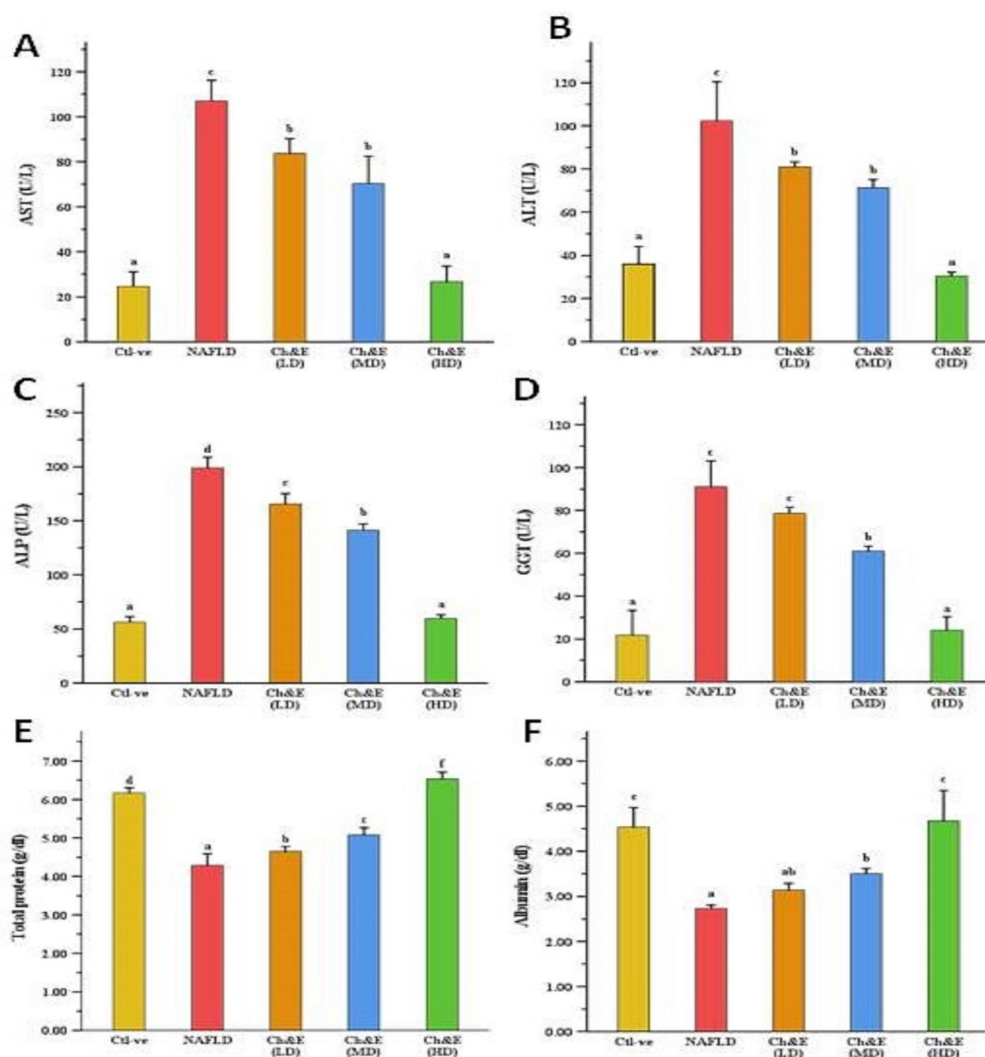


Fig. 1. Effect of choline and vitamin E combination on liver function tests in rats induced with NAFLD. (A) Aspartate aminotransferase (AST) (U/L), (B) Alanine transaminase (ALT) (U/L), (C) alkaline phosphatase (ALP) (U/L), (D) Gamma-glutamyl transferase (GGT) (U/L), (E) Total protein (g/dl) and (F) Albumin (g/dl). Values are mean  $\pm$  SEM. Means with different superscript were significantly different at  $p < 0.01$ .

ic mRNA expression level of PPAR $\alpha$  and CPT-1 was significantly higher ( $P < 0.01$ ) than NAFLD group (Figure 6A and B).

mRNA expression of hepatic lipogenesis genes (cholesterol biosynthesis)

Hepatic expression of SREBP-1c significantly increased ( $P < 0.01$ ) in NAFLD group in compared to control one. Furthermore, in choline and vitamin E combination treated groups, hepatic mRNA expression level of SREBP-1c was significantly higher ( $P < 0.01$ ) than NAFLD group (Figure 6C).

mRNA expression of hepatic PEMT

Hepatic expression of PEMT significantly decreased ( $P < 0.01$ ) in NAFLD group in compared to control one. Moreover, in choline and vitamin E combination treated groups, hepatic mRNA expression level of PEMT was significantly higher ( $P < 0.01$ ) than NAFLD group (Figure 6 D).

