

**Original Paper****Therapeutic potential and hepatoprotective activity of proanthocyanidin and clopidogrel in non-alcoholic fatty liver disease-induced rats**Samy A. Hussein¹, Omayma A. R. Abo zaid¹, Mohamed A. Azab², Soheir K. Mohamed^{1,*}¹Biochemistry Department, Faculty of Veterinary Medicine, Benha University, Egypt.²Physiology Department, Faculty of Veterinary Medicine, Benha University, Egypt.**ARTICLE INFO****ABSTRACT****Keywords**

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Non-alcoholic fatty liver disease (NAFLD) is a complex disease caused by a number of different pathogenic processes as a result of the systemic interaction between the liver and several other organs. Grape seeds proanthocyanidins extract (GSPE) potency in the liver is associated with improvement of the hepatic enzyme activities as their powerful antioxidant property results from its ability to directly scavenge free radicals and/or chelate metals. Thirty-two male albino rats were assigned into 4 equal groups of 8 rats as: Normal control group (G1): Rats fed ordinary normal diet for 12 weeks. NAFLD group (G2): Rats provided with HFD-diet for 6 weeks for NAFLD induction, followed by ordinary normal diet for another 6 weeks. NAFLD + GSPE treated group (G3): Rats fed HFD for 6 weeks (NAFLD) followed by administration of GSPE for another 6 weeks. NAFLD + GSPE + clopidogrel treated group (G4): Rats fed HFD for 6 weeks, followed by administration of GSPE and clopidogrel for additional 6 weeks. The results revealed that treatment with GSPE or in combination with clopidogrel (G3, G4) significantly decreased the higher activities of serum AST, ALT and γ GT in NAFLD induced rats (G2). Interestingly, the gene expressions of IL-1 β , PPAR α , TGF- β 1 and TIMP1 in liver tissue significantly down regulated in GSPE (G3) and GSPE + clopidogrel (G4) treated groups as compared with NAFLD group (G2). In conclusion, treatment with GSPE and in combination with clopidogrel is alternative therapies and powerful anti-inflammatory, protect liver cells against fatty liver disease via restoring the hepatocytes function and inflammatory mediators.

1. INTRODUCTION

The hallmark of NAFLD is excessive triglycerides (TG) accumulation of lipid droplets in the hepatocytes, which may be an isolated event of (NAFL) or accompanied by inflammation and cell injury with or without fibrosis (Oseini and Sanyal, 2017).

The “multi-hit” theory consider the imbalanced lipid metabolism and insulin resistance (IR) as “the first hit” to the liver, and oxidative stress as the “second hit” involved in the pathogenesis of NAFLD (Jian et al., 2018). However, GSPE has documented powerful antioxidant and anti-inflammatory properties (Long et al., 2016), through the ability to abrogate oxidative stress, inflammation (Hamza et al., 2018). Additionally, platelets play an important role in the establishment and progression of liver disease. Subsequently, anti-platelet strategies have a beneficial effect in animal models of chronic liver disease (Chauhan et al., 2016). Accordingly, this study aimed to evaluate the potential protective and therapeutic effects of GSPE or in combination with clopidogrel to NAFLD rats through determination of serum liver marker enzymes and the mRNA gene expression of the inflammatory mediators IL-1 β , PPAR α , TGF- β 1 and TIMP1 in rats livers.

2. MATERIAL AND METHODS**2.1. Experimental animals**

Thirty-two albino male rats, 4-5 week's old with ~150–200g weight were used in this experiment. All rats were acclimatized for two weeks before the experiment, and were fed regularly. The study protocol was approved by the Animal Care and Use commission of Benha University in compliance with the National Institute of Health Guide for the Care and Use of research Animals.

2.2. Antioxidant agent and drugs**2.2.1. Grape seed proanthocyanidin extract**

The antioxidant agent GSPE was purchased from Al Debeiky Pharma Company for Pharmaceutical industries, Al Obour, Cairo, Egypt. GSPE was freshly dissolved in 100% Dimethyl sulfoxide (DMSO) and diluted to the appropriate concentration by saline and administered orally at a dose of (200 mg/kg body weight b.wt./day) (da Costa et al., 2017).

2.2.2. Antiplatelet drug

The pharmacological anti-platelet drug (Plavix[®]) 75 mg film coated tablets with the active ingredient clopidogrel hydrogen sulfate, was obtained from Sanofi pharma Bristol-Myers Squibb SNC Paris-France. Clopidogrel was

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dissolved in saline and administered orally by intra-gastric tube at a dose of 6.75 mg/kg b.wt. /day (Ahn et al., 2019).

2.3. Experimental design

The rats were randomly divided into four equal groups, 8 rats each, placed in separate cage and classified as follows:

Group I: Normal control group (G1): Rats received no drugs or chemicals, provided with ordinary normal diet for 12 weeks, and served as a control group.

