Advanced Journal of Microbiology Research Vol. 2008
Available online at http://internationalscholarsjournals.org/journal/ajmr

Full Length Research Paper

Diethylnitrosamine-induced hepatocarcinogenesis in rats: possible chemoprevention by blueberries

***Nermin A.H. Sadik, Shohda A. EL-Maraghy, Manal F. Ismail**

Biochemistry Department, Faculty of Pharmacy, Cairo University, Cairo, Egypt.

Accepted 14 March, 2008

Nitrosamine compounds are known hepatic carcinogens. This study was designed to study the efficacy of dietary supplementation with blueberries (BB) on diethylnitrosamine (DEN)-initiated hepatocarcinogenesis in male wistar rats. Rats were divided into three groups. The first group served as normal control group, the second group received DEN at a dose of 10 mg/kg body weight five times a week for 15 weeks. The third one received DEN as in DEN-treated group simultaneously with 4% BB-supplemented diet. The results showed that BB caused significant decrease in the elevated serum levels of -fetoprotein (AFP), homocysteine (Hcy) along with levels of glutathione(GSH), deoxyribonucleic acid (DNA), ribonucleic acid (RNA)and activity of glutathione reductase (GR) in liver. Normalization of elevated 2-macroglobulin (2M) and total antioxidant capacity (TAC) levels in serum, hepatic glutathione-S-transferase (GST), glutathione peroxidase (GPx) activities and liver weight was achieved whereas body weight was significantly decreased. Moreover, no significant change was observed in elevated relative liver weight, hepatic glucose-6-P-dehydrogenase (G6PD), lactate dehydrogenase (LDH) along with serum aminotransferases, alkaline phosphatase (ALP) and -glutamyltransferase (-GT) activities. Significant increase in reduced hepatic activity of xanthine oxidase (XO) was achieved and histopathological damage was minimized in BB-treated group. It is suggested that BB suppress DEN- induced hepatocarcinogenesis and could be developed as a promising chemopreventive natural supplement for liver cancer.

Key words: Blueberries, diethylnitrosamine, hepatocarcinogenesis, 2-macroglobulin, -fetoprotein, homocysteine, glutathione, rats.

INTRODUCTION

Diethylnitrosamine (DEN), a hepatocarcinogen, is known to cause perturbations in the nuclear enzymes involved in deoxyribonucleic acid (DNA) repair/replication and is normally used as a carcinogen to induce liver cancer in animal models (Bhosale et al., 2002). DEN has been shown to be metabolized to its active ethyl radical metabolite, and the reactive product interacts with DNA causing mutation, which would lead to carcinogenesis (Anis et al., 2001; Chakraborty et al., 2007). Experimental, clinical and epidemiological studies have provided evidences supporting the role of reactive oxygen species in the etiology of cancer. Diethylnitrosamine has been suggested to cause oxidative stress and cellular injury due to the enhanced formation of free radicals (Ramakrishnan et al., 2006; Valko et al., 2006).

Nitrate and nitrite are added to meat and fish for the purpose of preservation, as colour fixatives and as flavouring. Ingestion of nitrite and nitrate can result in the endogenous formation of nitroso compounds, particularly in the presence of nitrosatable precursors, such as primary amines, in the acidic condition of the stomach (Lin et al., 2002). In addition, exposure of man to preformed nitrosamines occurs due to the use of tobacco products, cosmetics, pharmaceutical products and agricultural chemicals (Hecht, 1997). In humans, average intake of nitrosamines from food is approximately 1 mg/day (Scanlan, 1983).

Recent approach of chemoprevention serves as an attractive alternative to control malignancy (Guruswamy et al., 2008). Epidemiologic and clinical studies have shown that dietary intakes of blueberries (BB) may have

*Corresponding author. Email: nerminsadik@yahoo.com. Fax. 00202 3635140

potential to reduce various types of cancers (Pan et al., 2007; Wilms et al., 2007), including liver cancer (Yi et al., 2006). Blueberries, nature's only 'blue' food, are a rich source of polyphenols, potent antioxidants that include phenolic acids, tannins, flavonols and anthocyanins. The main anthocyanidins present are delphinidin, cyanidin, petunidin, peonidin and malvidin. Yi et al. (2006) have found that phenolic compounds in blueberries could inhibit HepG2 liver cancer cell population growth and induce apoptosis. Moreover, blueberry constituents are likely to act by mechanisms that counteract oxidative stress, decrease inflammation, and modulate macromolecular interactions and expression of genes associated with disease processes (Neto, 2007).

Several biochemical markers have been suggested for biomonitoring the actions of anticancer agents. Serum -fetoprotein (AFP) is a useful tumour marker for the detection and monitoring of liver cancer development although the false negative rate with AFP level alone may be as high as 40% for patients with small size tumour (Qin and Tang, 2002; Yao et al., 2006). Recently, 2-macro-globulin (2M), a homotetrameric major acute-phase glycoprotein has been suggested as a novel cytochemical marker characterizing preneoplastic and neoplastic rat liver lesions negative for hitherto established cyto- chemical markers (Sukata et al.,2004). It is only recently that homocysteine (Hcy) has been implicated in increased cancer susceptibility and development.

