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MODULATION OF MSH2 GENE EXPRESSION AND DNA GLOBAL METHYLATION IN DIETHYLNITROSAMINE INDUCED HEPATIC FIBROSIS MOUSE MODEL BY CISPLATIN-ANTHOCYANINS COMBINATION

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Abstract

Novel therapeutic approaches targeting the molecular pathways of hepatocellular carcinoma (HCC) development are highly demanded. We aimed at studying the effect of cisplatin alone and combined with cranberry extract on global DNA methylation and the MSH2 gene expression in diethyl-nitrosamine (DEN)-induced hepatic fibrosis model, as a landmark in HCC development pathway in mice. Forty male albino mice were divided into 2 groups: Group I (n=10): control, Group II (n=30): mice were injected intraperitoneal with DEN and sub-classified into 3 equal groups; IIa: untreated, IIb: intraperitoneal cisplatin, IIc: intraperitoneal cisplatin + oral cranberry-derived anthocyanin. Histopathological examination of liver, assessment of global DNA methylation and MSH2 gene expression were done. MSH2 gene expression was significantly decreased in DEN-group as compared to the control group, but significantly increased by cisplatin alone and furthermore when anthocyanin was added ($p<0.001$). Global DNA methylation significantly increased in DEN-group, significant decrease in cisplatin-group and further decrease when anthocyanin was added ($p<0.001$). A significant negative correlation was observed between global DNA methylation and MSH2 gene expression among all the studied groups ($p<0.001$). Histopathological examination showed improvement of liver fibrosis when anthocyanin was added to cisplatin. Improvement of histopathological findings of DEN-induced hepatic fibrosis by cranberry-derived anthocyanins was associated with global DNA hypomethylation and increased MSH2 expression. Further studies are warranted to confirm the applicability of adding anthocyanins to liver fibrosis and HCC treatment regimen.

Keywords:
Hepatic fibrosis, Global DNA methylation, MSH2 expression, Cisplatin, Anthocyanins, Cranberry, Hepatocellular carcinoma

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Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors globally, with the highest occurrences reported in Asia and Africa. The major risk factor for HCC is chronic viral hepatitis. It represents 20-40% of human cancers in countries endemic for viral hepatitis [1]. Other risk factors include aflatoxin, alcohol, and metabolic disorders. These factors induce chronic liver injury, regeneration, and cirrhosis which leads to malignant changes due to increasing cellular turnover [2]. Generally, HCC prognosis remains poor in spite of the advancements in its treatment [3]. Novel therapeutic approaches, targeting the molecular pathways of HCC development, may improve in the prevention and treatment strategies of HCC. Therefore, identification of prognostic molecular markers is crucial for a more effective therapy and clinical outcomes. [1-4].

As documented, the development of HCC is a long process of genetic mutations and epigenetic aberrations, such as unusual methylation and histone modification [2]. The identification of specific DNA methylation signatures which could be considered as diagnostic markers may help in early disease detection and add new strategies for the therapeutic regimens.

In hepatocytes, DNA methylation occurs at the 5′ position of the cytosine ring within CpG dinucleotides. The most common forms of abnormal CpG methylation in cancer include total hypomethylation that causes chromosomal instability and promoter hypo- or hyper methylation that leads to inappropriate activation of some genes as oncogenes or silencing of others as tumor suppressor genes (TSGs) [3].

It is noteworthy that genomic hypomethylation mainly affects the intergenic and intronic regions of DNA, particularly repeat sequences and transposable elements. Methyl cytosine (mC) is dispersed within the transposons, including short, interspersed nucleotide elements (SINEs) and long interspersed nucleotide elements (LINEs), which are comparatively rich in CpG dinucleotides. Thus, global hypomethylation is believed to be due to loss of DNA methylation in these sequences [4].

Another mechanism that may contribute to the development of HCC is defective DNA mismatch repair (MMR). [5] The DNA MMR system plays a crucial role in the preservation of genomic integrity as it corrects replicative mismatches that escaped DNA polymerase proofreading. DNA mismatch repair protein, MutS homolog 2 or MSH2 protein, joins with MSH6 or MSH3 to form complex dimer, which identifies locations on the DNA where errors have been made during DNA replication [6].

Consequently, cells with defective MMR mechanisms might be associated with the pathogenesis of tumors. Defects in genes encoding components of the MMR system lead to a genome-wide instability of the microsatellites that are involved in several human solid tumors [7,8]. When such defects occur in oncogenes or tumor suppressor genes, loss of control over cell growth and proliferation may develop [9]. Promoter methylation of the MMR genes in HCC has been documented. A high methylation frequency of two important MMR genes (hMSH2 and hMSH3) was observed in HCC [10,11].