### Histopathological findings

Normal histology of hepatic cells, portal triads and central vein were seen in control group and high dose of choline and vitamin E group (Figure 7 A and E). In the other hand, NAFLD group showed intense macrovesicular and microvesicular steato-

sis which distributed mainly within periportal areas and midzone of hepatic lobules. Moreover, Focal round cells aggregations and dilated vasculatures were also commonly seen (Figure 7 B). Fatty droplets in a moderate number of hepatocytes. In addition to, presence of albuminous degeneration in some hepatocytes were detected in low dose of choline vitamin E group (Figure 7C). While moderate dose of choline vitamin E group revealed mild distributed small fatty vacuoles within hepatic parenchyma (Figure 7 D). The histological features improved with increasing doses of choline and vitamin E, resulting in a decrease in the number of apoptotic figures, and the highest dose nearly restored the liver to its normal appearance in rats (Figure 7). The lesions were scored using a semiquantitative method as follows: 0 = no detectable histopathological changes, 1 = minimal or focal changes that were rare, 2 = multifocal changes, and 3 = patchy or diffuse changes (Table 2).

## DISCUSSION

The purpose of this study was to detect and compare the effect of different choline and vitamin E doses on the several dysfunctions associated with NAFLD by investigating body weight, liver histology, liver functions, glucose and lipid homoeostasis, antioxidant enzymes, lipid peroxidation, and hepatic gene expression of PPAR- $\alpha$ , CPT-1, PEMT, IL-1, TNF and SREBP-1c.

Elevated concentrations of serum AST and ALT served as indicators of possible liver damage (Preethi *et al.*, 2016). In this study,

