

Role of Grape Seed Proanthocyanidins in the Suppression of High Calorie Diet-Induced Hepatic Injury and Apoptosis

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Abstract: *Hepatocyte oxidative stress and apoptosis are considered to be the key components in the pathogenesis of nonalcoholic steatohepatitis. In this study we investigated the effect of grape seed proanthocyanidins (GSP) and metformin (MET), alone and in combination, on high fructose/fat diet (HFFD) induced steatohepatitis, with focus on oxidative stress and apoptosis markers. NASH was induced in male albino Wistar rats by feeding HFFD for 45 days. Either GSP (100 mg/kg b. w), MET (50 mg/kg b. w) or both were administered as therapeutic options. HFFD-feeding caused increase in lipid peroxides, protein carbonyls, proapoptotic proteins, (bax, caspases 3 and 9) and decline in anti-apoptotic protein (bcl2). Further, growth and DNA damage (GADD) 45 β , an NF- κ B regulated anti-apoptotic factor, was also reduced in HFFD-fed rats. These changes were reversed more effectively by GSP administration than by MET. Combined administration of GSP and MET showed synergistic effect in improving cell survival and hence could be considered for controlling HFFD-induced hepatocyte apoptosis.*

Keywords: Grape seed proanthocyanidins, hepatic injury, oxidative stress, apoptosis.

1. Introduction

The key events that contribute to the initiation and progression of NAFLD are summarized in a “multi-hit” model of NASH pathogenesis by Polyzos and others [1]. According to this model, dysregulated metabolism of free fatty acids (FFAs) is considered to be the “first-hit” of NAFLD pathogenesis, which leads to insulin resistance and fat accumulation in the liver. Inflammatory response, oxidative stress, and apoptosis, serve as “following-hits” that contribute to the ongoing inflammation that leads to non-alcoholic steatohepatitis (NASH). Emerging data suggest that oxidative stress and apoptosis plays a critical role in NAFLD-induced liver injury and in the progression from steatosis to NASH and then to cirrhosis [2, 3]. Moreover, the degree of apoptosis is closely associated with the severity of NASH and the stage of fibrosis [4]. Thus, inhibition of oxidative stress and apoptosis in the liver may be a useful treatment strategy of NAFLD.

Apoptosis is a morphologically distinct, gene-directed form of cell death characterized by cytoplasmic fragmentation and nuclear condensation that contributes to both physiological and pathological processes [5]. Emerging evidence suggest that high rate of apoptotic response is associated with oxidative stress occurring in NASH [6]. The process of apoptosis is regulated by several proteins with either inhibiting or promoting actions. Bcl2 family of proteins that reside in mitochondria are the key players in apoptosis. Bax protein, a member of the bcl-2 family acts as proapoptotic factor and promotes apoptosis whereas bcl2 protein inhibits apoptosis. Bcl-2 is a potent, evolutionarily conserved, anti-apoptotic protein which inhibits cell death by reducing the generation of reactive oxidants, which are requisite for the completion of the apoptotic program [7]. The ratio of bax to bcl-2 determines the susceptibility of a cell to apoptosis [8]. Bcl2 is transcriptionally regulated by nuclear factor-kappa B

(NF- κ B). The anti-apoptotic/ the protective effect of NF- κ B has been proposed to result from the inhibition of JNK signaling by an NF- κ B responsive gene product, growth and DNA damage (GADD)45 β [9].

Several protease families are implicated in apoptosis, the most prominent being caspases [10]. Opening of mitochondrion permeability transition pores due to oxidative stress results in activation of procaspase 9 to caspase 9 which then activates its key downstream molecule caspase 3 for executing apoptosis. The proapoptotic factor bax is responsible for the conversion of procaspase 9 to caspase 9 [11]. Recent evidence suggests that the antiapoptotic factor bcl-2 is a downstream death substrate for caspases and is inactivated by caspases [12].