It is noteworthy to mention that the pathogenesis of HCC has been described as an axis of sequential events, which is identified as hepatic inflammation-fibrosis-cancer (IFC) axis. We resorted to the fibrosis component in the current study. [12] currently reviewed liver fibrosis treatment modalities based on experimental studies include, physical activity, low carbohydrate-fat diet, simtuzumab, pegbelfermin, probiotics and hematopoietic stem cells therapy [13].

Moving on to HCC systemic treatment or hepatic artery chemoembolization regimens, targeting hepatic microenvironment by biological immunotherapy hand in hand with cancer cell signaling by conventional therapy improved the clinical outcomes. The conventional chemotherapy includes platinum compounds doxorubicin or fluoropyrimidine whereas sorafenib, lenvatinib, atezolizumab and bevacizumab are the biological agents with reported efficacy in clinical trials [14].

Cisplatin is one of the platinum drugs, which induces the production of reactive oxygen species leading to DNA damage. Cisplatin has antitumoral properties that are mainly based on DNA cross-links with the purine bases, forming cisplatin-DNA adducts inhibiting DNA synthesis. Moreover, it triggers the tumor suppressor protein p53 activation that eventually leads to apoptosis. Various mechanisms have been suggested to contribute in tumor cell cisplatin resistance through mutations in genes involved in DNA repair, drug uptake, cell cycle control, apoptosis and IGF signaling pathways [14]. In reference to MMR genes, it was postulated that loss of expression of the MMR genes leads to tumor cells 'resistance to the damage induced by some chemotherapeutic agents, including cisplatin. This acquired resistance could be achieved through several mechanisms, including failure to recognize DNA adducts formed by some chemotherapeutic agents or failure to activate signaling pathways that trigger apoptosis [15, 16].

The role of methylation changes in MMR genes has been well characterized, however for the majority of aberrant DNA methylation events it is not clear whether they are associated with response to chemotherapy or are occurring coincidentally due to a methylator phenotype or just as random methylation events during platinum selection or DNA damage induction [17].

In similarity to the concept of 'driver and passenger', methylation changes emerging during carcinogenesis could either represent 'drivers' of chemoresistance that provide the cell with a selective advantage or 'passenger' events, with no considerable influence on chemosensitivity [18].

Aberrant gene methylation during carcinogenesis can provide a rationale for the targeting gene demethylation for chemoprevention and cancer therapy**.** The effect of treatment on global methylation and mismatch repair enzymes expression in HCC pathogenesis has been scarcely studied.

The induction of hepatocellular carcinoma in animal models by diethyl nitrosamine(DEN) was widely studied. This model demonstrates sequence of events in favor of histopathological progression from chronic inflammation to liver fibrosis then hepatocellular carcinoma. The effects of DEN on the liver vary according to its dose and the study duration [12]. In the current study we used DEN -6 weeks' model.

The hepatoprotective effect of cranberry extract was studied in non-alcoholic fatty liver disease (NAFLD) in which cranberry prevented NAFLD, dose-dependently in rat model. It demonstrated antioxidant, anti-inflammatory activities, and anti-fibrotic effects. Several classes of natural compounds were detected in cranberry extract including phenolic acids, flavones, flavonoids, and organic acids, and anthocyanins, which we used in the current study. Structurally anthocyanins are polyphenols, they are water soluble pigments found in fruits and vegetables. The benefits of anthocyanins and cranberry extract in liver fibrosis were reported to be through reduction of free radicle production, and inhibition of inflammatory markers including IL-6, TNF-alpha, and TGF-beta [19]. These mechanisms may provide promising potential of using anthocyanin-rich cranberry extract in liver fibrosis treatment regimen.

Aim of the study:

Study the effect of cisplatin alone and combined with cranberry derived anthocyanins on global DNA methylation level and the expression of MSH2 genes in hepatic fibrosis induced by diethylnitrosamine (DEN) in mice.

Materials and methods

This study has been approved by the research ethics committee of Alexandria Faculty of Medicine, Egypt. Serial N.: 0304144-2018. The research was performed in strict accordance with internationally accepted standards.

Study design

The present study was carried out on 40 male albino mice obtained from the Animal Unit. They were housed in stainless steel cages at a constant temperature ($24 \pm 2\ ^{0}C$) with free access to food and water. Mice were randomly divided into two groups:

- ***Group I (10 mice):*** Served as normal control
- ***Group II (30 mice):*** Were injected intraperitoneally with diethylnitrosamine (DEN) 75 mg/Kg once a week for 3 weeks, then 100 mg/Kg for another successive 3 weeks (DEN 1 gm/vial, Sigma-Aldrich, USA, N0258) [20].