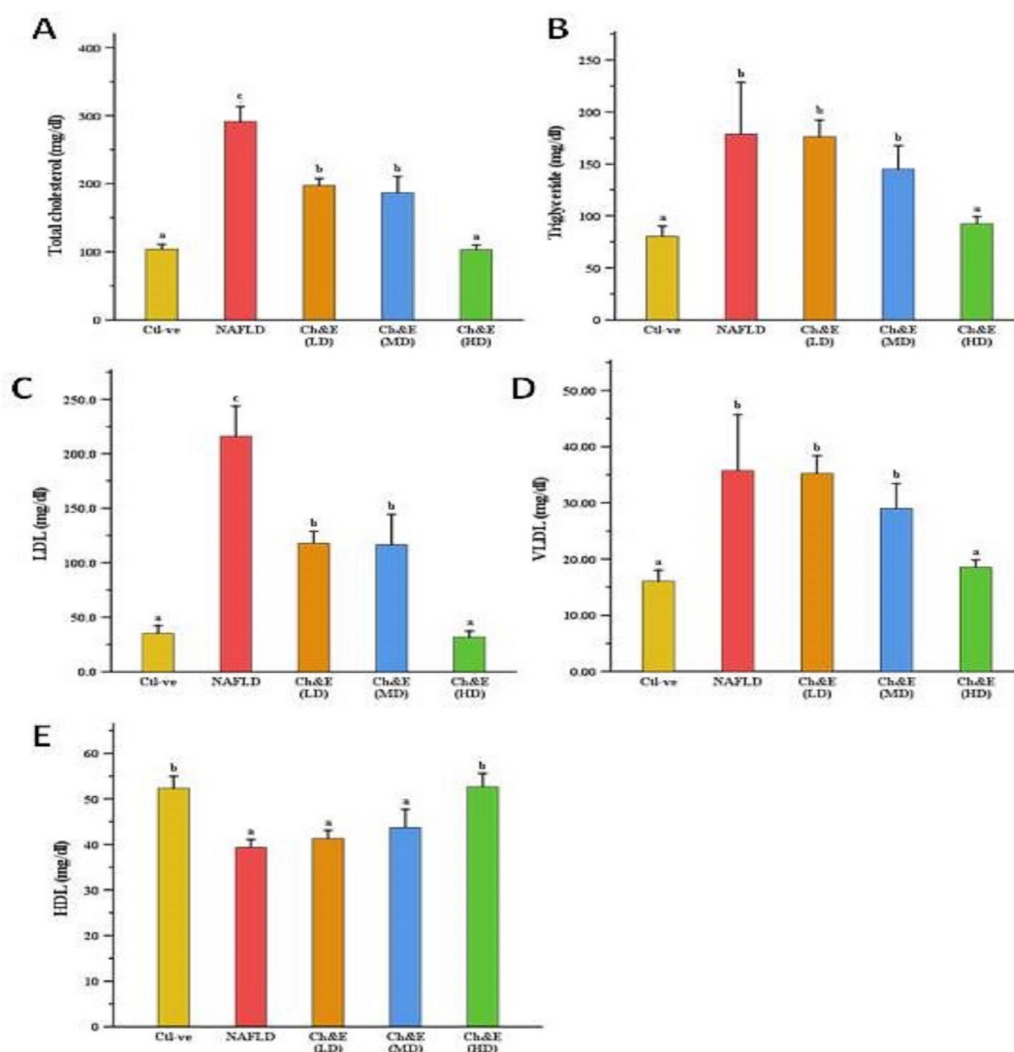


Fig. 2. Effect of choline and vitamin E combination on lipid profile in rats induced with NAFLD. (A) TC (mg/dl), (B) TG (mg/dl), (C) LDL (mg/dl), (D) VLDL (mg/dl) and (E) HDL (mg/dl). Values are mean  $\pm$  SEM. Means with different superscript were significantly different at  $p < 0.01$ .

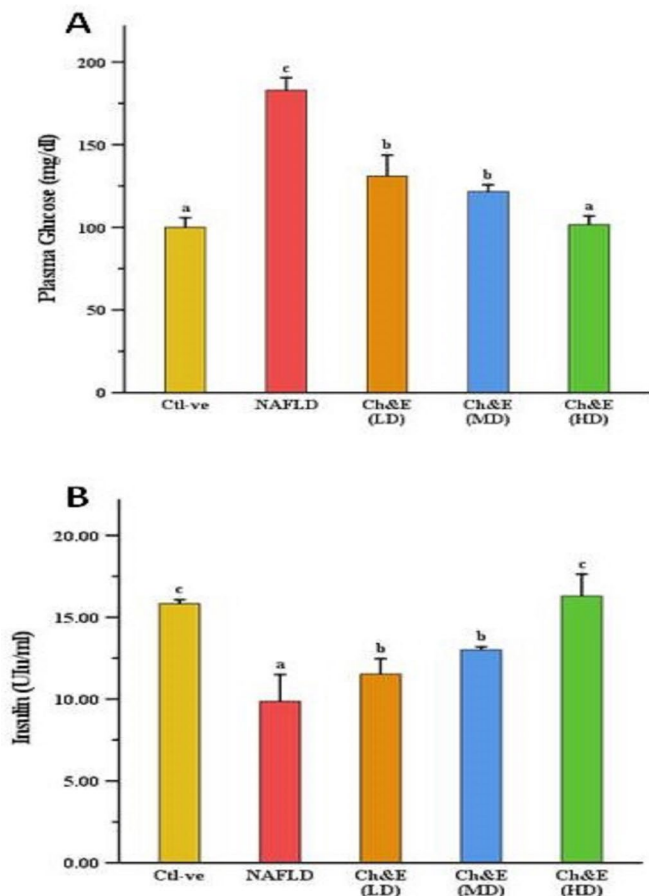


Fig. 3. Effect of choline and vitamin E combination on glucose homeostasis parameters in NAFLD rat induced by HFD. (A) Plasma Glucose (mg/dl) and (B) Insulin (U/μmol). Values are mean ± SEM. Means with different superscript were significantly different at p < 0.01.

