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Current concepts in pathophysiology and management of hepatocellular carcinoma

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Additional approaches to control malignancies are needed due to the emerging trends in the incidence of cancer of different organ sites. Due to the high frequency of hepatocellular carcinoma (HCC) and its poor prognosis, preventing HCC is an urgent priority. To explore the antioxidant and apoptotic pathways of grape seed extract (GSE) we induce HCC experimentally by diethylnitrosoamine (DEN) and treated the animals with low and high doses of GSE. The results indicate good therapeutic possibilities for GSE use in treatment of HCC., This was evidenced *via* regression of liver enzymes' function (ALT&AST), the HCC markers; α-fucosidase, α-fetoprotein and carcinoembrionic antigen (CEA) in HCC groups treated with the grape seed extract. Also, tumor necrosis factor (TNF-α) showed a significant decrease using GSE in HCC bearing animals. Regarding the apoptotic pathways of GSE, we found a significant down regulation of apoptosis enhancing nuclease (Aen), Bcl2-associated X protein (Bax), B-cell translocation gene 2(Btg2), Cyclin G1 (Ccng1) and Cyclin-dependent kinase inhibitor 1A (Cdkn1a) gene expression in HCC+GSE groups as compared to HCC bearing group. In the same trend, the necrotic/apoptotic rates were significantly higher in the HCC groups treated with GSE *vs*. the HCC bearing group. Finally, the 8-OHdG/2-dG generation decreased by 73.8% and 52.9% in HCC+GSE at low and high doses, respectively. Based on these encouraging observations, grape seed extract could be a promising natural remedy for attenuating hepatocellular carcinoma that has a great future in approaches directed towards control of HCC.

Key words: hepatocellular carcinoma, grape seed extract, apoptosis, antioxidant, HCC pathophysiology

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INTRODUCTION

An increased prevalence of hepatitis C virus (HCV) infections leads to increasing occurrences of hepatocellular carcinoma (HCC) (El-Serag, 2002). In Egypt, the prevalence of HCV infection among general population has been estimated to be around 14% (Heintges & Wands, 1997).

Hepatocarcinogenesis is a long-term, multistep process, and is associated with changes in gene expression profiles. There is a set of gene markers for tracking the progression of hepatitis C virus-induced liver carcinogenesis (Wurmbach *et al.,* 2007). Additionally, Liu and coworkers (2009) found that an expression of a total of 694 genes, especially the inflammatory response, apoptosis, immune response, and oxidative stress metabolism-related genes, was significantly changed during the development of diethylnitrosamine (DEN)-induced liver cancer. These studies could help to investigate the pathogenesis of hepatic cancer at the molecular level.

Natural products extracted from plants or traditional Egyptian medicine, exhibit a variety of biological activities. Many of them might be viable options for the treatment of liver dysfunctions. Grape seed extract (GSE) has been found to be a powerful free radical scavenger and possesses a broad spectrum of pharmacological, biological, and therapeutic effects (Li *et al.,* 2012). Grape seed extract accumulates a wide range of flavonoids including anthocyanins, flavan-3-ols, flavonols, and stilbenes, that are biologically active (Jo *et al.*, 2005). Epidemiological studies support a health benefit of polyphenol-rich fruits consumption in lowering cancer risk (Bianchini & Vainio, 2003). An *in vivo* study showed that resveratrol, a recognized chemopreventive agent present in grapes and wine, inhibited later stages of carcinogenesis in hepatoma-bearing rats (Miura *et al.,* 2003). Tannins and procyanidins, that grape seeds are rich in, have been shown to have an *in vivo* activity against prostate cancer in mice (Singh *et al.,* 2004). Selective cytotoxicity has been also observed towards various human cancer cell lines including breast, lung, prostate and leukemic cells (Chen *et al.*, 2005), and while protecting normal human gastric mucosal and murine macrophage cells (Ye, 1999).

In addition, Kweon and coworkers (2003) suggested that the reduction of fatty acid synthase activity in liver by grape seeds might be one of the contributing mechanisms of hepatic cancer prevention. In a cell culture study, grape seed extract was found to inhibit advanced human prostate cancer growth and to induce apoptosis (Dhanalakshmi *et al.,* 2003). Grape seed proanthocyanidin oligomers and polymers were

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Abbreviations: 2-dG, 2-deoxyguanosine; 8-OHdG, 8-hydroxy-2-deoxyguanosine; AO/EB, acridine orange/ethidium bromide; *ALP*, alkaline phosphatase; AFP, alpha-fetoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; Aen, apoptosis enhancing nuclease; Btg2, B-cell translocation gene 2; Bax, Bcl2-associated X protein; CEA, carcinoembrionic antigen; Ccng1, Cyclin G; Cdkn1a, cyclin-dependent kinase inhibitor 1A; DEN, diethylnitrosoamine; Doxo, doxorubicine; GSH, glutathione reduced; GPX, glutathione peroxidase; HCV, hepatitis C virus; HCC, hepatocellular carcinoma; GSE, grape seed extract; HPLC, high-performance liquid chromatography; MDA, malondialdehyde; NDEA, N-nitrosodiethylamine; PCR, polymerase chain reaction; ROS, reactive oxygen species; RT, Reverse transcription; TNF-α, tumor necrosis factor; TNF-α, Tumor necrosis factor alpha

more potent as antitumor promoters than green tea polyphenols, comprised of mostly monomers (Zhao *et al.,*1999). Both, grape seed proanthocyanidins and resveratrol, have demonstrated chemopreventive effects (Baliga & Katiyar, 2006).