The present study aims to carry out a systematic investigation of the protective influences of blueberries against DEN-induced hepatocarcinogenesis by analyzing serum -fetoprotein, 2-macroglobulin and homocysteine levels along with the non-enzymatic and enzymatic antioxidants as biochemical end points of chemoprevention.

MATERIAL AND METHODS

Chemicals

Blueberries were kindly provided as a gift from Dr Jeremy Spencer, School of Food Biosciences, University of Reading, UK. Blueberries are obtained from local market. Whole blueberries (500g) were lyophilized (freeze dried) for 72h using a Vickers accelerated freeze-drier, pilot plant model. HPLC analysis revealed that 1 g of freeze-dried blueberries contained 22 mg of anthocyanins (Spencer et al. unpublished data). Freeze-dried blueberries were kept at -20 °C. Blueberries were mixed with diet daily and presented fresh to rats. Diethylnitrosamine was purchased from Sigma-Aldrich Chemicals Co., St. Louis, USA. All other chemicals were of analytical grade.

Experimental design

Male Wistar rats weighing (140 – 160 g) were obtained from the animal house of the Faculty of Medicine, Cairo University, Cairo, Egypt and were used after a 1-week acclimatization period. Animals were divided into three groups. The first group (untreated control) received neither DEN nor BB. The second group (DEN) received DEN intragastrically at a dose of 10 mg/kg 5 times a week for 15 weeks (Karimov et al., 2003). The third group (DEN + BB) received DEN as mentioned in second group along with dietary supplementation of blueberries at a dose of 4 g/100g diet (Spencer et al. unpublished data).

Tissue sampling and biochemical assays

At the end of the experimental period, the body weight of each rat was taken before sacrifice. Blood was collected and serum was separated for the estimation of AFP, 2M, Hcy and total antioxidant capacity (TAC) levels and alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and -glutamyltransferase (-GT) activities.

The abdominal cavity of rats was dissected immediately after decapitation and the liver was rapidly removed, washed by ice-cold saline, weighed and blotted dry. A portion of the liver was homogenized in ice-cold saline using Potter–Elvejhem glass homogenizer and the homogenate was divided into three portions. A portion of the homogenate was mixed with 0.1M Tris buffer pH 8.1, containing 1 mM phenylmethylsulphonyl fluoride (PMSF) and centrifuged at 105000 xg at 4 °C for 40 min using Dupont Sorvall ultracentrifuge (USA) . The resulting cytosolic fraction was used for the determination of glutathione-S-transferase (GST), glutathione reductase (GR), glutathione peroxidase (GPx), glucose-6-P dehydrogenase (G6PD), lactate dehydrogenase (LDH) and xanthine oxidase (XO) activities. Another portion was mixed with sucrose/Tris buffer, pH 7.5, for subsequent estimation of DNA and ribonucleic acid (RNA) levels. The remaining portion was precipitated with 7.5% 5-sulfosalicylic acid ,centrifuged at 600xg for 15 min and the resulting protein- free supernatant obtained was used for the estimation of GSH level. Finally, portion of the liver from all the animals was formalin-fixed and paraffin-embedded for subsequent histopathological examination.

Serum level of AFP was estimated using ELISA kit supplied from Anogen (Mississauga, Ontario, Canada). Serum 2M was determined using radial-immuno diffusion kit (Biocientifica SA, Buenos Aires-Argentina), whereas serum Hcy level was measured using Axis Homocysteine EIA kit (Axis-Shield AS, Germany). Serum ALT and AST were assayed using commercially available kits provided by ProDia international, Germany, according to the method of Reitman and Frankel (1957). Serum ALP was estimated using kit supplied by Biolabo SA, France according to method described by Kind and King (1954) whereas serum -GT was measured by the method of Orlowski and Meister (1965) using kit supplied by Scalvo Diagnostics, Italy. TAC was measured in serum by the ferric reducing antioxidant power (FRAP) assay (Benzie and Strain,1996) which measures the ferric reducing ability of antioxidants in serum.

Measurement of liver cytosolic enzyme activities of GST (Habig et al., 1974), GR (Long and Carson, 1961), GPx (Paglia and Valentine, 1967), G6PD (Kornberg and Horecker, 1955) and XO (Prajda and Weber, 1975) were carried out. LDH activity was measured using commercially available kit provided by Stanbio, San Antonio, TX, USA, according to the method of Buhl and Jackson (1978). GSH was determined according to the method of Beutler et al. (1963). Hepatic levels of DNA and RNA were estimated according to method of Burton (1956) and Blobel and Potter (1968), respectively as modified by Giles and Myers (1965). The protein content was measured by the method of Lowry et al. (1951).

Statistical analysis

Data were expressed as the mean ± S.E.M. Means were compared by one – way analysis of variance (ANOVA) followed by Tukey-Kramer test, which was used to identify differences between groups. A value of $P< 0.05$ was accepted as significant.