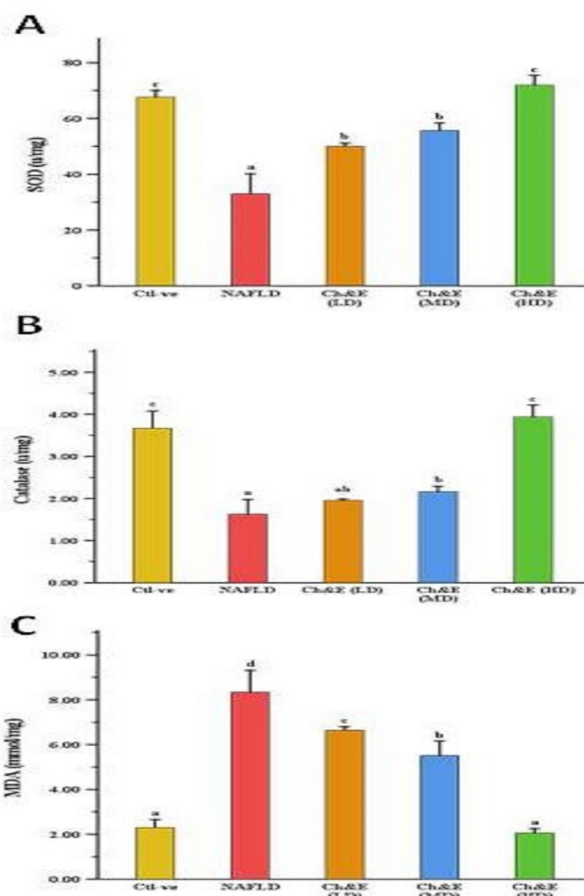


Fig. 4. Effect of choline and vitamin E combination on hepatic tissues antioxidant enzymes and lipid peroxidation in rats induced with NAFLD. (A) Super-oxide dismutase (u/mg), (B) Catalase (u/mg) and (C) Malondialdehyde (MDA) (mmol/mg). Values are mean ± SEM. Means with different superscript were significantly different at p < 0.01.

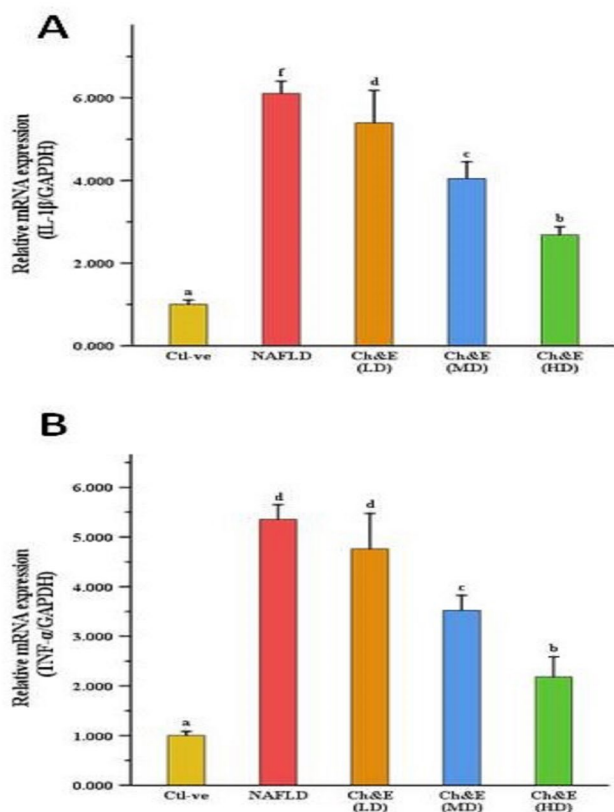


Fig. 5. Effect of choline and vitamin E combination on hepatic mRNA expression of inflammatory cytokines in rats induced with NAFLD. (A) Interleukin-1β (IL-1β) and (B) Tumor necrosis factor (TNFα). Values are mean ± SEM. Means with different superscript were significantly different at p < 0.01.

ALT, AST, ALP, and GGT concentration significantly increase, while total protein and albumin significantly decrease in NAFLD group. These results also agreed with previous studies in which, NAFLD is accompanied by increases in serum ALT, AST, GGT (Tahan *et al.*, 2008; Panchal *et al.*, 2011; Feng *et al.*, 2014). Moreover, choline and vitamin E groups show significant decrease in AST, ALT, ALP and GGT concentration with a significant increase of total protein and albumin compared to NAFLD group. These findings were consistent with previous study, which reported nearly complete normalization of liver enzyme level (ALT and AST) especially at the highest doses of choline. Another study (Khalaf *et al.*, 2019) agreed that vitamin E improved serum ALT and AST level when compared to the NAFLD group. In addition, vitamin E therapy significantly improves serum AST, ALT and ALP levels in NAFLD patients (Sato *et al.*, 2015).