Therefore, the goal of the present study was to evaluate the anti-tumor activity of grape seed extract against HCC induced experimentally through antioxidant and apoptotic mechanism. To achieve our goal, we performed several biochemical analyses, monitored gene expression levels of genes encoding apoptotic proteins, carried out apoptosis determination and assessment of 8-OHdG generation in hepatic cells in male rats.

MATERIALS AND METHODS

Preparation of grape seed extract. Dried grape seeds were powdered and underwent extraction with 80% methanol at room temperature for three times. The extracts were concentrated in a vacuum evaporator to obtain the crude aqueous methanolic extract (23% from the dried seeds).

Experimental animals. The current study was conducted on sixty male albino rats, the Sprague-Dawley strain, weighting 150–160 g. Animals were obtained from the Animal House Colony of the National Research Centre, Cairo, Egypt and acclimated for one week prior to the experiment. Animals were maintained under standard conditions of humidity (55%) and temperature (25±1) in a specific pathogen free barrier area. Rats had a free access to standard laboratory diet containing casein 10%, salt mixture 4 %, vitamin mixture 1%, corn oil 10 % and cellulose 5%, supplemented to 100 g with corn starch (AOAC, 1995). Treatment of animals and all experimental protocols were approved by the Ethical Committee of the National Research Center.

Experimental design. After the acclimatization period, rats were randomly divided into 5 groups (12 rats /group). Group (1), assigned as healthy control group received 1 ml of saline. Groups (2–5) received diethylnitrosamine DEN orally at a dose of 20 mg/kg body weight, five times a week during a period of 8 weeks, and 10 mg/kg body weight for another 7 weeks (total: 15 weeks) to induce hepatocellular carcinoma (HCC) (Karimov *et al*., 2003). Group (2) served as HCC bearing rats (positive control). Group (3) (HCC+GSE low) rats were orally treated with a low dose of grape seed extract, 2.25 mg/kg body weight, during the experimental period. Group (4) (HCC+GSE high) rats were orally treated with a high dose of grape seeds extract, 4.5 mg/kg body weight, during the experimental period. The selected doses of grape seed extract depend on the chronic toxicity study of GSE (unpublished data). Group (5) (HCC+Doxo), rats were orally treated with doxorubicine at a dose of 0.072 mg/rat which is equivalent to the human dose of 20 mg/m^2, according to Barnes and Paget (1965), once weekly for 3 weeks.

At the end of the experimental period, the rats were fasted overnight and subjected to diethyl ether anesthesia. Blood samples were immediately collected from the retro-orbital venous plexus in a tube free from any anticoagulant agent for separation of serum samples for biochemical analysis. Then the rats were sacrificed by cervical dislocation and the liver was dissected out and preserved at –80°C for molecular genetic analysis.

Biochemical analyses. In routine liver functions test, ALTand AST were assayed according to Reitman and Frankel (1957). Serum α-fetoprotein and α-fucosidase were measured according to the manufacturer instructions of assay kits from Glory Science Co., TX, USA. Serum carcinoembryonic antigen (CEA) level was detected by ELISA technique using CEA assay kit according to the method of Schwartz (1987). Furthermore, TNF-α was detected by using ELISA procedure as described by Corti and coworkers (1992).

Isolation of total RNA. Isolation of total RNA was carried out from liver tissue of male rats by the standard TRIzol® Reagent extraction method (Invitrogen, Germany).

Total RNA was treated with 1 U of RQ1 RNAse-free DNase (Invitrogen, Germany) to digest residual DNA, and then re-suspended in DEPC-treated water. Determination of the purity of the total RNA was made by the 260/280 nm ratio (between 1.8 and 2.1). Moreover, RNA integrity was assessed with ethidium bromide-stain analysis of 28S and 18S bands by formaldehyde-containing agarose gel electrophoresis. Thus obtained RNA aliquots were used immediately for reverse transcription (RT).