Increased apoptosis has been suggested in animal models of NASH [6] and NASH patients [13]. However, role of bax and bcl-2 as a causative factor in diet induced NAFLD have not been studied. Management of apoptosis is considered as an effective way to treat NAFLD. Grape seed proanthocyanidins (GSP) is known well for its antioxidant potential and has been investigated to combat various disease conditions including insulin resistance. In this study, we have investigated the alterations of bax/bcl-2 ratio in relation to changes in the apoptosis co-ordination enzymes, caspases-3 and 9, in the liver of rats with NAFLD. Also, we investigated the effect of GSP, a potent antioxidant and metformin (MET), a potent insulin sensitizer, alone and in combination on oxidative stress and apoptosis markers on HFFD-induced NAFLD rats.

2. Methodology

2.1 Animals Diet and Experimental design

Albino male Wistar rats of body weight 120-150 g were obtained from and maintained at the Central Animal House, Department of Experimental Medicine, Rajah Muthiah Medical College and Hospital, Annamalai Nagar. The animals were housed under a controlled temperature (22±2 °C) without limitation of access to water and chow. The study was conducted in accordance with the guidelines of the Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA). All procedures were approved by the Institutional Animal Ethical Committee (IAEC).

The animals were fed with either normal rat chow (control) or high fat-fructose diet (HFFD). Normal chow consisted of 60% starch, 22.08% protein and 4.38% fat, the caloric content of which was 382.61 cal/100 g. HFFD was prepared fresh in our laboratory every week which provided 471.25 cal/100 g. HFFD contained 45% fructose, 20% fat (10% beef tallow, 10% groundnut oil) and 22.5% casein.

A total of 6 groups were treated as follows: Group 1 were fed with normal rat chow (CON), group 2 were fed with HFFD, group 3 were fed with HFFD and supplemented with GSP by gastric intubation (100 mg/kg b. w/day, HFFD+GSP), group 4 rats were fed with HFFD and administered with MET through gastric intubation (50 mg/kg b. w/day, HFFD+MET), group 5 rats were fed with HFFD and administered GSP first followed by MET at an interval of 3-4 hrs once daily (HFFD+GSP+MET) through gastric intubation, and group 6 rats were fed with normal rat chow and administered GSP (CON+GSP). Food and water were provided ad libitum to the animals. The total experimental duration was 45 days. GSP and MET were given for the last 15 days of the experimental period. At the end of experimental period, the animals in each group (n=6) were fasted overnight and sacrificed by cervical decapitation to obtain blood and liver. Samples were stored at -80°C until further analysis.

2.2 Liver injury markers and oxidative stress

To assess the liver injury, activities of aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), gamma glutamyltransferase (GGT) and bilirubin levels were assayed using kits obtained from Agappe diagnostics, Kerala, India. Oxidative stress markers like thiobarbituric acid reactive substance (TBARS), lipid hydroperoxides (LHP) and protein carbonyl (PCO) were measured in plasma according to respective standard procedures [14, 15, 16]

2.3 Immunoblotting

Homogenates containing equal amounts of protein were resolved by 8-12% SDS-PAGE and processed for Western blot and electrotransferred onto polyvinylidene fluoride membranes. The membranes were then incubated overnight at 4°C with antibodies specific to bax, bcl2, caspase 3 and 9. The membranes were washed with TBST and incubated with

respective secondary antibody for 2 h at room temperature. Protein band detection was performed by enhanced chemiluminescence assay (Thermo Scientific Super Signal West Pico chemiluminescent substrate, Rockford, USA) as per the manufacturer's instructions. Blots were subsequently stripped, reprobed and processed for visualizing β -actin. The band density was normalized with that of β -actin. Quantitative comparisons of protein expression between various groups were performed using Image J software (from US National Institutes of Health).

