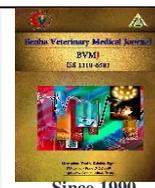




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### Original Paper

## Grape seed proanthocyanidin extract and anti-platelet drug (clopidogrel) alleviates non-alcoholic fatty liver disease via inhibition of hepatic inflammation in rats

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### ABSTRACT

The initiation and progression of nonalcoholic fatty liver disease (NAFLD) include imbalanced lipid metabolism, insulin resistance (IR) and oxidative stress. Consequently, grape seed proanthocyanidin extract (GSPE) showed very beneficial effect against the NAFLD metabolic disruptions, via its antioxidant, anti-inflammatory and lipolytic properties. Clopidogrel also can reduce the oxidative stress and inflammation. Thirty-two male albino rats were divided into four equal groups of 8 rats; Group I: Normal control group (G1): Rats fed ordinary normal diet for 12 weeks. Group II: NAFLD group (G2): Rats fed HFD-diet for 6 weeks for NAFLD induction, followed by ordinary normal diet for another 6 weeks. Group III: NAFLD+GSPE treated group (G3): Rats fed HFD for 6 weeks (NAFLD) followed by administration of GSPE for another 6 weeks. Group IV: NAFLD + GSPE + Clopidogrel treated group (G4): Rats fed HFD for 6 weeks (NAFLD), followed by administration of GSPE and clopidogrel for additional 6 weeks. The results showed that treatment with GSPE (G3) or in combination with Clopidogrel (G4) significantly decreased the higher serum total cholesterol, triacylglycerols and ferritin concentrations that were observed in NAFLD-induced group (G2). Interestingly, the hepatic inflammatory mediators VEGF, PDGF  $\alpha$  and MAPK gene expression levels were significantly down-regulated in GSPE (G3) or GSPE and Clopidogrel co-treated (G4) groups as compared with NAFLD-induced group (G2). In conclusion, treatment with GSPE alone or with Clopidogrel is a potent hypolipidemic and anti-inflammatory, restoring hepatic cell function and alleviates the oxidative stress and inflammation related to NAFLD in rats.

## 1. INTRODUCTION

Oxidative stress, lipotoxicity, and inflammation play key role in the progression of NAFLD (Yang et al., 2019). When IR occurs, liver becomes more vulnerable to hyperinsulinemia-induced “multi-hit” events involving the release of reactive oxygen species (ROS), pro-fibrogenic factors and pro-inflammatory mediators from impaired organelles (Xiao et al., 2013). Grape seeds extract (GSE) improved the antioxidant defense, and prevented the HFD-induced inflammation, through the reduction of pro-inflammatory cytokines (da Costa et al., 2017), while Clopidogrel is the most widely prescribed anti-platelet for the amelioration of oxidative stress, and endothelial dysfunction (Ramadan et al., 2014). Accordingly, the present study was designed to investigate the possible protective and therapeutic efficacy of GSPE or in combination with Clopidogrel by evaluation of serum lipids profile, ferritin in addition to the molecular analysis of some inflammatory mediators gene expression levels in liver tissues as VEGF, PDGF $\alpha$ , and MAPK in the experimental model of NAFLD in rats.

## 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. The 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.

*Preparation:* GSPE was freshly dissolved in 100% Dimethyl sulfoxide (DMSO) and diluted to the appropriate concentration by saline and administered orally in a dose of (200 mg/kgb.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

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hydrogen sulfate, was obtained from Sanofi pharma Bristol-Myers Squibb SNC Paris-France.

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

### 2.3. Experimental design:

Rats were randomly divided into four equal groups (8each) as follows:

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

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

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

Group IV: NAFLD + GSPE + clopidogrel treated group (G4): Rats fed HFD for 6 weeks (NAFLD), followed by administration of GSPE and Clopidogrel (6.75 mg/kg b.w./day) for additional 6 weeks.

### 2.4. Sampling

#### 2.4.1. Blood samples:

All rats were sacrificed, after 12 week of the experiment onset by cervical decapitation according to Animal Ethics Committees, and blood samples for serum separation were collected, then centrifuge for 15 minutes at 3000 rpm and the separated serum was kept in Eppendorf tubes and stored at -20 °C in deep freezer for determination of the of total cholesterol, triacylglycerols and ferritin concentrations according to the method described by NCEP expert panel, (1988),Stein, (1987),and Dawson et al. (1992), respectively.