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)-determination of expression of apoptosis genes (Aen, Bax, Btg2, Ccng1, Cdkn1a). Determination of rat cDNA copy number was assessed by QIAGEN's real-time PCR cycler (Rotor-Gene Q, USA). Primer sequences for Apoptosis enhancing nuclease (Aen, accession no: NM 001108487) were upstream: 5'-GCA CTG CAC AAT GAC TTC CAG-3'; and downstream: 5'-GCC AGG TCC TTA AGA GAG ACC C-3'), Bcl2-associated X protein (Bax, accession no: NM 017059) were upstream: 5'-GGC GAA TTG GAG ATG AAC TGG-3') and downstream: 5'-GTT GAA GTT GCC ATC AGC AAA C-3'), B-cell translocation gene 2, anti-proliferative (Btg2, accession no: NM 017259) were upstream: 5'-GAG AGG TGG CTC AAA GCT CCA G-3' and downstream: 5'-AGG ACC CAA CCG CAG GAA AG-3', Cyclin G1 (Ccng1, accession no: NM 012923) were upstream: 5'-TAA GGC AAA GCC TTC TGT GCT G-3'; and downstream: 5'-CTC GGC CAC TTA TCT TGG AAT G-3') and Cyclin-dependent kinase inhibitor 1A (Cdkn1a, accession no: NM 080782) were upstream: 5'-TTG TCG CTG TCT TGC ACT CTG G-3'; and downstream: 5'-GCG CTT GGA GTG ATA GAA ATC TG-3'). Normalization of the RT-PCR quantitative values of the mRNA of the above mentioned genes was done by dividing them by the expression values of the β-actin gene (β-actin-F: 5'-GTG GGC CGC TCT AGG CAC CAA-3', β-actin-R CTC TTT GAT GTC ACG CAC GAT TTC (Khalil & Booles, 2011)). Afterwards, a melting curve analysis was performed to test the quality of the specific primers.

Gene expression calculation. The efficiency (Ef) of the amplification was measured from the standard curve slope using the following formula found in the manufacturer's instruction pamphlet:

$$Ef=10^{-1/slope}$$

$$\text{Efficiency } (\%)=(Ef-1)\times 100$$

The relative quantification of the target to the reference was determined by using the $2^{-\Delta\Delta CT}$ method where Ef for the target (Aen, Bax, Btg2, Ccng1, Cdkn1a) and the reference primers (β-actin) were as follows:

$$\Delta C_{T(test)}=C_{T(target,\ test)}-C_{T(reference,\ test)},$$

$$\Delta CT(calibrator)=C_{T(target,\ calibrator)}-C_{T(reference,\ calibrator)},$$

$$\Delta\Delta CT=\Delta C_{T(Test)}-\Delta C_{T(calibrator)}.$$

The relative expression was calculated by $2^{-\Delta\Delta CT}$.

Apoptosis analysis-staining of hepatic cells with acridine orange/ethidium bromide. Apoptotic changes in hepatic cells were determined morphologically by fluorescent microscopy after labeling with acridine orange/ethidium bromide (AO/EB) according to Czene and coworkers (2002). Briefly, hepatic cells were washed with PBS, finely chopped and centrifuged at 7000 rpm for 5 min. The pellet obtained was suspended in trypsin-EDTA (0.25%, 53 mM) in PBS for 1 h at 37°C and smeared on clean glass slides. Finally, hepatic cell smears were air-dried and fixed in a solution of methanol/acetic acid (3:1). The slides were stained with 25 µl of the dye mixture (4 µg/ml AO and 4 µg/ml EB in PBS, pH 7.4). The hepatic cells observed were allocated to several categories in which living cells were classified by having a green nucleus; early apoptotic cells were classified by having a bright green nucleus with condensed or broken chromatin; late apoptotic cells were classified by having orange nuclei with condensed or broken chromatin; and necrotic cells were classified by having uniformly orange nuclei. In each group, a total of 100 cells were observed under fluorescent microscope using B2A filter and the apoptotic/necrotic ratio was calculated as a number of apoptotic and necrotic cells/total cell count (×100).

In addition, necrosis and necrotic index were measured according to Hoorens and Pipeleers (1999). The calculation of the indexes was performed using the following formula:

Necrosis index=[(% necrotic cells in test — % necrotic cells in control condition)/(100 — % dead cells in control condition)] 100%

The apoptosis index was obtained by replacing the percentage of necrotic cells by the percentage of apoptotic cells.

Assessment of DNA adducts (8-hydroxy-2-deoxyguanosine, 8-OHdG, and 2-deoxyguanosine, 2-dG). DNA was extracted from liver tissues to assess the 8-OHdG and 2-dG in rats. The tissues were homogenized in lysis buffer (1% sodium dodecyl sulfate, 10 mM Tris, 1 mM EDTA, pH 7.4), and incubated overnight with proteinase K (0.5 mg/mL) at 55ºC. Afterwards, the samples were incubated with RNase (0.1 mg/mL) at 50ºC for 10 min and the DNA was extracted with a mixture of chloroform/isoamyl alcohol. The DNA was precipitated using a mixture of 3 M sodium acetate and two volumes of 100% ethanol. To wash the DNA, the samples were treated twice with 70% ethanol, air-dried and dissolved in 100 µL of Tris/EDTA (10 mM Tris/1mM EDTA, pH7.4). DNA was digested as previously described (Khalil *et al.,* 2011; Girgis *et al.,* 2012). A high-performance liquid chromatography (HPLC) with a CoulArray system (Model 5600) was used to measure the 8-OHdG adducts. HPLC column C18 (260×4.6, particle size 5 µl) was used with mobile phase acetonitrile/methanol/phosphate buffer (25/10/965) v/v. The 8-OHdG and 2-dG adducts were detected using an electrochemical detector and phosphate buffer. The buffer was filtered 2 times before being used at a flow rate of 1 ml/min using electrochemical detector with cell potential of 600 mV.