Group II was further classified into three groups of 10 mice each:

Group II a: untreated DEN group, **Group IIb**: treated with single intraperitoneal injection of cisplatin 10 mg/kg in the beginning of the 3rd week (Cisplatine MYLAN 1 mg/mL (10mL) [21], **Group IIc:** received oral anthocyanins 100 mg/kg daily [22] from the 3rd week for 3 weeks (Cranberry EMA Pharma, Egypt). It was combined with cisplatin in the same dose as group IIb.

By the end of the 6th week, animals were anesthetized by thiopental sodium (50 mg/kg IP) and blood samples were collected by cardiac puncture. Blood samples were centrifuged and serum was stored at -20˚C for further biochemical analysis.

The liver was dissected immediately after mice scarification and was divided into two parts:

One part of the liver tissue was fixed in 10% formaldehyde and processed for paraffin embedding. Tissue sections were subsequently stained with hematoxylin-eosin (H&E) and Mallory's trichrome and examined microscopically [23]. The other part was frozen at -80^{0}C and used for later DNA and RNA extraction and molecular studies.

DNA extraction and assessment of global DNA methylation using ELISA [24]

Mice's liver tissue samples were used for DNA extraction, with a weight of liver tissue ranging between 25-30 mg. After being sliced, tissues were placed in clean microcentrifuge tubes and 180 µl of lysis buffer, provided in the extraction kit, was added. DNA purification from hepatic tissue was performed using Spin Protocol QIAamp DNA Mini Kit (Qiagen) according to manufacturer's instructions. Quantification of total DNA concentration was performed using Nanodrop. An optimal amount of 150 ng DNA extract per reaction was used. Extracted DNA was eluted using Tris EDTA (TE) buffer and stored at -20°C until the day of assay of DNA global methylation.

Global DNA methylation assessment was performed using Enzyme-linked immunosorbent method, to assess extracted DNA cytosine methylation in animals' livers using MethylFlash Kit (Epigentek). Briefly, the DNA samples were diluted using 30 µL of binding buffers, followed by incubation at 60°C. Capture and detection antibodies were used and optical densities were read at 450 nm. DNA methylation was quantified by calculation of 5-methylcytosine percentage, which was expressed relative to methylation in positive control (polynucleotide containing 50% of 5-methylcytosine) provided within the kit. The amount of DNA input in the positive control was 5 ng/µl.

Assessment of MSH2 gene expression: [11]

Total RNA extraction

30 mg of liver tissue sections were transferred to RNase-free round-bottomed tubes- on ice to be homogenized on the day of the assay. 0.6 mL of freshly prepared Qiazol lysis reagent (Qiagen, Germantown, MD, USA; cat. no. 79306) containing 1% 2-mercaptoethanol was added to each tissue sample. Homogenization was performed using a rotor-stator, according to the manufacturer's protocol. PureLink® RNA Mini Kit (Invitrogen, Waltham, MA, USA; cat. no. 12183018A) was used for total RNA extraction and purification.

Reverse transcription

Concentration of total RNA was estimated using the Nanodrop. Purified RNA was reverse transcribed using Applied Biosystems (Waltham, MA, USA) High-Capacity cDNA Reverse Transcription kits (cat. no. 4374966). Briefly, equivalent to 150 ng of total RNA was used per 20 µL reaction. The thermal cycler was programmed as follows: 25ºC for 10 min, 37ºC for 120 min, 85ºC for 5 min and 4ºC until the removal of samples. A minus reverse

transcription control was added in all experiments to rule out DNA contamination. Complementary DNA (cDNA) was stored at -20ºC until MSH2 expression assessment.

Measuring MSH2 gene expression using quantitative real time PCR (qRT-PCR) q RT-PCR reactions were performed using an Applied Biosystems Step-one Real-time system. Primer sequence for amplified genes was designed using primer- BLAST tool, NCBI (https://www.ncbi.nlm.nih.gov/tools/primer-blast) Primer sequence for MSH2 was (5-TTCTCCCGGCAATCTTTCTC-3) for forward primer and (5-TCCACATACCCAACTCCAACA-3) for reverse primer. PCR reaction master mix (SensiFAST SYBR Lo-ROX Mix) was prepared and the following volumes were used based on a standard 20ul final reaction mix volume: 2x SensiFast SYBR Lo-ROX Mix (10µl), 10µM forward primer (0.8µl), 10µM reverse primer (0.8µl) with a final concentration of 400 nM for each primer, template cDNA (50 ng) and H2O as required. GAPDH was used as the endogenous reference gene for data normalization. Primer sequence of GAPDH was as follows: Forward: 5-TAAGAGCAACTGGGGGTTTGG -3, Reverse: 5-GGGAGATGCTCAGTGTTGGG -3. RT-PCR settings were as follows: an initial two-minute hold cycle at 50ºC, 10 min hold 95ºC and 40 cycles of 15 secs at 95ºC and 1 min at 60ºC.