2.4 qPCR

Total cellular RNA was extracted from liver using TriZol reagent. The concentration and purity of RNA preparation were checked by measuring the absorbances at 260 and 280 nm. Total RNA (2.0 μ g) was reverse transcribed to cDNA in a reaction mixture containing 1 μ l of Oligo (dT) primer (0.2 μ g/ml), 1 μ l of RNase inhibitor (10 U/ml), 1 μ l of 0.1 M DTT, 4 μ l of RT Buffer (5X), 2.0 μ l of 30 mM dNTP mix (7.5 mM each), 0.5 μ l of M-MuLV Reverse Transcriptase (50 U/ μ l) and made up to 20 μ l with sterile water and kept at 37°C for 1 h and then heated at 95°C for 2 min. PCR amplification was performed in a mixture containing 100 μ g cDNA, 1 μ l each of 50 pM GADD45 β forward (3'-gctggccatagacaagaag-5') and reverse (3'-gagttcggtagggagcctct-5') primer (Sigma Aldrich, St Louis, MO, USA), 10 μ l SYBR green master mix with a final volume of 20 μ l made up using nuclease free water. The thermocycling conditions were as follows, initial denaturation 95°C for 10 min, denaturation 95°C for 15 s, annealing 60 ± 3°C for 30 s and extension 72°C for 30 s for 40 cycles. The reactions were run in triplicate for each sample. The Ct values obtained for each gene was normalized with that of GAPDH gene using the formula $2^{-\Delta\Delta Ct}$. The relative quantity was expressed in bar graphs as fold change with respect to control after normalization with GAPDH for each gene.

2.5 Histology

Histologic analysis of liver were performed after liver tissue samples were fixed at room temperature in formalin and embedded in paraffin. The liver was sectioned using a microtome (3-5 μ m) and mounted on glass slides,

Table 1: Activities of liver function enzymes in experimental animals

Parameters	CON	HFFD	HFFD+GSP	HFFD+MET	HFFD+GSP+MET	CON+GSP
AST (IU/L)	44.6±2.44 _a	120.9±8.96 ^b	64.8±3.95 ^c	78.6±4.30 ^d	53.9±3.27 ^e	45.3±2.51 _a
ALT (IU/L)	53.8±3.02 _a	101.5±5.00 ^b	71.6±4.43 ^c	86.5±3.89 _d	62.5±5.44 _e	52.5±2.88 _a
ALP (IU/L)	39.1±2.05 _a	155.1±10.5 ^b	75.1±4.30 ^c	92.3±5.88 _d	58.5±4.54 _e	36.6±2.21 _a
GGT (IU/L)	21.8±1.30 _a	45.1±2.64 ^b	32.6±1.39 ^c	36.1±1.72 _d	29.6±2.09 ^c	21.6±2.05 _a
Bilirubin (mg/dL)	0.66±0.04 _a	3.2±0.12 ^b	1.18±0.06 ^c	2.01±0.09 _d	0.99±0.04 _e	0.65±0.04 _a

CON- Control, HFFD- High fat, fructose diet, HFFD+GSP- High fat, fructose diet + grape seed proanthocyanidins, HFFD+MET - High fat, fructose diet + metformin, HFFD+GSP+MET - High fat, fructose diet + grape seed proanthocyanidins + metformin, CON+GSP - Control +

grape seed proanthocyanidins. Values are means ±SD of 6 rats from each group. Values that bear different alphabets in their superscript differ significantly from each other. One way ANOVA followed by TMRT. A value with p<0. 05 is considered statistically significant.

Table 2: Levels of oxidative stress markers in plasma of experimental animals

Parameters	CON	HFFD	HFFD+GSP	HFFD+MET	HFFD+GSP+MET	CON+GSP
TBARS(A)	0. 96±0. 03 ^a	3. 65±0. 19 ^b	1. 68±0. 07 ^c	2. 79±0. 13 ^d	1. 16±0. 13 ^e	0. 89±0. 02 ^a
LHP(B)	0. 99±0. 02 ^a	2. 92±0. 13 ^b	1. 36±0. 05 ^c	2. 12±0. 11 ^d	1. 05±0. 11 ^e	0. 91±0. 04 ^a
PCO(C)	2. 16±0. 10 ^a	8. 19±0. 57 ^b	4. 65±0. 18 ^c	6. 81±0. 58 ^d	4. 26±0. 39 ^e	2. 21±0. 12 ^a

Comparisons and significance as given in Table 1. A= μmoles/dL; B= nmoles/dL; C=nmol/dL.