Statistical analysis. All data were analyzed by one-way ANOVA and the significance of the differences between means was tested using Tukey's HSD (Honestly Significant Difference) test ($P<0.05$). The software used was SAS, Version 9.1 (Statsoft Inc.,Tulsa, USA). Values are expressed as means ± standard error.

RESULTS

Biochemical results

Data obtained from Table 1 illustrates that low and high doses of grape seed extract induced a significant decrease in ALT and AST levels as compared to positive control group ($P\leq0.05$). There was no significant difference between the two doses of grape seed extract used.

Groups of HCC animals treated with low and high doses of grape seed extract showed significant regression in α fucosidase and α fetoprotein levels as compared to HCC bearing group ($P\leq0.05$) as illustrated in Table 2. Also, it could be seen that the high dose of grape seed extract induced the lowest level of α fetoprotein.

Moreover, CEA and TNF-α concentration significantly decreased upon treatment of HCC bearing groups with low and high doses of grape seed extract ($P\leq0.05$), as illustrated in Table 3.

Results of gene expression analysis

The individual qPCR gene expression levels (Exp/Cont) were calculated for each group that included 12 rats. Expression of genes related to apoptotic cell cycle, such as Aen, Bax, Btg2, Ccng1 and Cdkn1a, was examined in liver tissues of rats induced to promote hepatocellular carcinoma (HCC) by diethylnitrosoamine (DEN).

The results indicate that expression of Aen, Bax, Btg2, Ccng1 and Cdkn1a genes in liver tissues of rats induced to promote hepatocellular carcinoma (HCC) by diethylnitrosoamine (DEN) treatment was significantly up-regulated compared to those in control healthy rats (Figs 1–5).

Table 1. Therapeutic effect of grape seed extract on liver enzymes' functions in hepatocellular carcinoma bearing rats. Data expressed as (mean ± S.E.) n=12

Parameters groups	AST (U/L)	ALT (U/L)
Healthy Control Group	140.43±5.80[b]	160.78±6.82
Cancer (HCC)	248.20±8.91[a]	238.40±10.04[a]
GSE (low) + HCC	152.42±15.38[b]	185.55±10.26[b]
GES (high) + HCC	148.40±17.50[b]	174.23±10.98[b]
Doxo +HCC	146.43±6.71[b]	166.76±11.92[b]

[a]Significant change at p< 0.05 in comparison with control group. [b]Significant change at $p<0.05$ in comparison with HCC induced group.

Table 2. Therapeutic effect of grape seed extract on α-fucosidase and α-fetoprotein in hepatocellular carcinoma bearing rats. Data expressed as (mean ± S.E.) n=12

Parameters Groups	α-Fucosidase (pg/ml)	α-Fetoprotein (ng/ml)
Healthy control group	76.2±0.84	25.65±0.632
Cancer (HCC)	97.7±1.98[a]	50.45±1.74[a]
GSE (low) + HCC	89.2±1.103[ab]	26.5±1.45[b]
GES (high) + HCC	78.4±1.79[b]	22.3±2.99[b]
Doxo +HCC	81.25±2.03[ab]	25.5±0.5[b]

[a]Significant change at $p<0.05$ in comparison with control group. [b]Significant change at $p<0.05$ in comparison with HCC induced group

Table 3. Therapeutic effect of grape seed extract on CEA and TNF-α in hepatocellular carcinoma bearing rats. Data expressed as (mean ±S.E.) n=12

Parameters groups	CEA (ng/ml)	TNF-α (ng/ml)
Healthy Control Group	3.21± .007	0.053 ± .0013
Cancer (HCC)	6.89 ± .237[a]	.087 ± .0053[a]
GSE (low) + HCC	4.32 ± .608[b]	0.062 ± 0 .0024[b]
GES (high) + HCC	3.92 ± .796[b]	0.058 ± .00249[b]
Doxo +HCC	4.256 ± .307[b]	0.049 ± .00221[b]

[a]Significant change at $p<0.05$ in comparison with control group. [b]Significant change at $p<0.05$ in comparison with HCC induced group

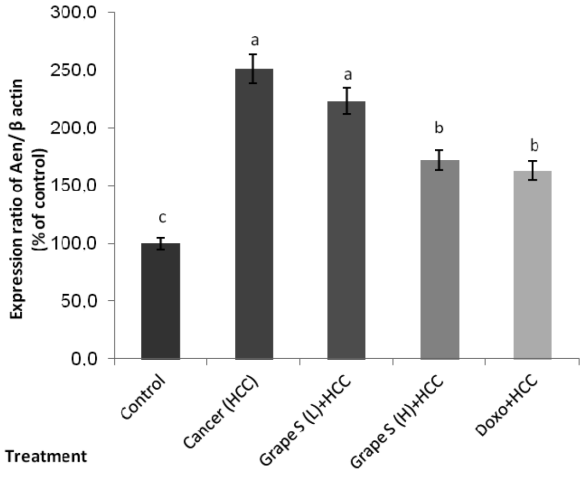

Figure 1. Semi-quantitative Real Time-PCR confirmation of Aen gene in liver tissues of HCC-rats treated with grape seed extract. "a" is significantly different from "b" and "c" ($p<0.05$).