#### 2.4.2. Tissue samples:

Liver of all rats 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 (qPCR) analysis of the hepatic gene expression levels of vascular endothelial growth factor (VEGF),platelet-derived growth factor- $\alpha$  (PDGF $\alpha$ ) and mitogen activated protein kinase (MAPK).

### 2.4.3. Molecular analysis

The mRNA expressions content of VEGF, PDGF  $\alpha$  and MAPK in the liver tissues of the rats were determined using qPCR. Samples were processed simultaneously (Bush et al., 2001), B-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 Aid™ 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  $\beta$ -actin by the  $2^{-\Delta\Delta Ct}$  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')
PDGF $\alpha$	CCAACCTGAACCCAGACCAT	AGGAGAACAAGACCGCACG
VEGF	GATCATGCGGATCAAACCTCAC	CCTCCGGACCCAAAGTGCTC
MAPK	AGGGCGATGTGACGTTT	CTGGCAGGGTGAAGTTGG
$\beta$ -actin	AAGTCCTCACCTCCAAAAG	AAGCAATGCTGTACCTTCC

### 2.5. 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 were analysed. Values were statistically significant at  $p \leq 0.05$ .

## 3. RESULTS

This study data in table (2) illustrate that, serum total cholesterol, triacylglycerols and Ferritin levels were significantly increased in the NAFLD group (G2),when compared with the control group (G1), while were significantly decreased in GSPE (G3), and GSPE + clopidogrel (G4) treated groups, comparing with the NAFLD rats (G2).Likewise, the obtained qPCR results in table (3) showed significant up-regulation ( $P \leq 0.05$ ) in VEGF,PDGF $\alpha$  and MAPK gene expression levels in the NAFLDrats (G2),when compared to the controls (G1), while were significantly downregulated in the GSPE (G3)and GSPE + clopidogrel (G4) treated groups, comparing with the NAFLD group (G2).However, no significant difference was noticed between (G3) and (G4) and their expression levels remained significantly higher than the controls (G1).

Table 2 Effect of GSPE or in combination with Clopidogrel treatment on serum Total cholesterol, Triacylglycerols and Ferritin concentrations in NAFLD-induced rats.

Animal groups	Total cholesterol (mg/dl)	Triglycerides (mg/dl)	Ferritin (mg/dl)
Control (G1)	90.43 $\pm$ 5.05	40.80 $\pm$ 2.2	10.42 $\pm$ 0.58
NAFLD (G2)	175.50 $\pm$ 7.82	107.37 $\pm$ 6.55	34.49 $\pm$ 2.11
NAFLD+GSPE treated (G3)	138.68 $\pm$ 6.11	74.29 $\pm$ 3.69	20.90 $\pm$ 1.11
NAFLD+GSPE+Clopidogrel treated(G4)	130.27 $\pm$ 6.03	72.33 $\pm$ 3.45	22.03 $\pm$ 1.02

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 VEGF, PDGF $\alpha$  and MAPK gene expression levels in NAFLD-induced rats.

Animal groups	VEGF Fold change $\pm$ SEM	PDGF $\alpha$ Fold change $\pm$ SEM	MAPK Fold change $\pm$ SEM
Control (G1)	1.00 $\pm$ 0.00	1.00 $\pm$ 0.00	1.00 $\pm$ 0.00
NAFLD (G2)	5.46 $\pm$ 0.26	4.72 $\pm$ 0.22	7.52 $\pm$ 0.43
NAFLD+GSPE treated (G3)	3.16 $\pm$ 0.14	2.62 $\pm$ 0.11	5.28 $\pm$ 0.30
NAFLD+GSPE+Clopidogrel treated(G4)	1.77 $\pm$ 0.10	1.28 $\pm$ 0.09	4.44 $\pm$ 0.26