Group II: NAFLD group (G2): Rats provided with HFD-diet (Li et al., 2014) for 6 weeks for the induction of NAFLD, followed by normal feeding for another 6 weeks.

Group III: GSPE treated group (G3): Rats fed HFD for 6 weeks (NAFLD) followed by administration of GSPE for another 6 weeks.

Group IV: GSPE + clopidogrel treated group (G4): Rats fed HFD for 6 weeks (NAFLD), followed by administration of GSPE and Clopidogrel for additional 6 weeks.

2.4. Sampling

Rats were sacrificed after the 12 week of the experiment by cervical decapitation according to Animal Ethics Committees.

Serum samples were collected and used immediately for determination of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and γ -glutamyl transferase (γ GT) activities, to evaluate the hepatic injuries.

Moreover, rats liver specimens were removed, cleaned by rinsing with cold saline, immediately kept in liquid nitrogen and stored at -80°C till RNA extraction for the real time quantitative polymerase chain reaction analysis (qPCR) of the gene expression levels of interleukin-1 β (IL-1 β), peroxisome proliferator activated receptor- α (PPAR α), transforming growth factor- β 1 (TGF β 1) and tissue inhibitor of metalloproteinase-1 (TIMP1).

2.5. Molecular analysis

The hepatic mRNA expressions levels of IL-1 β , PPAR α , TGF- β 1 and TIMP1 in the rats were determined using qPCR. Samples were processed simultaneously, as previously described (Bush et al., 2001). β -actin was used as

the load control. Total RNA was isolated using High Kit for pure RNA isolation (Thermo Scientific, Fermentas, #K0731). The produced cDNAs from the reverse transcribed template RNAs using Revert AidTM H Minus Reverse transcriptase kit (#EP0451, Thermo Scientific, Fermentas, USA) were amplified on Fast start Universal SYBR Green Master (Roche, GER). The target gene was normalized with β -actin by the $2^{-\Delta\Delta\text{Ct}}$ method (Livak and Schmittgen, 2001).

Table 1 Forward and reverse primers sequence for real time PCR

Gene	Forward primer(5'-----3')	Reverse primer(5'-----3')
IL-1 β	CACCTCTCAAGCAGAGCACAG	GGGTTCATGGTGAAGTCAAC
TGF- β 1	AAGAAGTCACCCGCTGCTA	TGTGTGATGCTTTGGTTTTGTCA
PPAR α	CCTGTCCTCGGGATGTCACAC	GTGACGGTCTCCACGGACATGC
TIMP1	CGCAGCGAGGAGTTCTCAT	GGCAGTATGTGCAAATTCC
β -actin	AAGTCCCTCACCTCCCAAAG	AAGCAATGCTGTCACTTCCC

2.6. Statistical analysis

Results were expressed as mean \pm SE using SPSS (18.0 software, 2011). Using one-way ANOVA followed by Duncan's test data was analyzed. Values were statistically significant at $p \leq 0.05$.

3. RESULTS

The obtained data in table (2) showed that, serum AST, ALT and γ GT activities were significantly increased in the NAFLD group (G2) as compared to the control group (G1), while significantly reduced in GSPE (G3) and GSPE + clopidogrel (G4) treated groups comparing with the NAFLD group (G2). Also, the qPCR results in the liver tissues of rats existing in table (3) revealed significant up-regulation ($P \leq 0.05$) in IL-1 β , PPAR α , TGF- β 1 and TIMP1 expression levels in the NAFLD rats (G2) when compared with the control rats (G1), however a significant down-regulation in IL-1 β , PPAR α , TGF- β 1 and TIMP1 gene expression levels were observed in GSPE (G3) and GSPE + clopidogrel (G4) treated groups comparing with the NAFLD rats (G2). However, no significant difference was noticed between G3 and G4 and the expression levels of (G3, G4) remained significantly higher than the controls (G1).

Table 2 Effect of GSPE or in combination with Clopidogrel treatment on serum AST, ALT and γ GT activities in HFD-induced NAFLD in rats.

Animal groups	AST (U/L)	ALT (U/L)	γ GT (U/L)
Control (G1)	41.80 ^a \pm 1.72	39.12 ^a \pm 1.20	8.94 ^a \pm 0.42
NAFLD (G2)	85.17 ^b \pm 3.85	94.48 ^b \pm 4.53	19.28 ^b \pm 0.83
NAFLD + GSPE treated (G3)	70.14 ^b \pm 2.90	70.85 ^b \pm 2.61	14.36 ^b \pm 0.58
NAFLD+GSPE+Clopidogrel treated (G4)	68.82 ^b \pm 2.76	67.25 ^b \pm 2.25	15.00 ^b \pm 0.60

Data are presented as (Mean \pm S.E). S.E = Standard error. Mean values with different superscript letters in the same column are significantly different at ($P \leq 0.05$).