The results showed that treatment of HCC bearing-rats with low dose of grape seed extract significantly decreased the expression of all examined genes when compared with those of HCC bearing-rats, except the Aen and Ccng1 genes where the concentration of Aen and Ccng1 mRNA was slightly decreased in HCC bearing-rats treated with low dose of grape seed extract (Figs 1–5).

Moreover, expression of Aen, Bax, Btg2, Ccng1 and Cdkn1a genes was significantly decreased in HCC bearing-rats treated with high dose of grape seed extract when compared with HCC induced-rats (Figs 1–5).

On the other hand, treatment of HCC bearing-rats with the Doxo drug significantly decreased the expression of Aen, Bax, Btg2, Ccng1 and Cdkn1a when compared with those in positive control rats. However, its effect on the mRNA of Aen, Bax, Ccng1 and Cdkn1a was similar to the effect of high dose of grape seed extract, while its effect on the mRNA of Btg2 was significantly lower than that of the high dose of grape seed extract (Figs 1–5).

Apoptosis determination

The alterations in the morphology of hepatic cells after double staining with AO/EB were investigated to assess the type of cell death induced by grape seed extract in HCC-rats.

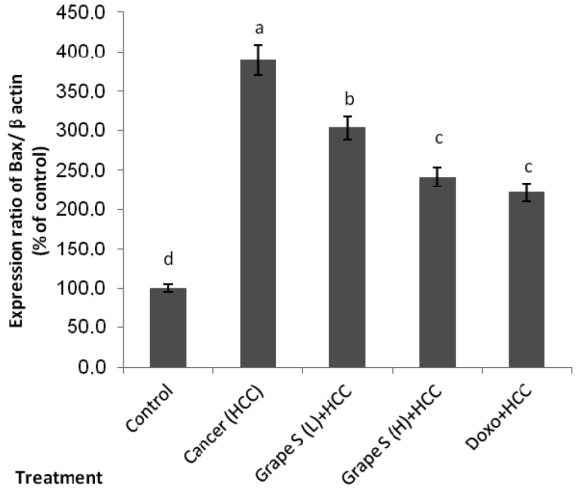

Figure 2. Semi-quantitative Real Time -PCR confirmation of Bax gene in liver tissues of HCC-rats treated with grape seed extract. "a" is significantly different from "b", "c" and "d" ($p<0.05$)

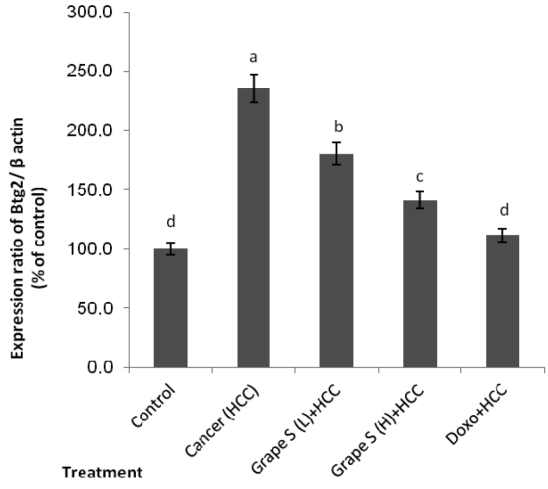

Figure 3. Semi-quantitative Real Time-PCR confirmation of Btg2 gene in liver tissues of HCC-rats treated with grape seed extract. "a" is significantly different from "b", "c" and "d" ($p<0.05$).

Figure 6 shows the necrosis and apoptotic indexes as well as necrotic/apoptotic rate in HCC-rats treated with grape seed extract. The results revealed that necrosis and apoptotic indexes, as well as the necrotic/apoptotic rate, were significantly lower in the positive control group when compared with healthy control rats. However, the highest number of damaged cells (necrosis and apoptosis) occurred in the HCC-bearing rats treated with a high dose of grape seed extract and the Doxorubicin drug.

In the same trend, the necrosis and apoptotic indexes, as well as necrotic/apoptotic rates, were also significantly higher in the HCC bearing rats treated with a low dose of grape seed extract when compared with control rats or rats treated with diethylnitrosoamine (DEN).