Choline affects beneficially on the lipid profile and improves liver steatosis. The hepatic export of TG via VLDL bundling requires the de novo synthesis of phosphatidylcholine, which is dependent on the presence of choline (Cole *et al.*, 2012). In this study, the levels of TC, TG, LDL-c, and VLDL-c were significantly higher in the NAFLD group compared to the control group, while HDL-c levels were significantly lower. Choline and vitamin E groups had significantly lower TC, TG, LDL-c, and VLDL-c levels, but significantly higher HDL-c levels. This results may be attributed to the restoration of VLDL production from choline, which facilitated the excretion of lipids from hepatocytes (Kitagawa *et al.*, 2015). The highest dose of choline and vitamin E resulted in a higher significant improvement in TC, TG, and LDL-c levels. A previous study on a NAFLD rat model found that choline treatment resulted in a reduction in serum TG, TC, and LDL levels, and an increase in serum HDL levels, as compared to untreated rats (Bakir *et al.*, 2019). In addition, according to Khalaf *et al.* (2019), vitamin E improves serum TG levels.

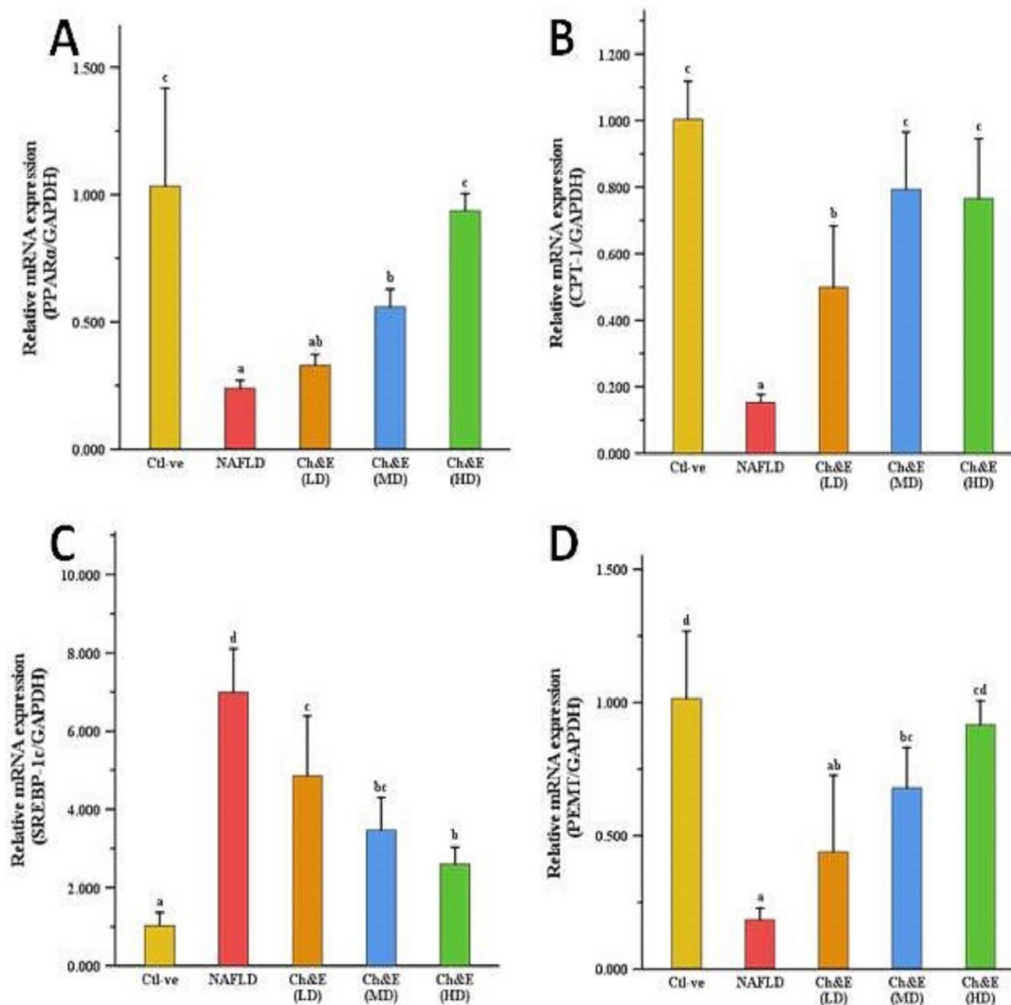


Fig. 6. Effect of choline and vitamin E combination on hepatic mRNA expression of lipid metabolism related genes in rats induced with NAFLD. (A) Peroxisome proliferator-activated receptor (PPAR- $\alpha$ ), (B) Carnitine palmitoyltransferase (CPT-1), (C) Sterol regulatory element-binding protein (SERBP1-c) and (D) Phosphatidylethanolamine N-methyltransferase (PEMT). Values are mean  $\pm$  SEM. Means with different superscript were significantly different at  $p < 0.01$ .

A high-fat diet (HFD) resulted in elevated blood glucose level in mice (Liu *et al.*, 2015; He *et al.*, 2020). Choline deficiency developed liver dysfunction and fatty liver (Zeisel *et al.*, 1991). In this study NAFLD group demonstrate a significant increase in blood glucose concentration and a decrease in insulin level as compared to control group. Choline and vitamin E groups had significant decrease plasma glucose level and significant increase insulin level compared to NAFLD groups. In previous studies, choline supplementation decreases blood glucose level in NAFLD and improve insulin resistance. Vitamin E also reduced the accumulation of hepatic lipid and glucose homeostasis resulting from a high-fat diet (He *et al.*, 2019).