RESULTS

General findings

Significant increase was observed in serum levels of AFP, 2M, Hcy along with activities of ALT, AST, ALP

Table1. Effect of blueberries on DEN-induced alteration in serum AFP, 2M, Hcy, liver function tests, body weight, liver weight and relative liver weight.

	Control	DEN	DEN + blueberries
AFP(ng/ml)	0.4 ±0.03	0.96 ±0.06 [a]	0.64±0.036 [ab]
2M(mg/dl)	132.9 ± 3.6	225.7 ± 5.3[a]	135.7 ± 2 [b]
Hcy(µmol/l)	4.2±0.26	9.6±0.6 [a]	6.4±0.25 [ab]
ALT(U/l)	80 ± 6.9	123.4 ± 6.2[a]	118.3 ± 8.5[a]
AST(U/l)	90.8 ± 4.4	129.7 ± 9.9[a]	112.4 ± 8.7[a]
ALP(U/l)	79.4 ± 3.1	121 ± 0.4[a]	113.9 ± 2.9[a]
-GT(U/l)	1 ±0.03	5.3 ±0.4[a]	5.9 ±1.1[a]
Body weight(g)	257.9 ± 11	237 ± 7.2	176.4 ± 10.3[ab]
Liver weight(g)	5.8 ± 0.08	7.3 ± 0.4[a]	4.7 ±0.44[b]
Relative liver weight (g %)	2.4± 0.06	2.95 ± 0.11 [a]	3 ± 0.21 [a]

Values represent mean ± S.E.M for seven rats. Comparisons are made as follows: (a) with control group; (b) with DEN group. Values are statistically significant at $P < 0.05$.

Table 2. Effect of blueberries on DEN-induced alteration in antioxidant status.

	Control	DEN	DEN +blueberries
GST(U/mg protein)	415.6 ± 20.6	847.3 ± 68.7[a]	546 ± 59.2[b]
GR(U/mg protein)	131 ± 7.1	255.7 ± 15.8[a]	194.1± 4.1[ab]
GPx(U/mg protein)	278.2 ± 29.9	436.4 ± 16.2[a]	335.4 ± 17.4[b]
G6PD(mU/mg protein)	16.2 ± 1.1	44.9 ± 6.1[a]	61 ± 10.9[a]
GSH(mg/g)	2 ± 0.1	3.3 ± 0.1[a]	2.7 ± 0.16[ab]
TAC(µmol/l)	702.9 ± 13.3	1182 ± 87.8[a]	675.7 ± 56.8[b]

Values represent mean ± S.E.M for seven rats. Comparisons are made as follows: (a) with control group; (b) with DEN group. Values are statistically significant at $P < 0.05$.

and -GT in the DEN group as compared with the control group. No significant change was observed in body weight in the DEN- treated group whereas increased liver weight and relative liver weight was shown as compared with the control group. BB supplementation resulted in normalization of serum 2M and significant decrease of AFP and Hcy levels whereas no significant change in activities of ALT, AST, ALP and -GT was observed as compared with DEN group. Significant decrease in body weight was detected in BB-group as compared with either control or DEN-group. Blueberries supplementation was able to restore liver weight whereas no significant change was obtained in relative tissue weight as compared with DEN-group as shown in Table (1).

Antioxidants status

As shown in Table (2), significant increase was observed in the hepatic activities of GST, GR, GPx and G6PD along with level of hepatic GSH, serum TAC in DEN-treated group. Blueberries supplementation caused normalization of hepatic GST and GPx activities and serum TAC level. Significant decrease was observed in the elevated hepatic GSH level and GR activity where as no significant change was observed in the hepatic activity of G6PD as compared with the DEN-treated group.

Hepatic levels of DNA, RNA and activities of XO and LDH

As shown in Table (3), significant increase was observed in levels of DNA and RNA and activity of LDH whereas significant decrease was observed in XO activity in liver as compared with the control group. Blueberries supplementation caused significant decrease in hepatic levels of DNA and RNA and increase in XO activity whereas no change was detected in LDH activity.

Histopathological findings

Histopathological examination of liver sections from control animals revealed normal architecture. DEN group showed appearance of hepatocellular carcinoma with enlarged hyperchromatic nuclei and scattered mitosis. Restoration of most of the normal hepatocytes architecture with regular dark nuclei and dysplasia was shown in BB group (Figure 1a-c).

Table 3. Effect of blueberries on DEN-induced alteration in hepatic levels of DNA and RNA along with activities of LDH and XO.

	Control	DEN	DEN + blueberries
DNA(mg/g)	1.77 ± 0.03	2.14 ± 0.06[a]	1.4 ± 0.13 [ab]
RNA(mg/g)	1.3 ± 0.07	2.1 ± 0.1[a]	1.7 ± 0.07 [ab]
LDH(U/mg protein)	2 ±0.09	2.56 ± 0.08[a]	2.6 ± 0.19[a]
XO(U/mg protein)	6.3±0.3	4.3±0.1 [a]	5.2±0.19 [ab]

Values represent mean ± S.E.M for seven rats. Comparisons are made as follows: (a) with control group; (b) with DEN group. Values are statistically significant at $P < 0.05$.