Generation of 8-Hydroxy-2-deoxyguanosine (8-OHdG)

Figure 7 represents generation of 8-OHdG in rat hepatic DNA following grape seed extract treatment as a protector against oxidative stress-induced DNA damage. The results obtained reveal that 8-OHdG levels in HCC-

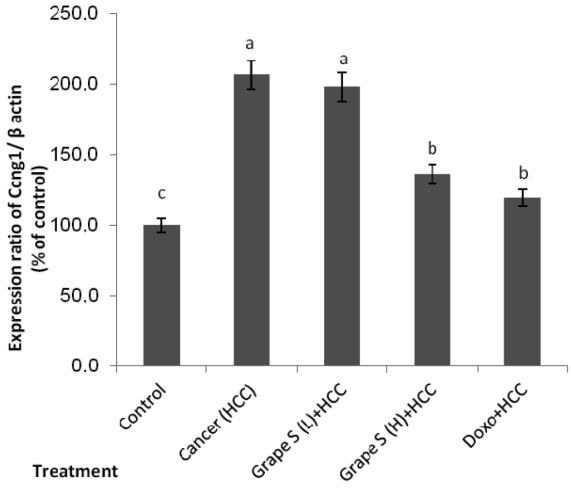

Figure 4. Semi-quantitative Real Time-PCR confirmation of Ccng1 gene in liver tissues of HCC-rats treated with grape seed extract.
"a" is significantly different from "b" and "c" ($p<0.05$).

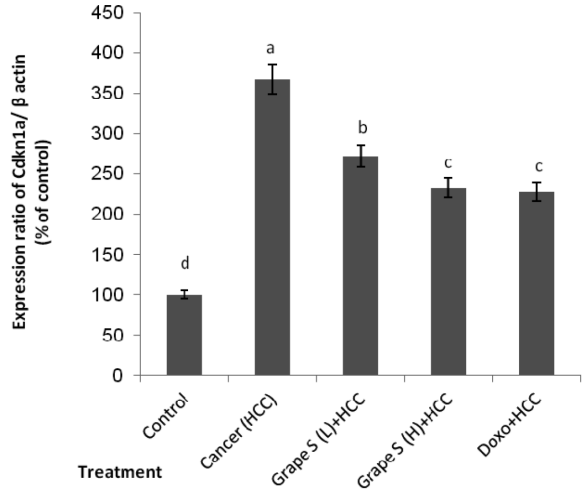

Figure 5. Semi-quantitative Real Time-PCR confirmation of Cdkn1a gene in liver tissues of HCC-rats treated with grape seed extract.
"a" is significantly different from "b" , "c" and "d" ($p<0.05$)

bearing rat liver tissues reached a significantly higher generation value of 8-OHdG (17.2±0.6 8-OHdG per 10^5 dG) when compared with healthy control rat value (5.1±0.4 8-OHdG per 10^5 dG).

Treatment of HCC- bearing group with grape seed extract significantly decreased the ratio of 8-OHdG/2-dG generation when compared with positive control group, where 8-OHdG/2-dG generation decreased by 73.8 and 52.9% in HCC+ grape seed extract at low and high doses, respectively, when compared with positive control group (Fig. 7). In addition, the ratio of 8-OHdG/2-dG generation induced in HCC, decreased by 48.3% in HCC+Doxo group compared with positive control group (Fig. 7).

DISCUSSION

In this study we investigate new concepts of grape seed extract in attenuating and management of hepatocellular carcinoma (HCC) induced by DEN injection in male rats.