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

## 4. DISCUSSION

Currently, a beneficial treatment of NAFLD may be through the targeting of NAFLD by treating obesity and treating NAFLD by targeting fat redistribution (Niccolai et al., 2019).The obtained data in table (2) revealed significant

increase in serum total cholesterol and triacylglycerols concentrations in HFD-induced NAFLD rats (G2), in comparison with the control rats (G1). Likewise, Oriquat et al. (2018) showed that, hepatic tissues of NAFLD rats showed significantly higher levels of triacylglycerols (~225%) and total cholesterol when compared with control

rats. Actually, serum lipid profile correlates significantly with NAFLD severity (Francque et al., 2016), as the obese state in the liver promotes lipogenesis and elicits mitochondrial dysfunction resulting in hepatic fatty acids and lipid overload (Leonetti et al., 2018).

Interestingly, treatment of the NAFLD rats with GSPE (G3) showed effective improvement in the serum triacylglycerols and total cholesterol levels illustrated by their significant decrease, comparing with the NAFLD untreated rats group (G2). Similarly, GSPE (25 or 200 mg/kg/b.wt. /day) decreased the plasma triacylglycerols level by 28% or 25%, respectively in obese rats (Pascual-Serrano et al., 2018). GSPE treatment reduced adiposity, plasma triacylglycerols, and oxidative stress in the liver (Leonetti et al., 2018), in which the most recognized mechanism of action include inhibition of LPO and avoiding the associated ROS production (Rodríguez-Pérez et al., 2019).

Moreover, administration of Clopidogrel with GSPE co-treatment to the NAFLD-induced rats (G3), showed significant decrease in the serum levels of triacylglycerols and total cholesterol, compared to the NAFLD untreated rats (G2). The obtained data agree with Hadi et al. (2013), who observed non-significant decrease of serum triacylglycerols and total cholesterol concentrations after Clopidogrel treatment in comparison with atherogenic control rabbits fed on HFD. Referring that antithrombotic treatment slows down progression of acute or chronic liver failure in animal models (Fujita et al., 2008), the clinical benefit of Clopidogrel may be attributed to improvement of oxidative stress and reducing of inflammation (Ramadan et al., 2014).

The obtained data shown a significant increase in serum ferritin concentration in the NAFLD-induced rats (G2) as compared to control (G1). Similarly, Ryan et al. (2018) stated that, serum ferritin was higher in NAFLD patients compared to controls. Ferritin plays a pro-inflammatory role during the progression of the liver disease, in which its elevated serum level is related to IR and hepatocyte damage (Du et al., 2017).

Furthermore, a clear protective role was observed following administration of GSPE to NAFLD induced rats (G3), compared to the NAFLD untreated group (G2), clarified by the significant decrease in serum ferritin concentration. Likewise, Lafay et al. (2009) showed that, after supplementation of grape extract (400 mg) ferritin were significantly decreased in male athletes. Regarding that high iron stores is reflected by increased circulating ferritin (Basuli et al., 2014), the antioxidant property of GSPE is closely related to its iron-chelating effects (Bagchi et al., 2014), and/or its ability to directly scavenge free radicals, or indirectly modulating pro-oxidant enzymes (Fraga and Oteiza, 2011).

Additionally, GSPE and clopidogrel co-treated NAFLD induced rats (G4) also showed significant improvement of ferritin concentrations, comparing with the NAFLD untreated rats (G2). Clopidogrel can ameliorate the endothelial dysfunction (Ramadan et al., 2014), caused by release of ROS (Ostad et al., 2011), in which toxic effect of increased iron resulted in altering of endothelial function and decreasing of vascular reactivity (Sullivan, 2005).