Calculation of MSH2 gene expression

Values obtained at cycle threshold (Ct) were used to calculate MSH2 gene expression using relative quantification method ($RQ= 2^{-\Delta\Delta Ct}$).

Histopathological assessment:

Livers from all mice were harvested and fixed in formalin. Sections were obtained and processed as formalin-fixed paraffin-embedded material. Five-micron-thick sections were obtained.

Statistical analysis of the data

Data were fed to the computer and analyzed using IBM SPSS software package version 20.0. (Armonk, NY: IBM Corp). Non-parametric quantitative data were expressed as range (minimum and maximum) and median. Kruskal Wallis test was used for comparing the four studied groups and followed by Dunn's test for pairwise comparison. Spearman's rank correlation coefficient was used to correlate between abnormally distributed quantitative variables. Significance of the obtained results was judged at the 5% level. GraphPad Prism -10 software was used for the charts.

Results:

Comparison between the studied groups according to MSH2 gene expression and global DNA methylation

Results showed a statistically significant difference in MSH2 gene expression among the studied groups, with a significant decrease in the expression shown in DEN-treated group as compared to the control group, followed by significantly increased MSH2 expression in groups treated by cisplatin alone and further increased expression when anthocyanin was added, (Table 1, Figure 1).

Regarding global methylation, there was again a statistically significant difference between the control group and the DEN-treated group where methylation was significantly increased in the latter group, followed by a significant decrease in the level of global methylation in groups treated by cisplatin and furthermore in the group treated by anthocyanins combined with cisplatin, (Table 1, Figure1).

Correlation between MSH2 gene expression and global DNA methylation among the studied groups

Results showed a statistically significant negative correlation between MSH2 gene expression and global methylation among all the studied groups, (Table 2).

Histopathological examination of liver sections of studied groups:

Microscopic examination of sections obtained from mice in the control group revealed preserved liver architecture showing neither portal nor lobular inflammation. No fibrosis was detected by trichrome stain [Figure 2 a, b]. Sections from the DEN-treated group revealed marked hepatocellular injury in the form of feathery degeneration and foci of lytic necrosis. Severe portal inflammation, expansion and bridging fibrosis were noted [Figure 2 c-e]. Sections from the Cisplatin-treated group revealed mild hepatocellular injury in the form of feathery degeneration without evident lytic necrosis. Trichrome stain revealed mild portal expansion with focal bridging fibrosis [Figure2 f, g]. Cisplatin + anthocyanin-treated group showed a notable amelioration whereby only mild lobular inflammation and focal hepatocellular degeneration were noted. Neither portal expansion nor bridging fibrosis was noted by trichrome stain [Figure 2 h, i].

Discussion

The pathogenesis of HCC is described as an axis of sequential events which is known as hepatic inflammation-fibrosis-cancer (IFC) axis. DEN-induced HCC in mice was reported to result from a stepwise histopathological progression similar to that in humans, which follow the IFC axis. Early interventions during hepatic inflammation and fibrosis stages may confer a promising option for HCC prevention [12].

HCC, like any cancer, is associated with defects in DNA methylation and its machinery [25]. Three types of alterations in DNA methylation are involved in cancer: Hyper methylation of the CpG islands in the promoter regions of tumor suppressor genes, abnormal expression of DNA methyl transferase (DNMTs), and global hypomethylation of genes and repetitive sequences leading to genomic instability and oncogene activation [26].

Hypomethylation usually leads to microsatellite instability (MSI), a phenomenon linked to genomic instability, which is characteristic of cancer cells [27]. On the other hand, signature mutations in the form of C → T transitions at methylated CpG sites are the hallmark of hydrolytic deamination of 5-methylcytosine and commonly produce mutations in tumor suppressor genes such as p53 [28].

Genome stability is mainly maintained by the mismatch repair system (MMR). Abnormally functioning MMR leads to alterations of microsatellites, consequently increasing the overall mutational rate of a given cell. Therefore, MMR plays a vital role in cancer etiology and influences its biological behavior [27].

In the current study, we aimed to examine global methylation pattern and MSH2 expression in the liver of mice in which hepatic fibrosis was induced by DEN and compared to cisplatin administration alone and when combined with cranberry-derived anthocyanins. Cisplatin is considered as a reference or a standard drug, with widely studied impact of epigenetics on its efficacy including the DNA methylation mechanisms [27].

Our results indicated an increase in global DNA methylation following induction of hepatic fibrosis. This is consistent with the results indicating global DNA hyper methylation in HCC from patients with Indigenous American ancestry. Integrative characterization in their study revealed that Peruvian HCC seems to be controlled by the PRC2 complex, which mediates cell reprogramming resulting in massive DNA methylation hence modulating gene expression [29]. A group of hyper methylated genes was identified in HCC, which could serve as diagnostic biomarkers [30].