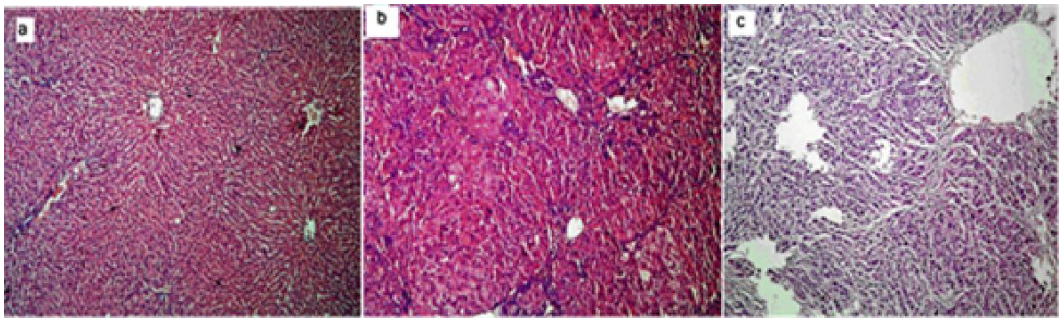

Figure 1. Light photomicrographs of rat liver: (a) control group; (b)DEN-group; (c)DEN+BB-treated group (100 × , HE)

DISCUSSION

It is well known that liver cancer is one of the most important cancers in the world, resulting in more than 1 million patients and over 260,000 deaths per year (Liu et al., 2006). Therefore, the chemoprevention and treatment of liver cancer is very important. AFP, 2M, Hcy, ALT, AST, ALP, -GT, liver weight and relative liver weight are valuable references, widely used in animal studies to diagnose and observe the development of hepatocarcinogenesis (Thirunavukkarasu et al., 2005). In the present study, the values of previously mentioned parameters showed sharp increase in DEN-group as compared with that of the normal control group (Table1).

AFP, a tumour-associated fetal protein, has long been employed as a serum fetal tumour marker to monitor disease progression (Abelev, 1971; Liu et al., 2006). The observed significant increase of serum 2-macroglobulin in DEN-induced rats is in harmony with Sukata et al. (2004) who stated that 2M might be tightly linked to the rat hepatocarcinogenesis from the initial stage to tumour progression even in conditions, which are undetectable, by established cytochemical markers such as placental glutathione-S-transferase (GST-P) and -GT-positive lesions. Sukata et al. (2004) also confirmed that the observed increases in serum 2M concentrations during hepatocarcinogenesis and in animals, bearing hepatic tumours was not a result of secretion by the host liver of 2M as an acute-phase reactant in response to inflammatory injury. 2M functions as a carrier protein and regulator for various growth factors and cytokines such as transforming growth factor- (known to be involved in the onset of hepatocyte apoptosis) (James, 1990). Furthermore, 2M partially counteracts the inhibitory effects of transforming growth factor- on proliferation of neoplastic hepatocytes, suggesting that under some conditions, 2M can promote hepatocarcinogenesis by perturbing transforming growth factor- -induced apoptosis (Wollenberg et al., 1991).

Increased serum Hcy and hepatic DNA and RNA levels along with decreased XO activity seem to be interrelated in the present study. Animal and human studies have increasingly demonstrated associations between folate deficiency, serum Hcy elevations, and a variety of cancers. The observed increase in serum Hcy in our study is suggested to reflect inhibition of homocysteine metabolism due to folate deficiency reported in other studies (Eichholzer et al., 2001; Davis and Uthus, 2004). Folate is important for normal DNA synthesis, repair, and converting homocysteine to methionine (Davis and Uthus, 2004). Therefore, increased demand of folate is postulated to be a result of increased hepatic levels of DNA and RNA (Table 3) and might indicate increased DNA and RNA synthesis and proliferation of cancer cells in response to growth stimulation.

Metabolically active and proliferating tissues require large amounts of purine nucleotides for transmission of metabolic energy and synthesis of nucleic acids. Xanthine oxidoreductase (XOR) catalyzes the final reactions of the purine catabolic pathway, oxidizing hypoxanthine

to xanthine, and xanthine to uric acid. The decrease in XO activity which depresses the ability to degrade purines was thus reported to provide purines which can be recycled for nucleic acid biosynthesis in the salvage pathway (Linder et al., 2005). Progressive decrease of XOR activity has been shown in all hepatomas irrespective of growth rate or differentiation as compared with the corresponding normal tissues (Prajda and Weber, 1975).The observed decrease in liver cytosolic XO activity shown in Table(3) may be attributed to down-regulation of xanthine oxidoreductase which might include loss of heterozygosity at chromosome 2p where XOR is located, decreased gene promoter activity, increased XOR mRNA degradation, or posttranslational changes (O'Connell et al.,1998; Linder et al., 2005).