The obtained qPCR results in table (3) elucidate significant upregulation of the hepatic expression levels of the inflammatory mediators VEGF, PDGF $\alpha$  and MAPK in the NAFLD-induced rats (G2), in comparison with the control (G1). Similarly, Miura et al. (2019) observed that, fat overload by HFD increase liver Kupffer cells and macrophages VEGF expression. Moreover, when liver

damage occurs, PDGF may be highly-expressed in activated hepatic stellate cells (HSC), injured endothelial cells and macrophages (Ying et al., 2017). Furthermore, Yan et al. (2017) had stated that, obesity promotes NAFLD, in a rat model of NAFLD, by over-expression of c-Jun NH2-terminal kinase (JNK)-1. VEGF may be used as an independent predictor of NAFLD (Wu et al., 2015), as it is a key factor in angiogenesis and tissue remodeling, and has a role in IR and inflammation, that are important characteristics in the development of NAFLD (Hong et al., 2019). Additionally, VEGF and PDGF are produced and released by several liver cells during chronic liver diseases progression (Novo et al., 2014), in which binding of PDGF to their receptors- $\alpha$  and - $\beta$  activates predominantly the Ras-MAPK pathway leading to the cellular reactions and molecular changes (Ying et al., 2017). Actually, the MAPK signaling pathway plays a key role in the NAFLD development (Ye et al., 2019), and MAPKs participate in the hepatic metabolism process, notably, the hepatic MAPKs are activated by stress responses, as p38 MAPKs and JNKs can be activated by ROS (Lawan and Bennett, 2017).

Interestingly, treatment of NAFLD-induced rats with GSPE resulted in significant down-regulation of the hepatic expression levels of VEGF, PDGF $\alpha$  and MAPK (G3), compared to the NAFLD untreated rats (G2). Likewise, Huang et al. (2012) had reported that GSPE inhibit VEGF, as one of multiple modulating signaling pathways to exhibit anti-angiogenic effects against cancer. Also healing repair effects by GSPE, via VEGF expression blocking can be a possible mechanism that can be mediated through inhibition of protein kinase (Akt) activation (Lu et al., 2009), and the inhibitory effect of PDGFR signaling by procyanidin-B2 may be via inhibition of ligand binding to the receptor and/or inhibition of the receptor's intrinsic tyrosine kinase activity (Rosenkranz et al., 2002), namely  $\alpha$ - and  $\beta$ -PDGF receptors (Rosenkranz and Kazlauskas, 1999). Additionally, GSPE pre-treatment suppressed the activation of Akt and MAPKs (JNK, Extracellular signal-regulated kinase (ERK) and p38) (Lee et al., 2017). In fact, GSPE may be a novel treatment of hepatic inflammation, exerting its anti-inflammatory effects by the attenuation of MAPK, via suppression of the JNK, and nuclear factor- $\kappa$ B (NF- $\kappa$ B) signaling pathways, in which MAPK/ERK-nuclear factor erythroid 2-related factor (Nrf2) is crucial to proanthocyanidin-mediated antioxidation and anti-inflammation (Nie and Stürzenbaum, 2019).

Furthermore, GSPE and clopidogrel co-treatment to the NAFLD-induced rats (G4) showed significant down-regulation of VEGF, PDGF $\alpha$  and MAPK hepatic expression levels, comparing with the NAFLD untreated rats (G2). Similarly, Coimbra et al. (2014) clarified that, Clopidogrel enhanced angiogenesis by reducing PDGF expression during periodontal repair, thereby, reducing the inflammation. Moreover, Clopidogrel can directly reduce the synthesis of VEGF (Kou et al., 2018). Interestingly, transforming growth factor (TGF $\beta$ ) pathway can mediate the activation of the JNK and p38/MAPK pathways (Papageorgis and Stylianopoulos, 2015), and the inhibition of MAPK cascade leads to reduction of JNK activity, which may improve IR (Prattali et al., 2005), thereby the down-regulation of TGF $\beta$  by Clopidogrel treatment (Joshi et al., 2016) in NAFLD may explain its positive effect. Additionally, Clopidogrel exerts beneficial vascular effects via the marked oxidative stress, reduction and abolishing of the vascular inflammatory responses and remodeling (An et al., 2018).

## 5. CONCLUSIONS

It could be concluded that, treatment with GSPE or with antiplatelet drug alleviates the hepatic oxidative stress and inflammation associated with NAFLD clarified by improving hyper-lipidemia and reduction of the inflammatory mediators. In fact, GSPE may be a novel treatment of hepatic inflammation in fatty liver disease, exerting its anti-inflammatory effect by the attenuation of MAPK, through suppression of the VEGF and PDGF $\alpha$  signaling pathways.

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