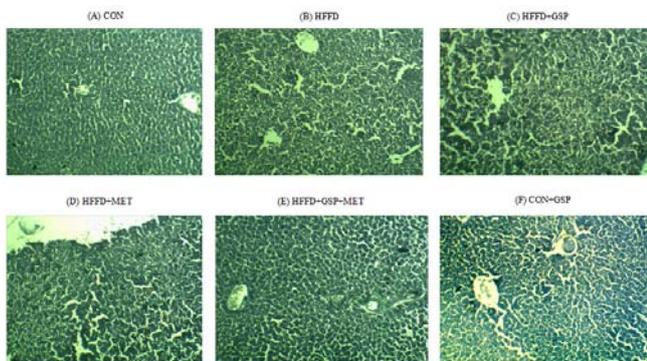
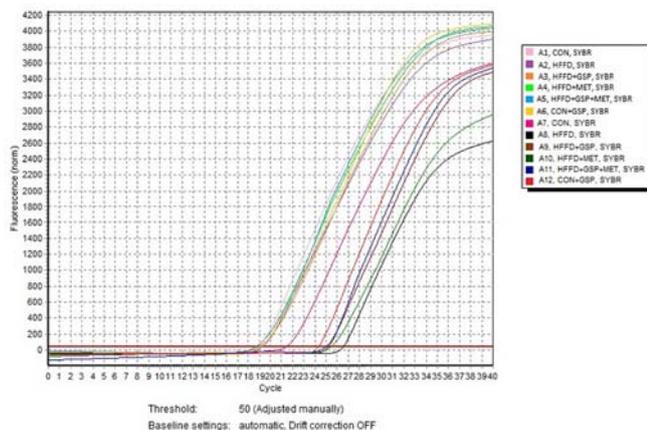
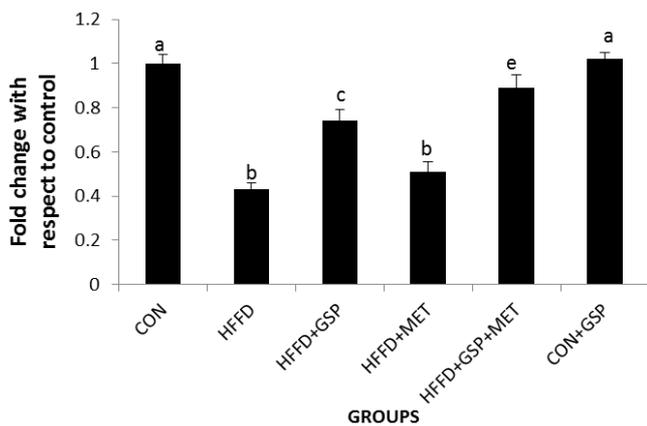


Figure 1: Histology Using Reticulin



(A)



(B)

Figure 2: mRNA expression of GADD45β, (A) qPCR qualification graph. (B) Fold change with respect to control calculated using. Data are expressed as means + S. D. of 4 rats from each group. Statistical significance between the groups, denoted by different alphabets, was determined by one-way ANOVA of significance set at P<0. 05

2. 6 Statistical analysis

Values are presented as means ± SD of 6 rats for biochemical analysis and 4 rats for histology, qPCR and immune blotting analysis. Data analysis was performed with the use of SPSS statistical software 17. 0. The statistical significance of differences between groups was determined by one-way analysis of variance (ANOVA). ANOVA followed by the Turkey’s multiple comparison tests (TMRT). Value with p<0. 05 was considered statistically significant.