HFD increases oxidative stress in mammals resulting in NAFLD initiation (Yan *et al.*, 2015). MDA amplifies oxidative damage (Serviddio *et al.*, 2013). Oxidative stress in NAFLD evidenced by increased MDA and diminished SOD and catalase (Świdarska *et al.*, 2019). Moreover, increased SOD and catalase with vitamin E supplementation (Ahsan *et al.*, 2014; Tabei *et al.*, 2015; Debbabi *et al.*, 2016).

In this study, the NAFLD group had significantly lower SOD and catalase levels and a significantly higher MDA level than the control group. The choline and vitamin E groups had significantly higher levels of SOD and catalase and significantly lower levels of MDA in comparison to the NAFLD group. The highest dose of choline and vitamin E resulted in a greater improvement in hepatic levels of SOD, catalase, and MDA. Vitamin E can increase antioxidant enzymes such as superoxide dismutase and catalase (Perumpail *et al.*, 2018). Also, choline can decrease ROS level in NAFLD patients (Zhu *et al.*, 2014). A lack of choline can result in changes to the composition of mitochondrial membranes, caus-

ing mitochondrial dysfunction in hepatocytes and an excessive production of free radicals (Zeisel, 2006; Corbin and Zeisel, 2012). Furthermore, previous studies suggested that  $\alpha$ -tocopherol supplementation can reduce hepatic concentrations of lipid peroxidation biomarkers while increasing the concentrations of antioxidant enzymes in the liver (Podszun and Frank, 2021).

In NAFLD, oxidative stress induces the release of proinflammatory mediators, particularly TNF- $\alpha$  and interleukins (Alcala *et al.*, 2015; Wong *et al.*, 2017). In this study, NAFLD group showed a significant increase in hepatic mRNA expression of TNF- $\alpha$  and IL-1 $\beta$  compared to the control group. This data consistent with previous studies showed a significant increase in TNF- $\alpha$  in NAFLD (Stojsavljevic *et al.*, 2014; Lin *et al.*, 2015; Wong *et al.*, 2017). Moreover, our data in choline and vitamin E groups had significant decrease in hepatic mRNA expression of TNF- $\alpha$  and IL-1 $\beta$  when compared to the NAFLD group. Vitamin E can reduce the inflammatory response in NAFLD by inhibiting the expression of cytokines, specifically TNF- $\alpha$  and IL-1 $\beta$  (Ahsan *et al.*, 2014; Khalaf *et al.*, 2019). In vivo studies in obesity models and human studies have indicated that  $\alpha$ -tocopherol supplementation can reduce inflammation by reducing the expression of IL-1 $\beta$  and TNF- $\alpha$ , while increasing antioxidant constituents (Wong *et al.*, 2017).