On the other hand, opposite results reported, where mean methylation level of DNA was significantly lower in HCC tissues compared to adjacent non-tumor tissues [4]. Similarly, the presence of global hypo methylation in HCC was found [31]. It was reviewed that, both DNA hyper- and hypomethylation were found to occur in virtually all types of cancer, and can occur in early or late stage of cancer. DNA sequences

displayed cancer associated hyper- or hypo-methylation and these methylation changes can be dynamic [32].

Regarding mismatch repair enzyme MSH2 expression, our study showed a lower level of gene expression in DEN-treated group as compared to control group. In concordance with the current study, the cause of microsatellite instability-high (MSI-H) in HCC using the TCGA database was attributed to the downregulation of both MSH2 and MSH6 mRNAs. MSH2 gene hyper methylation together with the downregulation of its mRNA were confirmed [33].
Dysregulation of MSH2 was reported to be induced by tumor necrosis factor–α (TNF-α) stimulation and this was suggested as a mechanism of genetic alterations involved in hepatocarcinogenesis [34]. Our observations are also consistent with promoter methylation in HCC cases and hyper methylation of MMR genes, leading to its downregulation. [11] In line with this, reduced hMLH1 and hMSH2; MMR protein expression in HCC and in adjacent tissue were also reported [35,36].

Deficient mismatch repair (dMMR) is generally caused by somatic mutations, miRNA-mediated downregulation, or hypermethylation in MMR protein (MLH1, MSH2, MSH6, and PMS2) genes. These genes recognize and correct mismatched nucleotides. Errors in MMR lead to the accumulation of mutations in DNA microsatellites; MSI-H. MSI-H tumors have a distinctive feature, as they express a large number of neoantigens due to their high number of somatic mutations [37].

Our study demonstrated that global methylation significantly decreased, while MSH2 gene expression significantly increased with cisplatin treatment and further enhanced when anthocyanin was added. This was associated with chronic inflammatory microenvironment and fibrosis in DEN-treated group, hepatocellular degeneration and focal fibrosis in cisplatin-treated group, transitioning to remarkable mild lobular inflammation without portal fibrosis when anthocyanin was added.

The state of global DNA hypomethylation, induced by cisplatin, seems important for its therapeutic effectiveness. The relation of cisplatin to DNA methylation in germ cell tumor was studied, and hyper methylation of selected gene promoters, such as MGMT, CALCA, and RASSF1A was associated with cisplatin-resistant phenotype. A similar finding was reported in lung cancer, where the relation between cisplatin and methylation status was further elucidated, as cisplatin resistance was associated with downregulation of key genes by DNA methylation in non-small cell lung carcinoma [38-40].

Such findings can be explained in the study highlighting the ability of tumor cells to grow and survive under different circumstances, through modulating protein expression by alternating demethylation and methylation patterns. These alternations in methylation patterns are rapid, flexible and efficient [41].

In a phase II clinical trial, treatment with low-dose decitabine in patients with heavily pretreated ovarian cancer caused alteration in DNA methylation of genes and cancer pathways, restored sensitivity to carboplatin, and resulted in a high response rate and prolonged progression-free survival [42]. The increased MSH2 gene expression in cisplatin-treated group as shown in our results can be explained by the fact that, cisplatin has the ability to form covalent interstrand crosslinks (ICLs) between guanines on opposite strands of DNA, besides the majority of the lesions generated by cisplatin are intra-strand adducts between adjoining purines on the same strand of DNA.

However, it has been suggested that cells can use multiple DNA repair pathways of cisplatin ICLs [38]. ICL sites are more likely to undergo oxidative deamination to uracil. The generation of mismatches leads to increased recruitment of MMR proteins, which have been shown to efficiently remove G/U mismatches [43]. Subsequently, MMR plays a role in blocking productive cisplatin specific ICL repair.

Cranberry-derived constituents have antioxidant and anti-inflammatory functions. Cranberries are excellent dietary source of phytochemicals that

include proanthocyanidins (condensed tannins) and ursolic acid that has antiproliferative activity against several cancer cells from oral cavity, stomach, colon, hepatic, breast, cervical, lung, prostate and renal cancer cell lines. Cranberry organic soluble extracts have been described as modulators of cell cycle progression by the induction of G_1 cell cycle arrest, while the flavonoid rich extract induce G_2-M cell cycle arrest [44-46],

Our study showed improvement of histopathological picture when anthocyanin was added to cisplatin. It had been reported that cisplatin, when combined with resveratrol (a polyphenol in cranberry), had additive and/or synergistic effects leading to increased chemo sensitization of cancer cells [44]. The exact mechanisms by which resveratrol increases the chemosensitivity of cisplatin are still to be determined. Resveratrol was found to down regulate some of the proteins and enzymes involved in glucose metabolism such as the glucose transporter GLUT1, enzymes of glycolysis and hexose monophosphate shunt, thus limiting that Warburg effect required for tumor survival [44,47].