3. Results

3. 1 Liver injury markers

The activities of AST, ALT, ALP and GGT and the level of bilirubin were found to be significantly increased in the HFFD rats when compared with that of CON and treatment groups. All the three treatment groups showed significant reduction of injury markers of which the combination treatment showed the maximum reduction compared to that of HFFD group of rats. The values did not differ significantly between CON and CON+GSP groups (Table 1).

3. 2 Oxidative stress markers

The levels of TBARS, LHP and PCO in plasma were found to be significantly increased in HFFD fed rats, compared to CON rats. GSP and MET significantly reduced these oxidative stress markers compared with HFFD. Combined administration of GSP and MET showed better reduction in oxidative stress than individual treatments (Table 2).

3. 3 Histological analysis of fibrosis

Figure 1 depicts the histological sections of liver upon reticulin staining for observing fibrotic changes. Fig. 1 (A) and (F) represent sections from CON and CON+GSP respectively showing normal network of reticulin between hepatocytes. HFFD fed rats show severe changes with largely accumulated reticular fibers along the cells (B). GSP administration to HFFD fed rats reduced these fibers effectively as compared to HFFD fed rats (C). (D) represents liver sections from HFFD+MET group of rats showing moderate deposition of reticulin. HFFD+GSP+MET treatment have reduced the deposition of fibrils to near normal range (E).

3. 4 mRNA expression of GADD45β

mRNA expression analysis of the gene GADD45β showed decrease in HFFD fed rats compared to CON rats. HFFD fed rats showed decrease by 0. 43 fold compared to CON. All the three treatments showed improved expression of GADD45β

(0.74 fold by GSP, 0.51 fold by MET and 0.89 fold by GSP+MET administration) (Figure 2).

3. 5 Protein expression of bax and bcl2

Bax, the proapoptotic marker of bcl2 family was found to be increased by 3.17 fold in HFFD fed rats compared to CON rats. GSP, MET and combined treatment reduced the expression as compared to HFFD but the levels were higher by 2.07, 2.73 and 1.42 fold respectively compared to CON. Meanwhile, the anti-apoptotic marker bcl2 was decreased by 0.5 fold upon HFFD feeding considering CON as 1. All the three treatment groups showed increased expression as compared to HFFD. HFFD+GSP, HFFD+MET and HFFD+GSP+MET group of rats showed an near normal expression (0.76, 0.63 and 0.88 folds respectively) compared to CON group of rats (Figure 3B).

3. 6 Bax/Bcl2 ratio

Bax/Bcl2 ratio was calculated to find out the susceptibility of the cells to apoptosis. HFFD fed rats showed maximum susceptibility to apoptosis with an increase of 6.34 fold expression considering control as 1. GSP, MET and combination reduced this susceptibility and showed an expression level of 2.76, 4.33 and 1.61 folds respectively (Figure 4)

3. 7 Protein expression of caspase 3 and 9

HFFD fed rats showed significant increase in the expression of caspase 3 (Figure 5A) and 9 (Figure 5B) to 2.78 and 3.21 fold respectively as compared with CON. Supplementation of GSP, MET and combination treatment reduced the expression of caspase 3 to 1.51, 1.84 and 1.25 fold respectively and caspase 9 by 1.77, 2.58 and 1.43 fold respectively compared to CON.

4. Discussion

Our study reports for the first time that hepatocyte apoptosis is induced in rats by HFFD feeding in rats. HFFD consumption resulted in increased proapoptotic Bax, decreased anti-apoptotic Bcl2, which further increased the expression of caspase 3 and 9. GADD45 β , an NF- κ B regulated anti-apoptotic factor was found to be reduced indicating that NF- κ B has been switched to proapoptosis mode. Upon GSP administration, expression of Bax was decreased with a simultaneous improvement in Bcl2 expression. MET was less efficient than GSP in shifting this balance but showed significant improvement towards anti-apoptosis. Combined administration of GSP with MET shown synergistic effects in improving the cell survival rate as evidenced by the decreased Bax/Bcl2 ratio.