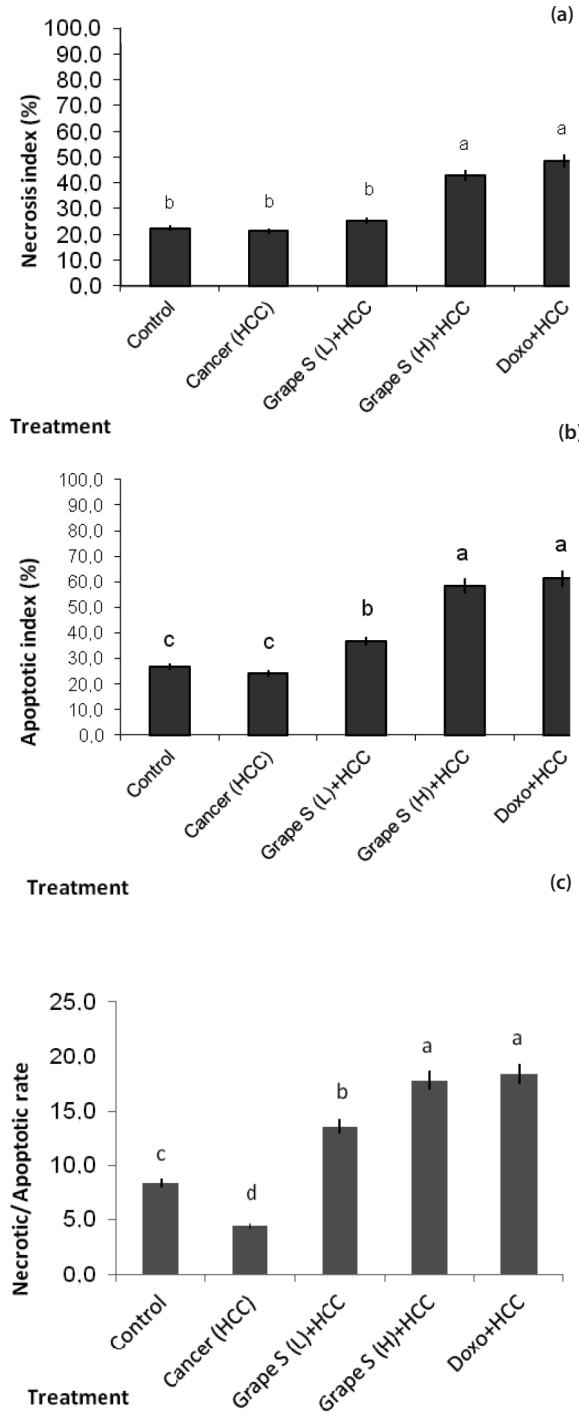

Figure 6. Effect of grape seed on necrosis (a) and apoptotic (b) indexes as well as necrotic/apoptotic rate (c) in HCC-bearing rats.
Results are expressed as the mean ±S.E. of data from at least ten samples. "a" is significantly different from "b", "c" and 'd' ($p<0.05$).

Results of the present study indicate a significant decrease in ALT and AST levels following administration of low and high doses of grape seed extract as compared to hepatocellular carcinoma bearing group. In the present study, elevation of the serum marker enzymes reflected the liver damage. Hepatopathy could lead to the leakage

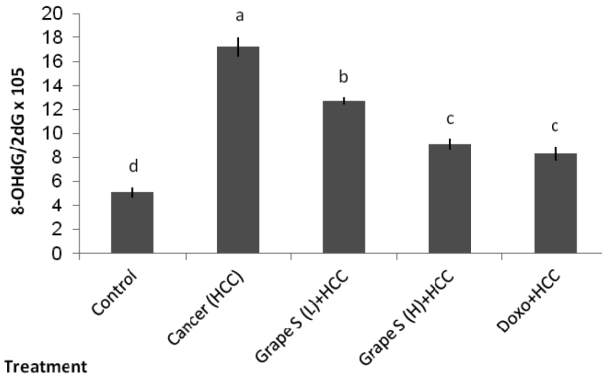

Figure 7. Generation of 8-OHdG in hepatic HCC-rat genome following grape seed treatment.
DNA damage was expressed as the ratio of oxidized DNA base (8-OHdG) to non-oxidized base (2-dG) in liver DNA. Results are expressed as the mean ± S.E. of data from at least ten samples. "a" is significantly different from "b", "c" and "d" ($p<0.05$).

of marker enzymes AST and ALT into the bloodstream, confirming the extent of liver damage. Also, their decreased levels in the liver tissues and increased serum levels could be due to toxic compounds affecting the integrity of liver cells (Senthikumar *et al.,* 2006). Prolonged destruction of hepatic cells results in more hepatic release that causes an elevation ALP level in the serum. Puiggros and coworkers (2005) reported that grape seed extract prevents oxidative injury by modulating the expression of antioxidant enzyme systems. This could exert protection towards hepatocytes leading to the decline in serum marker enzymes, revealing the ameliorating effect of GSE. These results agree with those obtained by Tanabe and coworkers (2008) who found that activities of AST, ALT and ALP were significantly increased following the nitroso-compound treatment in rats due to substantial liver damage. These findings were in agreement with Weber and coworkers (2003) who declared that some chemical agents may induce hepatic injury causing an increase in ALT and AST serum activities. The changes in serum ALT and AST were significantly improved after GSE administration to Tamoxifen-induced liver injury in rats.

Groups of HCC animals treated with low and high doses of grape seed extract showed significant regression in α-fucosidase and α-fetoprotein levels as compared to HCC bearing group. Also, it could be seen that high dose of grape seed extract induced the lowest level of fetoprotein. The α-L-fucosidase enzyme is usually found as a soluble component of the lysosome and functions as an acid hydrolase in the degradation of a diverse group of naturally occurring fugoglycol conjugate. Increased level of this enzyme is an early indication of HCC. However, it has been proposed as a sensitive tumor marker (Othman *et al.,* 2011).

A study by Sivaramakrishnan and coworkers (2007) found that the serum α-L-fucosidase activity level in patients with HCC is higher than in patients with liver cirrhosis. These findings suggest that an increase in serum α-L-fucosidase activity in patients with cirrhosis is the primary risk factor for developing HCC. α-fetoprotein is a glycoprotein primarily produced by the fetal yolk sac, liver and intestine. Its production is almost totally suppressed after birth, with concentration decreasing to <10 ng/ml. Low grade elevation of AFP is seen in benign liver disease, including acute and chronic hepatitis and cirrhosis (Yao *et al.,* 2007). Appearance of several malignancies, such as HCC and chronic liver disease, strongly correlates with α-fetoprotein elevation up to the pathological range (Baig *et al.,* 2009).