Table 3 Effect of GSPE or in combination with Clopidogrel treatment on hepatic IL-1 β , PPAR α , TGF-1 β and TIMP1 gene expression levels in NAFLD-induced rats.

Animal Groups	IL-1 β Fold change \pm SEM	PPAR α Fold change \pm SEM	TGF- β 1 Fold change \pm SEM	TIMP1 Fold change \pm SEM
Control (G1)	1.00 ^a \pm 0.00	1.00 ^a \pm 0.00	1.00 ^a \pm 0.00	1.00 ^a \pm 0.00
NAFLD (G2)	5.13 ^b \pm 0.26	4.23 ^b \pm 0.21	9.06 ^b \pm 0.58	8.82 ^b \pm 0.45
NAFLD + GSPE treated (G3)	3.16 ^b \pm 0.16	3.23 ^b \pm 0.15	4.76 ^b \pm 0.26	4.32 ^b \pm 0.21
NAFLD+GSPE+Clopidogrel treated (G4)	2.77 ^b \pm 0.12	3.01 ^b \pm 0.18	4.47 ^b \pm 0.22	4.38 ^b \pm 0.17

Mean values with different superscript letters in the same column are significantly different at ($P \leq 0.05$).

4. DISCUSSION

As a consequence of the lack of treatment and the growing global epidemic of obesity, a wide range of drugs and supplements, including antioxidants and anti-inflammations, have been applied in NAFLD experimental models as alternative therapies (Eslamparast et al., 2015).

The obtained results showed significant increase in serum ALT, AST and γ GT activities in HFD-induced NAFLD rats as compared with controls. Similarly, Swetha and

Thykadavil (2019) observed significant increase in serum AST, ALT and γ GT values in NAFLD cases than in controls, in which higher values of AST and ALT activities reflects oxidative stress, cell injury and steatosis, and free radicals elevation leads to reduction of glutathione and induces γ GT to protect glutathione level. Actually, ALT, AST and γ GT are markers of liver injury and may be useful surrogate measures of NAFLD (Sanyal et al., 2015).

Interestingly, GSPE treatment in the NAFLD rats caused significant decrease in the elevated serum ALT, AST and γ GT activities in comparison with the untreated NAFLD

rats group. Similarly, Rajput et al. (2017) noticed that supplementation of GSPE (250 and 500mg/kg b.wt.) significantly decrease serum ALT, AST and γ GT activities, by 31%, 16% and 16%, respectively, indicating the antioxidative and anti-inflammatory effects of GSPE, as the improvement in liver function may be attributed to the reduction of reactive oxygen species (ROS), maintaining the cell membrane integrity and restoring the hepatocytes function (Mohammed and Safwat, 2019).

Moreover, Clopidogrel and GSPE co-treatment to NAFLD rats showed potential therapeutic effect against oxidative stress illustrated by the significant decrease in serum ALT, AST and γ GT activities, comparing with the NAFLD untreated rats. The obtained data agree with An et al. (2018) who showed that, clopidogrel treatment markedly reduced platelet vascular deposition and oxidative stress, produced by Angiotensin-II (Ang-II)-infusion. Notably, HFD intake increased the susceptibility of liver to inflammatory stimuli through the induction of pro-coagulation state (Nanizawa et al., 2020), and platelets have important roles at every stage during the liver injury and healing processes (Chauhan et al., 2016).

In the current study the qPCR results showed significant up-regulation of the hepatic IL-1 β and PPAR α expression levels of the NAFLD rats as compared to the control. Likewise, Parafati et al. (2018) observed a clear up-regulation of IL-1 β gene expression in cafeteria-fed livers compared to control, as hepatic lipid accumulation leads to sub-acute hepatic inflammation via Nuclear factor-kappa β (NF- κ β) activation by releasing pro-inflammatory cytokines as IL-1 β , and tumor necrosis factor (TNF- α) (Martins and Oliveira, 2018). Surprisingly, ROS stimulate TNF- α which is a NF- κ β -dependent gene (Mohammed and Safwat, 2019). Regarding PPAR α , the obtained results agree with the reported data of Li et al. (2018) who found that, during the development of hepatic steatosis (8–16 weeks), the hepatic expression of PPAR α and its target genes were increased in high-fat feeding, referring to a role of PPARs in mice models of NAFLD, as PPAR α is highly expressed in oxidative tissues in the liver (Fougerat et al., 2020), and upon challenge with HFD, hepatic PPAR α levels increase acutely as an adaptive or protective response (Patsouris et al., 2006). Specifically, PPAR α regulates lipid metabolism in the liver, and its ligand may exhibit antisteatotic effects and anti-inflammatory (Wang et al., 2020).