Consumption of westernized diets high in fructose and fat leads to severe oxidative injury that culminates in apoptosis. Many studies in animal models have proved that feeding high fructose and/or fat diet for over a month results in hepatic steatosis which further progresses to steatohepatitis with the involvement of cellular injury and apoptosis [6, 17]. Liver injury on marker enzymes (AST, ALT, ALP and GGT) and

bilirubin were higher in plasma of HFFD-fed rats compared to control diet fed rats. Administration of GSP and MET, Either alone or in combination reduced the levels of these markers in plasma suggesting that cellular injury has been repaired.

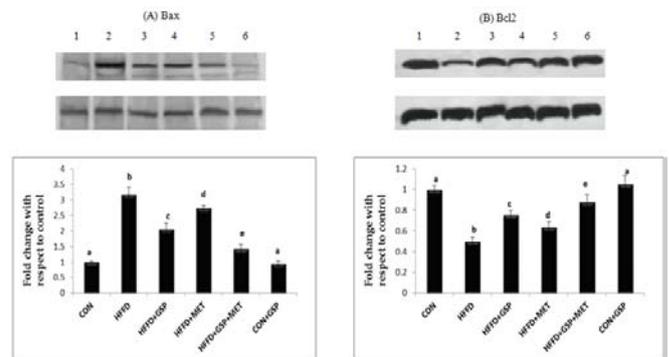


Figure 3: Protein expression of Bax and Bcl2. Lane 1-CON, Lane 2- HFFD, Lane 3- HFFD+GSP, Lane 4- HFFD+MET, Lane 5- HFFD+GSP+MET, Lane 6- CON+ GSP. Data are expressed as means + S. D. of 4 rats from each group.

Statistical significance between the groups, denoted by different alphabets, was determined by one-way ANOVA of significance set as P<0.05

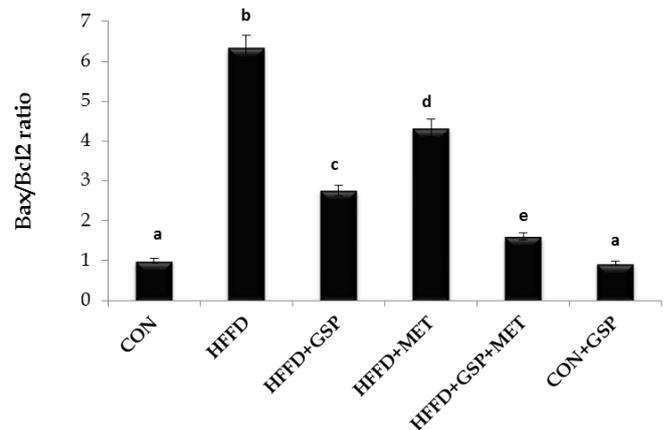


Figure 4: Effect of GSP and MET on Bax/Bcl2 ratio. Data are expressed as means + S. D. of 4 rats from each group.

Statistical significance between the groups, denoted by different alphabets, was determined by one-way ANOVA of significance set at P<0.05

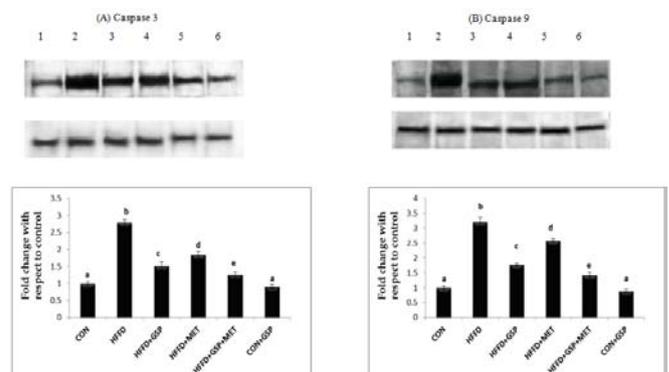


Figure 5: Effects of GSP and MET on protein expression of caspase 3 and 9 in liver of experimental animals. Lane 1- CON, Lane 2- HFFD, Lane 3- HFFD+GSP, Lane 4- HFFD+MET, Lane 5- HFFD+GSP+MET, Lane 6- CON+ GSP. Data are expressed as means + S. D. of 4 rats from each

group. Statistical significance between the groups, denoted by different alphabets, was determined by one-way ANOVA of significance set at $P < 0.05$