Also, tumor necrosis factor alpha (TNF-α) is produced by macrophages and it plays an important role under tumor conditions. It has been reported that TNF-α is an essential factor in tumor promotion. TNF-α is one of the major inflammatory mediators secreted by activated macrophage and is involved in the initiation of acute and chronic inflammation (Lutsiak *et al.,* 2005).

Also, CEA is an important biomarker of liver cancer which is synthesized mainly in the fetal stage; practically no production of this marker occurs in the normal adult. However, CEA is re-synthesized when adult cells are transformed into cancer cells. Our results show that CEA and TNF-α concentration significantly decreased upon treatment of HCC groups with low and high doses of grape seed extract as compared to HCC group. Increased CEA and TNF-α levels were the consequences of diethylnitrosoamine DEN oral intoxication (Cao *et al.,* 2008). On the other hand, GSE down-regulated the concentration of DEN and reduced the severity of injury. This may be explained on the basis of GSE interfering with cancer cell growth and proliferation, as well as inducing cell death that appears to be one of its main pathways, which may be contributing to some of the clinical benefits achieved by the extract (El-Beshbishy *et al.,* 2010).

Regarding gene expression, we observed alteration in the expression of Aen, Bax, Btg2, Ccng1, Cdkn1a genes. Recently, Suenaga and coworkers (2013) also reported that changes in gene expression levels were observed in HCC-rats induced by diethylnitrosamine DEN treatment. Additionally, dose-dependent alterations in gene expression were demonstrated for 31 genes, including apoptosis related genes, after the administration of DEN (3, 9, 27 and 80 mg/kg bw) as determined by qPCR (Watanabe *et al.,* 2009).

The present study indicates that necrotic/apoptotic rate and 8-OHdG generation in hepatic cells are significantly higher than those in control group.

The results of several studies generally support our results of the apoptosis and gene expression of apoptotic genes. Watanabe and coworkers (2012) reported that the DNA damage in HCC-rats was accompanied by gene-mediated signaling pathway involved in apoptosis response. Additionally, Suenaga and coworkers (2013) reported that levels of apoptosis in hepatic cells and changes in the protein expression of Cdkn1a were observed in HCC rats induced by DEN treatment. Moreover, El Mesallamy and coworkers (2011) reported that induction of HCC in male rats cause a decline in the antioxidative defense as indicated by an increase in the level of oxidative stress marker (MDA) and decrease of free radical scavenging antioxidants. The biological actions of cytochrome p450 enzymes and reactive oxygen species (ROS) are the main metabolites inducing the oxidative stress in livers of HCC rats (El-Mesallamy *et al.*, 2011).

Furthermore, for elucidation of the mechanism that modulates the gene expression and apoptosis in HCC rats, it may help to note that using DEN or NDEA drugs to induce HCC in rats produces bio-activated ethyldiazonium ions that may work as alkylating agents reacting with the nitrogen and oxygen atoms of DNA bases. These reactions between the ions and the DNA molecules reproduce damage of the DNA due to expulsion of promutagenic adducts such as O^6-ethyldeoxy-

guanosine and O^4 and O^6-ethyldeoxythymidine (Verna *et al*., 1996).

Moreover, decline in the levels of GSH and GSH dependent enzymes, GPx and GR, in rats due to NDEA treatment might be caused by one or both of the following reasons: (a) decline in the biosynthesis of these antioxidants during hepatocellular damage and/or (b) extreme reduction of the antioxidants due to their utilization during the metabolism of NDEA as scavenging agents against the free radicals formed. Additionally, the depletion of the levels of GSH antioxidant enzyme might have induced a decrease in the activities of GSH dependent enzymes such as GPx and GR, because GSH is a main co-factor for synthesis of these enzymes (Pradeep *et al.,* 2007).

To prevent such stress actions of the drugs inducing HCC in rat liver, various plant-derived materials have been previously evaluated, including grape seed proanthocyanidin extract (GSE) which possesses a variety of pharmacological and therapeutic effects, including antitumor, anti-inflammatory and antiallergic, besides free-radical scavenging and antioxidant activities (Tang *et al.,* 2012).

Khoshbaten and coworkers (2010) reported that resveratrol is one of polyphenols present in grape seeds, and it may interfere with cancer cell growth and proliferation, as well as induce apoptosis. In agreement with such observations, our results revealed that high dose of grape seed extract was able to increase the rate of apoptosis in hepatic cells of HCC induced-rats which may be attributed to the polyphenolic compounds. Moreover, a recent study indicated that grape seed extract (GSE), has anti-inflammatory and antioxidant effects and therefore GSE is able to prevent oxidative damage and hepatic dysfunction (Pan *et al.,* 2011).

Thus, based on these encouraging observations, we conclude that polyphenolic compounds and/or GSE may be greatly helpful in maintaining the disturbed oxidant– antioxidant balance to restrain the HCC. Also, the apoptotic effect of GSE was well documented in this study.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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