In HCC, numerous cranberry derived compounds have been shown to enhance cancer cells apoptosis and suppress cancer cell migration by suppression of PI3K/Akt signaling. It also caused downregulation of cyclin D1, p38, Akt, MAP kinase and Pak1, indicating the growth inhibitory activity is linked to sensitization to apoptosis, cell proliferation and survival pathways. Resveratrol has an inhibitory effect on angiogenesis and reduces ROS formation in hepatic stellate cells [48]. In a study testing the differential protection by resveratrol and anthocyanin-rich bilberry extract against intracellular oxidative stress. However, anthocyanin but not resveratrol decreased mitochondrial ROS generation [49].

In similar studies DEN–induced liver tumorigenesis model in rats, carcinogenesis was prevented by suppressing oxidative stress and inflammatory response mediated in part by hepatic nuclear factor E2–related factor 2 (Nrf2) [50]. In a DEN /carbon tetrachloride -induced fibrosis associated hepatocarcinogenesis model, anthocyanins-rich powder of myrtaceae fruits attenuated liver fibrosis [51]. Similarly, delphinidin (anthocyanidins derived from berries) modulated transcription of Nrf2 target genes in cancer cells [52].

In another study using dimethylnitrosamine (DMN) rat model, anti-fibrotic effect of the anthocyanins isolated from the purple-fleshed sweet potato was reported [53]. Anthocyanins lead to inhibition of DMN-induced activation of collagen expression and reduction of TNF-α and TGFβ [53]. Similarly, the anti-fibrotic activity of lingonberry-derived anthocyanins was through modulation of TGFβ/Smad/ERK signaling pathway which led to the inhibition of hepatic stellate cell activation and liver fibrosis [54].

The anti-proliferative effect and apoptosis signaling modulation of Hep G2 cells highlight berry- derived anthocyanin as promising natural agent against HCC pathogenesis and alcohol-induced liver disease [55].
In accordance with our study demonstrating decreased level of global DNA methylation with the introduction of anthocyanins, it was reported that anthocyanins were able to modulate DNA methylation within promoters of tumor suppressor genes in colon cancer cells. This was achieved through the inhibition of DNMT1 and DNMT3B [56]. Furthermore, the intake of juice rich in anthocyanins resulted in reducing DNA strand-breaks in lymphocytes through genome stabilization mediated via DNA repair factors [57].

Conclusion

Cisplatin treatment of DEN-induced hepatic fibrosis although did not improve liver fibrosis but it favorably decreased global DNA methylation and increased MSH2 gene expression. This was further accentuated with addition of anthocyanins with improvement of the histopathological picture reversing IFC axis. Anthocyanins- cisplatin combination maintains DNA in hypomethylated state and enhances repair genes expression, thus may overcome one of important molecular mechanisms of cisplatin resistance. Besides, adding Anthocyanins to cisplatin may reduce liver fibrosis as an adverse

effect during treatment of HCC. Further studies are warranted to confirm the applicability of adding anthocyanins to the treatment regimen of hepatic fibrosis and HCC in clinics.

Data availability statement

The data that support the findings of this study are available on request from the corresponding author (E.I.A)

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Conflict of interest

The authors report there are no competing interests to declare.

Ethical considerations

This study has been approved by Alexandria faculty of Medicine research ethics committee. The research was performed in strict accordance with internationally accepted standards and regulations.

The Authors' Contribution:

All authors have contributed to conception of idea, acquisition of data, drafting, writing, revising and editing the manuscript, in addition to statistical analysis. E.I.A and S.Z.H were responsible for animal experimental work. Y.A.I and R.A.G were responsible for biochemical and molecular biology assays. M.M.A was responsible for histopathological examination. E.I.A and E.A.H were responsible for final draft revision and data interpretation.