The existing results showed significant upregulation of hepatic TGF- β 1 and TIMP1 gene expression levels of the NAFLD rats compared to the control. The obtained result are nearly similar to Dewidar et al., (2019) who observed that, during acute and chronic liver injury, TGF β is activated from deposits in the extracellular matrix (ECM) and expressed and released from various cell types, as quiescent hepatic stellate cells (HSC) express up-regulated TGF- β after liver injury, in which TGF β mediate the pro-inflammatory effects, and participates in all phases of liver disease development (Dooley and Ten Dijke, 2012), as the chronic hepatocyte injury induces the recruitment and Toll-like receptor-dependent activation of inflammatory cells, mainly liver macrophages or kupffer cells, which amplifies inflammation (Fougerat et al., 2020).

Regarding hepatic TIMP1 gene expression level, the obtained results agree with Abdelaziz et al. (2015) who observed that, obese patients with elevated liver enzymes showed significantly elevated levels of TIMP1, 2, as compared to those with normal liver enzymes and the control. Upregulation of matrix metalloproteinases (MMPs) expression enhance the destruction of the hepatic tissue, with increased expression of tissue inhibitors of matrix

metalloproteinases (TIMPs) (Roderfeld (2018), as ROS may be involved in the imbalance in MMPs and their inhibitors TIMPs and participate in the observed extracellular matrix (ECM) remodeling (Doridot et al., 2019).

Furthermore, GSPE treatment of the NAFLD rats showed significant down regulation of IL-1 β , PPAR α , TGF- β 1 and TIMP1 expression levels in the hepatic tissues when compared with the NAFLD rats. Similarly, Lu et al. (2020) declared that GSPE significantly attenuated the expression of IL-1 β . Moreover, Nie et al. (2017) found that, trimer gallate of oligomeric proanthocyanidins down-regulated the nuclear hormone receptor-49 (an ortholog of the human PPAR α), a key regulator of fat metabolism. Interestingly, the evident reduction of oxidative stress and inflammatory markers as IL-1 β by GSPE can be owing to its beneficial effects of scavenging ROS, and increasing the epithelial barrier integrity, and decreasing inflammation (Nallathambi et al., 2020), while as the significant down regulation of PPAR α expression in this study may be explained as a positive feedback for alleviating of oxidative stress and inflammation by GSPE.

Regarding TGF- β 1 and TIMP1, the existing results agree with the data of Bao et al. (2015) who observed that GSPE (500 mg/kg/b.wt.) treatment of diabetic rats reduced the expression of TIMP1, comparing with diabetic controls. Attia et al. (2016) also stated that, the significant downregulation of hepatic TIMP1 gene expression level by extract of date fruits may be due to inhibition of HSC activity, and/or formation of TGF β , as also achieved by grape proanthocyanin, thus offering protection against oxidative stress (Zhen et al., 2016). Moreover, TGF- β 1 plays a key role in modulating the extracellular matrix (ECM) levels via inhibiting MMPs, while simultaneously up-regulates expression of TIMPs (Gomes et al., 2012).

Moreover, hepatic gene expression levels of IL-1 β , PPAR α , TGF β 1 and TIMP1 were significantly down-regulated, following administration of clopidogrel and GSPE to the NAFLD rats in comparison with the untreated NAFLD rats. These data are in line with An et al. (2018) who observed a significant decrease in IL-1 β mRNA levels by clopidogrel treatment. Likewise, clopidogrel significantly decreased the hepatic expressions of TIMP1 and TGF- β 1, compared to α -naphthylisothiocyanate (xenobiotic)-exposed protease activated receptor-4 in mice treated with vehicle (Joshi et al., 2016). Regarding PPAR α , rarely data linking PPAR α with clopidogrel, but contrarily to the present study, Massimi et al. (2018) showed that, cells treated with Aspirin, an anti-platelet drug, had up-regulated PPAR α mRNA. Anti-platelet treatment by clopidogrel in NAFLD ameliorated the hepatic inflammation (Sitia et al., 2013) and abolished vascular inflammatory responses and remodeling (An et al., 2018), as platelet granules contain a variety of factors including TGF β that are secreted upon platelet activation (Denslow et al., 2017), and a linear correlation of plasma TIMP1 levels and platelet count has been observed by the lower circulating TIMP1 levels in patients on clopidogrel treatment (Nagy et al., 2016).

5. CONCLUSION

We can conclude that, treatment with GSPE alone or with antiplatelet drug can improve the metabolic disruptions associated with the induction of NAFLD in rats elucidated by improving the liver biomarkers and down-regulation of inflammatory mediators expression.

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