Oxidative insults emanating from within the cell can threaten homeostasis if they are not appropriately resolved and hence prolonged oxidative injury is usually followed by apoptosis. Excessive levels of ROS can lead to cellular injury and death by altering cellular macromolecules including DNA, proteins and lipids. In the present study, HFFD feeding resulted in lipid and protein damage as evidenced by increased peroxidative products like TBARS, LHP and PCO. Results obtained with respect to treatment studies show that all the three treatments are effective in removing the oxidative insult and to protect the cells from further injury and apoptosis.

In the current study, there is a strong increase in bax/bcl-2 ratio that favors apoptosis with advancing NAFLD. The functionally linked enzyme to apoptosis, caspase-3, was also increased upon HFFD feeding. These results showed that in NAFLD induced by HFFD feeding, caspase-3 may activate cell death via the mitochondrial signals, i. e., bax activation and bcl2 suppression. GSP and MET inhibit the activation of bax and also improve expression of bcl2 thus decreasing the bax/bcl2 ratio. This was accompanied by decreased caspase 3 and 9 activation and thus GSP and MET act by suppressing the mitochondrial triggered apoptosis.

NF- κ B/Rel transcription factors are also involved in controlling apoptosis. Inactive form of NF- κ B resides in the cytosol of resting cells translocates to nucleus upon activation by various factors like oxidative stress and induce the transcription of various proteins involved in both pro and anti-apoptosis [18]. The anti-apoptotic activity of NF- κ B involves suppression of c-Jun N-terminal kinase (JNK). GADD45 β , one of the direct targets of NF- κ B, binds directly to a JNK-inducing kinase MKK-7 and blocks the catalytic activity of MKK-7 which is necessary for activation of JNK [19]. HFFD feeding caused decrease in the mRNA expression of GADD45 β indicating that anti-apoptotic effect of NF- κ B is suppressed by HFFD feeding. Interestingly, GSP significantly improved the expression of GADD45 β . Combined administration of both has showed synergistic effects. These results suggested that GSP reduces JNK mediated killing.

Matrix accumulation or fibrosis of the liver occurs in response to activated stellate cells that produces matrix molecules in response to oxidative stress and inflammation [20]. Increased matrix accumulation leads to cirrhosis, a condition that reflects unsuccessful repair of NASH. In our study, HFFD feeding to rats for 45 days resulted in increased fibrous network along the lining of hepatocytes which was significantly reduced in all three treatment groups.

In conclusion, the key findings of our study are that HFFD induces liver cell injury and apoptosis that can be alleviated by GSP and MET. Decrease in the expression of bax/bcl2 ratio and the suppression of caspases 3 and 9 could be the mechanisms involved. GSP also improved GADD45 β , a NF- κ B regulated anti-apoptotic protein factor showing that GSP acts via NF- κ B signals to suppress apoptosis. On the other hand, MET reduces the bax/bcl2 ratio significantly but not as

effectively as GSP. The mild suppression of GADD45 β induced by MET was also not significant. Hence it is evident that MET induces cell survival by inducing bcl2 and not by GADD45 β . Combination of GSP and MET showed maximum improvement in cell survival by reducing bax/bcl2 ratio and by improving GADD45 β . Combined administration of GSP and MET holds better promise for developing anti-apoptotic agents against NASH, but the underlying mechanisms are yet to be determined.

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