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Table 1: Comparison between the studied groups in terms of MSH2 gene expression and global DNA methylation

	Control (n = 10)	**DEN (n = 10)**	**DEN+ cisplatin (n = 10)**	**DEN +cisplatin + anthocyanins (n = 10)**	**H**	**P**
MSH2 gene expression Median (Min – .max.)	0.73 (0.41 – 0.97)	0.06 (0.01 – 0.09)	0.30 (0.13 – 0.51)	0.8 (0.41 – 0.99)	55.848*	<0.001*
Sig. bet. Groups		p_1=0.002*, p_2<0.001*, p_3<0.001*				
Global DNA methylation Median (Min – max.)	13.64 (10.70–20.14)	17.27 (10.57–19.30)	7.03 (4.52–10.12)	1.95 (0.19– 3.55)	74.778*	<0.001*
Sig. bet. Groups		P_1<0.001*, p_2<0.001*, p_3<0.001*				

SD: Standard deviation
H: H for Kruskal Wallis test, Pairwise comparison bet. each 2 groups were done using Dunn test p: p value for comparing between the studied groups p_1: p value for comparing between Control and each other groups p_2: p value for comparing between DEN and DEN + cisplatin p_3: p value for comparing between DEN + cisplatin and anthocyanins
*: Statistically significant at $p \leq 0.05$

Table 2: Correlation between MSH2 gene expression and global DNA methylation among different studied groups

	MSH2 gene expression					Total (n = 40)
Groups		**Control**	**DEN**	**DEN + cisplatin**	**DEN +cisplatin +anthocyanins**	
Global methylation	**ρ**	0.934*	-0.951*	-0.974*	-0.942*	-0.986*
	p	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*

ρ: Spearman's rank correlation coefficient

*: Statistically significant at $p \leq 0.05$

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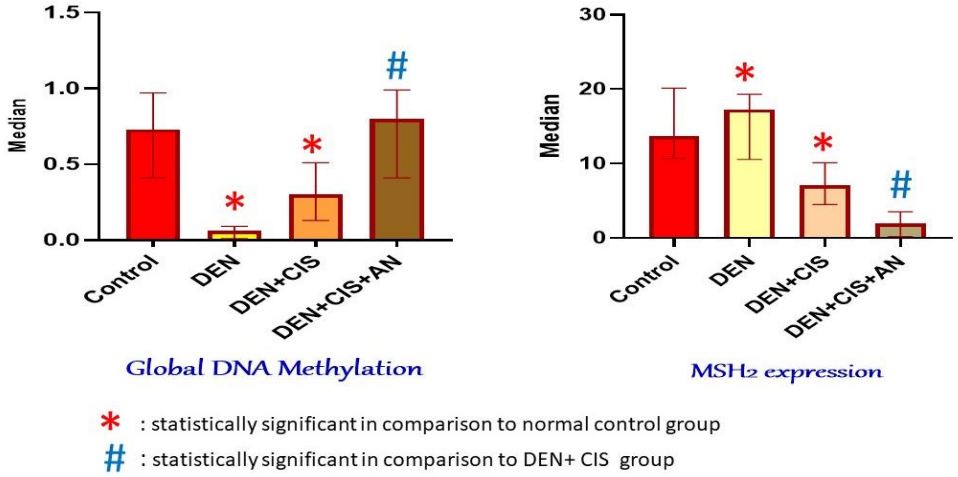

Figure (1): Comparison of Global DNA methylation and MSH2 gene expression in control versus drug- treated groups. DEN: diethylnitrosamine, CIS:cisplatin , AN:anthocyanins