Results of the present study revealed significant increase in liver cytosolic GST activity in DEN -received group. This increase in hepatic GST activity may be attributed to the fact that induction of GSTs, phase II enzymes that detoxify certain carcinogens, is regarded as a potential mechanism of blockade of the early stages of carcinogenesis. Sato (1989) has found that, of the preneoplastic marker enzymes, rat placental form of glutathione-S-transferase is markedly and specifically inducible in preneoplastic foci. Recently, immunohistochemical studies have revealed significant increase in the number of hepatocellular enzyme altered foci, which stained positive for GST-P (Sakata et al., 2004).

The significant increase of hepatic GSH level observed in the present study is in harmony with Marinho et al. (1997) who also reported that increased GSH concentration in DEN-treated rat liver is probably due to the simultaneous observed two-fold increase in the activities of -GT and - glutamylcysteine synthetase (- GCS), two main enzymes involved in GSH formation. The ubiquity of elevated -GT levels in many rodent and human hepatic and extrahepatic carcinomas (Hanigan and Pitot, 1985) have led to the hypothesis that -GT provides a growth advantage to focal cells during carcinogenesis. A plausible hypothesis is that the advantage may be due to the role of -GT in the transport of GSH constituents, leading to increase in cellular GSH. The latter is required for proliferation and resistance (Komlosh et al., 2002). -GT is reported to act by circumventing the rate- limiting - GCS and its feedback inhibition by GSH and may thus participate in promotion of growth. Previous studies have shown that an increased GSH level was associated with an early proliferative response and was essential for the cell to enter the S phase. The requirement for increased GSH or thiols prior to DNA synthesis may be related to the fact that proliferating cells require increased amounts of pentoses and thiols. DNA synthesis depends absolutely on the formation of pentoses and on their conversion into deoxyribose by ribonucleotide reductase. The activity of this rate-limiting enzyme in DNA synthesis requires reduced glutaredoxin or thioredoxin, which are maintained by GSH. An increase in the cellular GSH content may change the thiol-redox status of the cell that activates genes essential for G_1 to S transition (Holmgren, 1981; Lu, 1999).

GR mediates the reduction of oxidized glutathione (GSSG) to GSH. This reduction reaction requires NADPH, which is supplied by the enzyme G6PD in the pentose phosphate pathway. G6PD is significantly increased in DEN-treated group, which increases the availability of NADPH, which in turn enhances the activity of GR shown in Table 2. The activity of GPx, which is another constituent of GSH redox cycle, also showed a significant increase in DEN-treated rats as reported previously (Sarkar et al., 1995). All these events eventually lead to increase hepatic level of GSH observed in the present study. Increased serum TAC level in DEN- group (Table 2) might be regarded as an adaptive response to oxidative stress imposed by DEN. TAC depicts the synergistic interaction among different antioxidants present in plasma, thus providing an insight into the delicate in vivo balance between oxidants and non-enzymatic antioxidants (Serafini et al., 2000).

The elevated hepatic activity of G6PD and LDH in this study may be related to enhanced glucose metabolism. It was discovered in the 1920s that cancer cells constitutively up regulate glucose metabolism (Warburg, 1930). Thus, cancer cells tend to synthesize ATP mainly through 'glycolysis', a metabolic state that is linked to high glucose uptake and local acidification owing to lactate production. Gatenby and Gillies (2004) and Zu and Guppy (2004) have reported that when glycolysis prevails, pyruvate is reduced to lactate in order to reoxidize NADH to NAD that is required for sustained glycollysis. Increased glucose breakdown provides build-ing blocks for the synthesis of nucleotides via the pentose phosphate pathway. In addition, local acidification of the tumour microenvironment may facilitate tumour invasion (Weber, 2001; Kroemer, 2006). Glycolytic enzymes are induced by oncogenes (Plas and Thompson, 2005) or by the hypoxia-inducible transcription factor (Carew and Huang, 2002; King et al., 2006) or a dysfunctional tricarboxylic acid cycle owing to loss of function of mitochondrial tumour suppressor genes (Eng et al., 2003; Gottlieb and Tomlinson, 2005).

In recent years, considerable attention has been paid to anthocyanins due to their abilities to inhibit oxidative stress and cell proliferation (Joseph et al., 2007) . In our study, BB was able to restore serum 2M and to reduce AFP and Hcy levels significantly. The antioxidant potential of blueberries was clarified in the present study from its modifying effect of GSH and its related enzymes. Blueberries supplementation was able to restore hepatic activities of GST and GPx along with serum TAC level. Significant decrease was also observed in hepatic GR and GSH level. The regulations of apoptosis and phase II enzymes such as GST are other potential mechanisms through which flavonoids such as anthocyanins may prevent cancer as reported previously (Boateng et al., 2007;

Srivastava et al., 2007). The results of Srivastava et al. (2007) indicated that apoptosis was confirmed in colon cancer cell line (HT-29 cells) when treated with anthocyanins from blueberries at 50 - 150 µg/ml with consequent decrease of GST activity rather than induce it.