Hepatic steatosis is related to impaired genes expression of that participate in lipid metabolism. It decreases gene expression for fatty acid oxidation, such as PPAR $\alpha$ , CPT-1 and gene expression of PEMT that is responsible for biosynthesis of phosphatidylcholine, while increases of gene expression for de novo lipogenesis, such as SREBP-1c (Wang *et al.*, 2013; Nakatsuka *et al.*, 2016). PPAR $\alpha$  controls the transcription of several genes involved in hepatic lipid catabolism including CPT-1 which enhances the

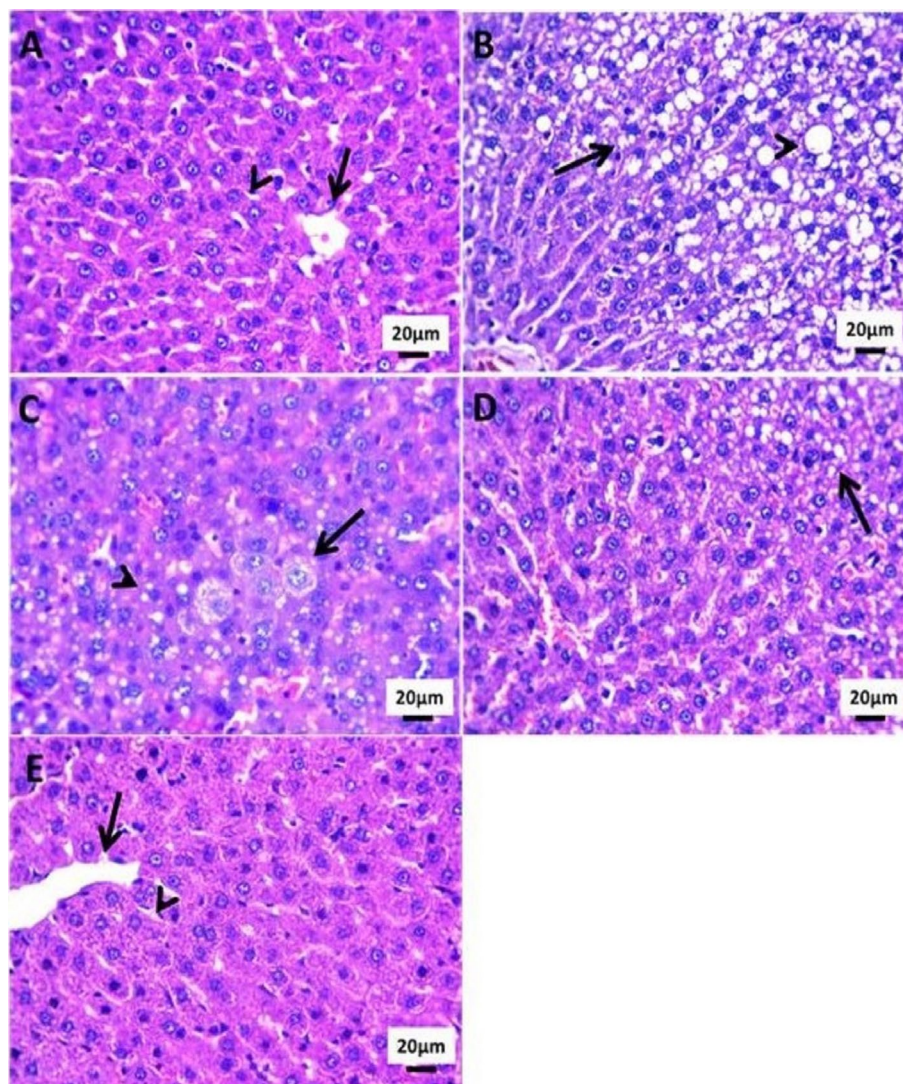


Fig. 7. Effect of choline and vitamin E combination on histological level of liver in rats induced with NAFLD. Photomicrograph of H&E stained sections from liver showing: (A) Control group and (E) High dose of choline and vitamin E combination group: Both groups showed normal histology of hepatic cells (arrowhead) and central vein (arrow). (B) Intense macrovesicular (arrowhead) and microvesicular (arrow) steatosis in NAFLD group. (C) Fatty droplets in a moderate number of hepatocytes (arrowhead) and albuminous degeneration in some hepatocytes (arrow) in low dose of choline and vitamin E combination group. (D) Mild distributed small fatty vacuoles (arrow) in medium dose of choline and vitamin E combination group. Scale bar 20µm