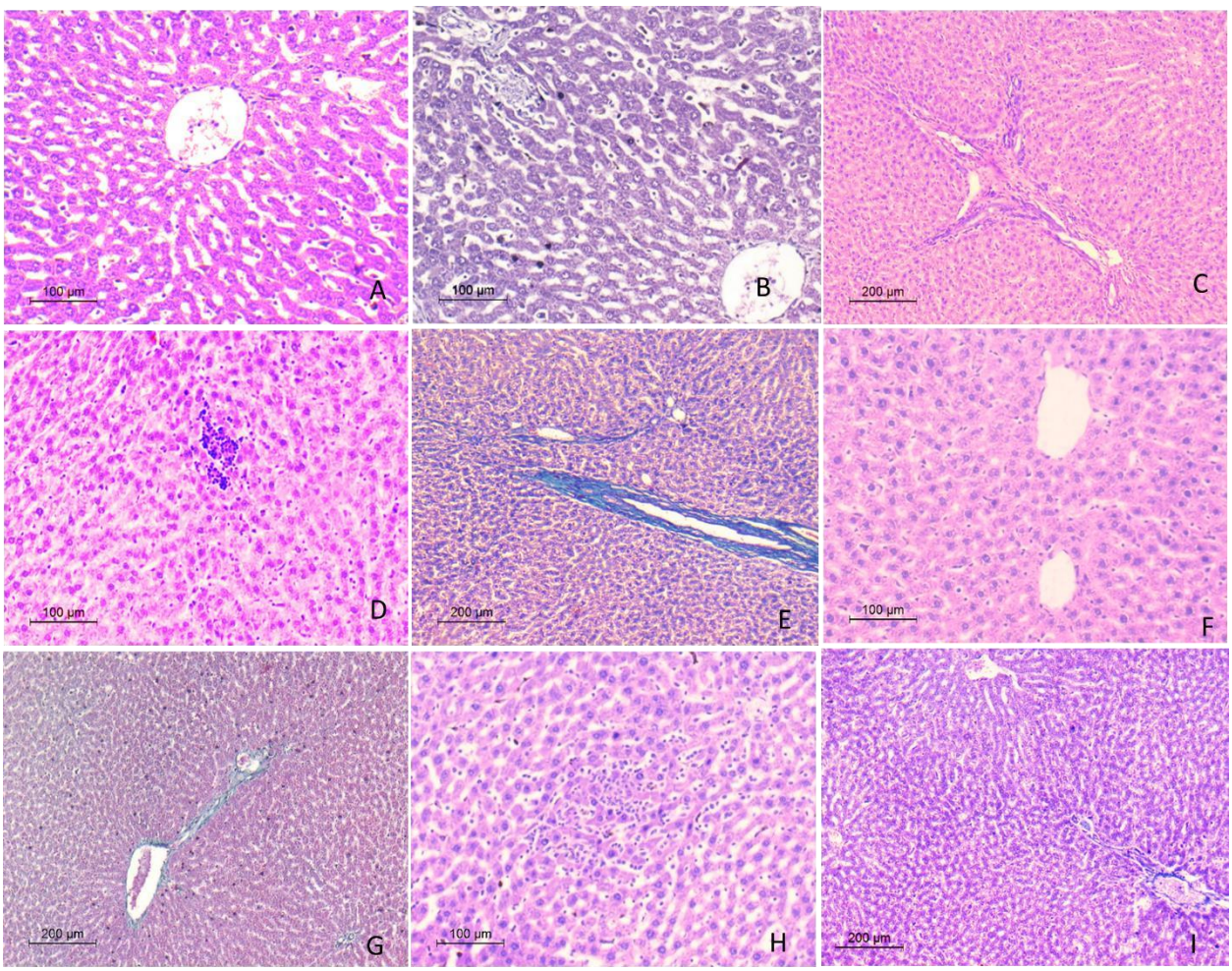

Figure (2) : Histopathological examination of liver sections of studied groups.

Group I [Control] shows normal liver architecture without signs of hepatocellular or lobular injury (a: H&E) and without fibrosis (b: Trichrome). DEN-treated group shows marked portal inflammation (c: H&E), foci of lytic necrosis among hepatocytes showing evident feathery degeneration (d: H&E), portal expansion and fibrosis (e: trichrome). Cisplatin-treated group showing mild hepatocellular degeneration without lytic necrosis (f: H&E) with focal fibrosis (g: Trichrome). Cisplatin+ anthocyanins -treated group shows mild lobular inflammation with mild feathery degeneration in hepatocytes (h: H&E) but without portal fibrosis (i: trichrome).

List of abbreviations

APAF-1: Apoptotic protease activating factor-1
cDNA: Complementary deoxyribonucleic acid
CpG: Cytosine-Phosphate-Guanine (dinucleotide sequence)
Ct: Cycle Threshold
DEN: Diethyl-nitrosamine
DNMTs: DNA Methyltransferases
ELISA: Enzyme-Linked Immunosorbent Assay
GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase
H&E: Hematoxylin and eosin (staining technique)
HCC: Hepatocellular Carcinoma
ICL: Inter strand Cross-Link
IL: interleukin
LINEs: Long interspersed nucleotide elements
MAPK: Mitogen-Activated Protein Kinase
MMR: Mismatch Repair
MSH2: MutS homolog 2 (a gene involved in DNA mismatch repair)
MSH6: MutS Homolog 6
MSI: Microsatellite Instability
MSI-H: Microsatellite Instability-High
NADPH: Nicotinamide Adenine Dinucleotide Phosphate
NAFLD: Non-alcoholic fatty liver disease
Nrf2: Nuclear Factor Erythroid 2-Related Factor 2
p: Probability value (statistical significance level)
p53: Tumor suppressor protein 53
PRC2: Poly comb Repressive Complex 2
qPCR: Quantitative Polymerase Chain Reaction
q RT-PCR: Quantitative Real-Time Polymerase Chain Reaction
RNA: Ribonucleic Acid
ROS: Reactive oxygen species
SINEs: Short interspersed nucleotide elements
SNP: Single Nucleotide Polymorphism
SPSS: Statistical Package for the Social Sciences
TE Buffer: Tris-EDTA Buffer
TGFβ/Smad/ERK: Transforming Growth Factor Beta/Smad/Extracellular Signal-Regulated Kinase
TGFβ: Transforming Growth Factor Beta
TNF: tumor necrosis factor
TSGs: Tumor suppressor genes