Blueberries and other berries, notably raspberries and strawberries, contain a potent anticarcinogenic compound called ellagic acid. Polyphenols exert antiproliferative and an anti-angiogenic effects that are particularly pronounced in tumour cells (Ray, 2005; Larrosa et al., 2006). Interestingly, adding blueberries, red wine and other sources of polyphenols to the diet is thought to be one reason for the greater slenderness of the French compared with other Europeans. Therefore, it is not surprising that our study showed significant decrease in body weight in BB-group and that this decrease counteracted any significant change in relative tissue weight despite of the normalization of liver weight caused by blueberries. The decrease in body weight in BB- supplemented group may be attributed at least partly to the slowing down of digestion by inhibiting enzymes such as amylase, protease and lipase activities at levels, which could affect carbohydrates, protein, and fat digestion and absorption (McDougall and Stewart, 2005).

In our study, BB was observed to decrease levels of DNA and RNA and to increase activity of XO in liver. Pterostilbene, an active constituent of blueberries, was found to induce apoptosis through activating the caspase cascade via the mitochondrial and Fas/FasL pathway, growth arrest DNA damage- inducible gene (GADD) expression, and by modifying cell cycle progress and changes in several cycle-regulating proteins (Pan et al., 2007). In another study, it was demonstrated that anthocyanidins contribute to the inhibition of tumourigenesis by blocking activation of the mitogen-activated protein kinase (MAPK) pathway. These findings provide the first molecular basis for the anticarcinogenic action of anthocyanidins (Hou et al., 2004).

The data provided so far by the current study and from other laboratories warrants further investigation into the chemopreventive and chemotherapeutic effects of blueberries in clinical studies. In sum, our results indicate that dietary intake of blueberries high in antioxidant activity may be an important component of a healthy living strategy designed to increase our resistance to liver cancer.

Acknowledgment

The authors are thankful to Dr Jeremy Spencer and Dr Manal M Abd El-Mohsen, School of Food Biosciences, University of Reading, UK for kindly providing the freeze-dried blueberries used in the current study.

REFERENCES

Abelev GI (1971). Alpha - fetoprotein in association with malignant tumors. Adv. Cancer Res. 14:295–357.

Anis KV, Rajesh Kumar NV, Kuttan R (2001). Inhibition of chemical carcinogenesis by biberine in rats and mice. J. Pharm. Pharmacol. 53: 763–768.

Benzie IF, Strain JJ (1996). The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. Anal. Biochem. 239(1):70-76.

Beutler E, Duran O, Kelly BM (1963). Improved method for the determination of blood glutathione. J. Lab. Clin. Med. 61: 882-888.

Bhosale P, Motiwale L, Ignle AD, Gadre RV, Rao KVK (2002). Protective effect of Rhodotorula glutinisNCIM3353 on the development of hepatic preneoplastic lesions. Curr. Sci. 83(3): 303–308.

Blobel G, Potter VR (1968). Distribution of radioactivity between the acid-soluble pool and the pools of RNA in the nuclear, non-sedimentable and ribosome fractions of rat liver after a single injection of labelled orotic acid. Biochem. Biophys. Acta. 166: 48-57.

Boateng J, Verghese M, Shackelford L, Walker LT, Khatiwada J, Ogutu S (2007). Selected fruits reduce azoxymethane (AOM)-induced aberrant crypt foci (ACF) in Fisher 344 male rats. Food Chem. Toxicol. 45(5):725-732.

Buhl SN, Jackson KY (1978). Optimal conditions and comparisons of lactate dehydrogenase catalysis of the lactate to pyruvate and pyruvate to lactate reactions in human serum at 25, 30 and 37ºC. Clin. Chem. 24:828-831.

Burton K (1956). A study of the conditions and mechanism of diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem. J. 62: 315-323.

Carew JS, Huang P (2002). Mitochondrial defects in cancer. Mol. Cancer. 9:1-9.

Chakraborty T, Chatterjee A, Rana A, Dhachinamoorthi D, Kumar P A, Chatterjee M (2007). Carcinogen-induced early molecular events and its implication in the initiation of chemical hepatocarcinogenesis in rats: chemopreventive role of vanadium on this process. Biochim. Biophys. Acta. 1772(1):48-59.

Davis CD, Uthus EO (2004). DNA methylation, cancer susceptibility, and nutrient interactions. Exp. Biol. Med. (Maywood). 229(10):988-995.

Eichholzer M, Lüthy J, Moser U, Fowler B (2001). Folate and the risk of colorectal, breast and cervix cancer: the epidemiological evidence. Swiss Med. Wkly. 131(37-38):539-549.

Eng C, Kiuru M, Fernandez MJ, Aaltonen LA(2003). A role for mitochondrial enzymes in inherited neoplasia and beyond. Nat. Rev. Cancer. 3(3):193-202.

Gatenby RA, Gillies RJ (2004). Why do cancers have high aerobic glycolysis?. Nat. Rev. Cancer. 4(11): 891–899.

Giles KW, Myers A (1965). An improved diphenylamine method for the estimation of deoxyribonucleic acid. Nature. 4979-4993.

Gottlieb E, Tomlinson IP (2005). Mitochondrial tumour suppressors: a genetic and biochemical update. Nat. Rev. Cancer. 5(11):857-866.