$\beta$ -oxidation of fatty acids and reduces cellular lipids (Zheng *et al.*, 2010; Tailleux *et al.*, 2012). Furthermore, PPAR $\alpha$  has an anti-inflammatory role in the liver of mice fed a high-fat diet (Stienstra *et al.*, 2007). SREBP-1c promotes de novo lipogenesis by controlling the expression of lipogenesis enzymes, causing steatosis and progression of NAFLD (Neuschwander-Tetri, 2017). The inhibition of PPAR $\alpha$  can result in the upregulation of SREBP-1c expression due to high levels of FFAs, leading to the accumulation of lipids and oxidative stress (Qin *et al.*, 2016).

In our data, PPAR $\alpha$  and CPT-1 had significant decrease and SREBP-1c had significant increase in NAFLD group compared to control group. Choline and vitamin E group show significant increase in PPAR $\alpha$  and CPT-1 levels and significant decrease in SREBP-1c level compared to NAFLD group. Choline had direct effect on PPAR $\alpha$  expression, which was upregulated (Zhu *et al.*, 2014), along with the expression of genes that it targets such as CPT-1. Choline can increasing hepatic fat export, enhancing fatty acid catabolism, and reducing oxidative stress, in part through raising PPAR and CPT1 expression via decreased methylation of the PPAR gene promoter (Wang *et al.*, 2013; Zhu *et al.*, 2018). Previous experiments on HepG2 cells demonstrated that PPAR $\alpha$  was reduced by inflammatory mediators such as TNF and IL-1, and that TNF- $\alpha$  suppresses PPAR $\alpha$  mRNA expression (Lim *et al.*, 2013). Moreover, PPAR  $\alpha$  and CPT-1 expression were downregulated and SREBP-1c expression was upregulated in NAFLD mice model (He *et al.*, 2019; Tokoro *et al.*, 2021) and show that PPAR $\alpha$  and CPT-1 expression were upregulated and SREBP-1c expres-

sion was downregulated in NAFLD mice treated with vitamin E supplementation led to a decrease in the synthesis of TG and cholesterol (Sanyal *et al.*, 2010; He *et al.*, 2019), which may be a direct effect of vitamin E on fat accumulation in liver. In another study demonstrated that hepatic expression of CPT-1 increased when exposed to low-dose (50mg/kg) of  $\alpha$ -Tocopherol, but not in high dose (200mg/kg) and may be correlated with the level of oxidative stress (Tokoro *et al.*, 2021).

Reduced expression of hepatic PEMT increases severity of NAFLD (Nakatsuka *et al.*, 2016; Piras *et al.*, 2022). In the present study, PEMT mRNA expression significant decreases in NAFLD group in comparison with control group. Choline and vitamin E group have significant increase in PEMT mRNA expression in comparison with NAFLD group. The highest dose of choline and vitamin E resulted in a greater improvement in PEMT expression.

NAFLD group showed intense macrovesicular and microvesicular steatosis as well as focal round cells aggregations and dilated vasculatures. With choline and vitamin E, fatty droplets and albuminous degeneration that were detected at low doses completely disappeared as the dose increased. Our results were in harmony with Borges Haubert *et al.* (2015) which is that choline was beneficial in decreasing hepatic steatosis and increasing the vitamin E hepatic concentrations. In accordance with He *et al.* (2019), histopathological examination of NAFLD mice treated with vitamin E revealed that liver steatosis was significantly alleviated.



## CONCLUSION

NAFLD is one of the most common health problems that affect the liver worldwide. The combination between choline and vitamin E has potential ameliorative effect on lipid profile, liver function enzymes, antioxidant status, inflammatory cytokines genes (IL-1 $\beta$  and TNF $\alpha$ ) and hepatic lipid metabolism-related genes in HFD-induced NAFLD in rats. Therefore, the synergistic effect between lipotropic effects of choline and antioxidant properties of vitamin E would be one possible therapeutic approach for NAFLD.

## CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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