Guruswamy S, Swamy MV, Choi CI, Steele VE, Rao CV (2008). S-adenosyl L-methionine inhibits azoxymethane-induced colonic aberrant crypt foci in F344 rats and suppresses human colon cancer Caco-2 cell growth in 3D culture. Int. J. Cancer. 122(1):25-30.

Habig WH, Pabst MJ, Jakoby WB (1974). Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. J. Biol. Chem. 249(22):7130-7139.

Hanigan MH, Pitot HC (1985). -Glutamyl transpeptidase: its role in hepatocarcinogenesis. Carcinogenesis. 6: 165–172.

Hecht SS (1997). Approaches to cancer prevention based on an understanding of N-nitrosamine carcinogenesis. Proc. Soc. Exp. Biol. Med. 216 (2):181–191.

Holmgren A (1981). Regulation of ribonucleotide reductase. Curr. Top. Cell. Regul. 19:47-76.

Hou DX, Kai K, Li JJ, Lin S, Terahara N, Wakamatsu M, Fujii M, Young MR, Colburn N (2004). Anthocyanidins inhibit activator protein 1 activity and cell transformation: structure-activity relationship and molecular mechanisms. Carcinogenesis. 25(1):29-36.

James K (1990). Interactions between cytokines and 2-macroglobulin. Immunol. Today. 11:163–166.

Joseph JA, Shukitt-Hale B, Lau FC (2007). Fruit polyphenols and their effects on neuronal signaling and behavior in senescence. Ann. N Y Acad. Sci. 1100:470-485.

Karimov KhY, Inoyatova FKh, Mukhamedova MT (2003). Changes in some indices of the synthesis of nitric oxide during the early stages of hepatocarcinogenesis. Exp. Toxicol. Pathol. 55(1):17-19.
Kind PRN, King EJ (1954). Estimation of plasma phosphatase by determination of hydrolysed phenol with amino antipyrine. J. Clin. Path. 7:322-326.
King A, Selak MA, Gottlieb E (2006). Succinate dehydrogenase and fumarate hydratase: linking mitochondrial dysfunction and cancer. Oncogene. 25(34):4675-4682.
Komlosh A, Volohonsky G, Porat N, Tuby C, Bluvshtein E, Steinberg P (2002). gamma-Glutamyl transpeptidase and glutathione biosynthesis in non-tumorigenic and tumorigenic rat liver oval cell lines. Carcinogenesis. 23(4):671-678.
Kornberg A, Horecker, BL (1955). Methods Enzymol, (Colowick SD, Kaplan NO (eds), Acad. Press, New York, 1:823.
Kroemer G (2006). Mitochondria in cancer. Oncogene. 25, 4630–4632.
Larrosa M, Tomás-Barberán FA, Espín JC (2006). The dietary hydrolysable tannin punicalagin releases ellagic acid that induces apoptosis in human colon adenocarcinoma Caco-2 cells by using the mitochondrial pathway. J. Nutr. Biochem. 17(9):611-625.
Lin K, Shen ZY, Lu SH, Wu YN (2002). Intake of volatile N-nitrosamines and their ability to exogenously synthesize in the diet of inhabitants from high-risk area of esophageal cancer in southern China. Biomed. Environ. Sci. 15:277-282.
Linder N, Lundin J, Isola J, Lundin M, Raivio KO, Joensuu H (2005). Down-regulated xanthine oxidoreductase is a feature of aggressive breast cancer. Clin. Cancer Res. 11(12):4372-4381.
Liu JG, Zhao HJ, Liu YJ, Wang XL (2006). Effect of selenium -enriched malt on hepatocarcinogenesis, paraneoplastic syndrome and the hormones regulating blood glucose in rats treated by diethylnitrosamine. Life Sci. 78(20):2315-2321.
Long WK, Carson PE (1961). Increased erythrocyte glutathione reductase activity in diabetes mellitus. Biochem. Biophys. Res. Commun. 5:394-399.
Lowry OH, Rosebrough NJ, Farr AL, Randall RG (1951). Protein measurement with folin reagent. J. Biol. Chem. 193: 265-275.
Lu SC(1999). Regulation of hepatic glutathione synthesis: current concepts and controversies. FASEB J. (10):1169-1183.
Marinho HS, Baptista M, Pinto RE (1997). Glutathione metabolism in hepatomous liver of rats treated with diethylnitrosamine. Biochim. Biophys. Acta. 1360(2):157-168.
McDougall GJ, Stewart D (2005). The inhibitory effects of berry polyphenols on digestive enzymes. Biofactors. 23(4):189-195.
Neto CC (2007). Cranberry and blueberry: evidence for protective effects against cancer and vascular diseases. Mol. Nutr. Food Res. 51(6):652-664.
O'Connell P, Pekkel V, Fuqua SA, Osborne CK, Clark GM, Allred DC (1998). Analysis of loss of heterozygosity in 399 premalignant breast lesions at 15 genetic loci. J. Natl. Cancer Inst. 90: 697-703.
Orlowski M, Meister A (1965). Isolation of -glutamyl transpeptidase from hog kidney. J. Biol. Chem. 240:338– 347.
Paglia DE, Valentine WN (1967). Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. J. Lab. Clin. Med. 70:158-169.
Pan MH, Chang YH, Badmaev V, Nagabhushanam K, Ho CT (2007). Pterostilbene induces apoptosis and cell cycle arrest in human gastric carcinoma cells. J. Agric.Food Chem. 55(19):7777-7785.
Plas DR, Thompson CB (2005). Akt-dependent transformation: there is more to growth than just surviving. Oncogene. 24(50):7435-7442.
Prajda N, Weber G (1975). Malignant transformation-linked imbalance: decreased xanthine oxidase activity in hepatomas. FEBS Lett. 59(2):245-249.
Qin LX, Tang ZY (2002). The prognostic molecular markers in hepatocellular carcinoma. World J. Gastroenterol. 8 (3):385-392.
Ramakrishnan G, Raghavendran HR, Vinodhkumar R, Devaki T(2006). Suppression of N-nitrosodiethylamine induced hepatocarcinogenesis by silymarin in rats. Chem. Biol. Interact. 161(2):104-114.
Ray A (2005). Cancer preventive role of selected dietary factors. Indian J. Cancer. 42(1):15-24.
Reitman S, Frankel SA (1957). Colorimetric method for determination of serum glutamic oxalacetic and glutamic pyruvic transaminases. Am. J. Clin. Pathol. 28: 56–63.
Sakata K, Hara A, Hirose Y, Yamada Y, Kuno T, Katayama M (2004). Dietary supplementation of the citrus antioxidant auraptene inhibits N,N-diethylnitrosamine-induced rat hepatocarcinogenesis. Oncology. 66(3):244-252.
Sarkar A, Mukherjee B,Chatterjee M(1995). inhibition of 3' -methyl-4-dimethylaminoazobenzene-induced hepatocarcinogenesis in rat by dietary beta-carotene: changes in hepatic anti-oxidant defense enzyme levels. Int. J. Cancer. 61(6):799-805.
Sato K (1989). Glutathione transferases as markers of preneoplasia and neoplasia. Adv. Cancer Res. 52:205–255.
Scanlan RA (1983). Formation and occurrence of nitrosamines in food. Cancer Res. 43(5 Suppl): 2435s-2440s.
Serafini M, Natella F, Scaccini, N (2000). Total antioxidant capacity to assess redox status: critical view and experimental data. Free Radic. Biol. Med. 29:1106–1114.
Srivastava A, Akoh CC, Fischer J, Krewer G (2007). Effect of anthocyanin fractions from selected cultivars of Georgia-grown blueberries on apoptosis and phase II enzymes. J. Agric. Food Chem. 55(8):3180-3185.
Sukata T, Uwagawa S, Ozaki K, Sumida K, Kikuchi K, Kushida M (2004). alpha(2)-Macroglobulin: a novel cytochemical marker characterizing preneoplastic and neoplastic rat liver lesions negative for hitherto established cytochemical markers. Am. J. Pathol. 165(5):1479-1488.
Thirunavukkarasu C, Premkumar K, Jagadeeswaran R, Sakthisekaran D (2005). The inhibitory effect of sodium selenite on N-nitrosodiethylamine-induced and phenobarbital promoted liver tumourigenesis in rats based on the modulation of polyamine levels. Mol. Cell Biochem. 280(1-2):165-172.
Valko M, Rhodes CJ, Moncol J, Izakovic M, Mazur M (2006). Free radicals, metals and antioxidants in oxidative stress-induced cancer. Chem. Biol. Interact. 160(1):1-40.
Warburg O (1930). The metabolism of tumours. Constable: London.
Weber G (2001). Ordered biochemical program of gene expression in cancer cells. Biochemistry (Mosc). 66(10):1164-1173.
Wilms LC, Boots AW, de Boer VC, Maas LM, Pachen DM, Gottschalk RW(2007). Impact of multiple genetic polymorphisms on effects of a 4-week blueberry juice intervention on ex vivo induced lymphocytic DNA damage in human volunteers. Carcinogenesis. 28(8):1800-1806.
Wollenberg GK, LaMarre J, Semple E, Farber E, Gauldie J, Hayes MA (1991). Counteracting effects of dexamethasone and alpha 2-macroglobulin on inhibition of proliferation of normal and neoplastic rat hepatocytes by transforming growth factors-beta type 1 and type 2. Int. J. Cancer. 47:311–316.
Yao DF, Wu W, Yao M, Qiu LW, Wu XH, Su XQ (2006). Dynamic alteration of telomerase expression and its diagnostic significance in liver or peripheral blood for hepatocellular carcinoma. World J. Gastroenterol. 12(31):4966-4972.
Yi W, Akoh Casimir C, Fischer J, Krewer G (2006). Effects of phenolic compounds in blueberries and muscadine grapes on HepG2 cell viability and apoptosis. Food Res. Int. 39(5): 628-638.
Zu XL, Guppy M (2004).Cancer metabolism: facts, fantasy, and fiction. Biochem. Biophys. Res. Commun. 313(3): 459–465.