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Evaluation of the Possible Antioxidant Effects of Soybean and Nigella Sativa During Experimental Hepatocarcinogenesis by Nitrosamine Precursors.

[Nitrozamin Öncülleri ile Deneysel Hepatokarsinojenez Oluşumunda Soya Fasulyesi ve Nigella Sativa'nın Muhtemel Oksidasyon Engelleyici Etkilerinin Değerlendirilmesi]

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ABSTRACT

This study was designed to evaluate the possible antioxidant and antihepatocarcinogenic effects of soybean (SB) and Nigella sativa (NS) in rats administered nitrosamine precursors dibutylamine and sodium nitrate in the drinking water.
Rats were randomly divided into four groups, each containing 48 animals. Group I (control), group II (dibutylamine (DBA) + $NaNO_3$), group III (DBA/$NaNO_3$ + SB treated) and group IV (DBA/$NaNO_3$ + NS treated). Markers of oxidative stress [reduced glutathione (GSH) and nitric oxide (NO)] and the levels of HER-2/neu, bcl-2 and p53 proteins were investigated in rats liver after 2, 4, 6, 8, 10 and 12 months. The data revealed an improvement in the status of oxidative stress represented as the compensation of GSH levels after 2 and 4 months and reduction of NO levels after 2 months; a significant improvement in the levels of HER-2/neu protein after 6 and 4 months in rats fed SB and NS, respectively. Although, the level of p53 protein was ameliorated compared with the treated with nitrosamine precursor rats, it was maintained in a lower levels compared with the time matched points of the normal controls until 12 months Both SB and NS improved the levels of bcl-2 after 2 months in both groups. The data suggested that oral feeding of the diet containing SB and NS antagonized the oxidative stress effects induced by DBA/$NaNO_3$.

Key Words: Soybean, Nigella sativa, Antioxidant, Nitrosamine, Oxidative Stress, Hepatocarcinogenesis, Her-2/neu, p53, bcl-2, GSH, NO.

ÖZET

Bu çalışmada soya fasulyesi (SB) ve nigella sativa (NS)'nın içme suyu ile nitrozamin öncülleri dibutilamin ve sodyum nitrat verilmiş sıçanlardaki antioksidan ve anti hepatokarsinojenik etkilerinin araştırılması hedeflenmiştir.
Bu amaçla sıçanlar, her biri 48 hayvandan oluşan gelişigüzel dört gruba ayrılmıştır: Grup I (kontrol), grup II (DBA/$NaNO_3$+ NS verilmiş) grup III (DBA/$NaNO_3$+ SB verilmiş). Sıçan karaciğerinde 2, 4, 6, 8, 10 ve 12 ay sonra oksidatif stres belirteçleri indirgenmiş glutatyon (GSH) ve nitrik oksit (NO) ile HER-2/nem, bcl-2 ve p53 proteinlerinin düzeyleri incelenmiştir. Elde edilen sonuçlar, SB verilen sıçanların oksidatif durumlarında, 2 ve 4 ay sonra GSH düzeyinde düzelme ve 2 ay sonra NO düzeyindeki düşmeyle belirlenen iyileşme olduğunu göstermiştir. HER-2/neu proteininde de anlamlı değişiklik görülmüştür. Ayrıca, p53 protein düzeyi nitrozamin öncülü verilmiş sıçanlarda artmakla birlikte normal kontrollere oranla 12 ay süreyle düşük kalmıştır. Bulgular besinle alınan SB ve NS, DBA/$NaNO_3$ ile oluşturulan oksidatif stresin etkilerini bertaraf ettiğini göstermektedir.

Anahtar Kelimeler: Soya Fasulyesi, Nigella Sativa, Antioksidan (oksidan karşıtları/oksidasyon engelleyici), Nitrozamin, oksidatif stres, hepatokarsinojenez, Her-2/neu, p53, bcl-2, GSH, NO

INTRODUCTION

Nitrosoamines play an important role in hepatocarcinogenesis. They are predominant compounds in human environment found in food (such as cured meat and fish) and tobacco (1,2) in addition to the occupational exposure (3). The endogenous production of these compounds takes place in both stomach and colon due to the reaction of their precursors (amines and nitrosating agents), which is catalyzed by acid or bacteria (4,5). Nitrosation of dibutylamine (DBA), for example, forms di-n-butylnitrosamine (DBN), which is known for its carcinogenic effect in different species (6) and organs (7). DBN induced pathological changes in lungs (8) developed nasal epithelial hyperplasia and squamous metaplasia, inflammation and necrosis in rats (9). On the cellular level, DBN developed a structural chromosomal aberrations and increased mitotic indices (10,11) and severe changes in the structure of the hepatic cells (12).

Many trials have been undertaken to investigate the chemopreventive effects of natural products against the carcinogenic effect of nitrosamines. Soybean (SB) and Nigella sativa (NS) have been used in Asia, Middle East and Africa to promote health and protected form diseases (13,14).

Oxidative stress during carcinogen metabolism seems to participate in liver tumor production in rats (15). The inclusion of both bcl-2 and p53 was attributed to the previous reports indicating that the apoptotic effect of thymoquinone, the most active constituent in NS, was modulated by bcl-2 protein and were linked to and depended on p53 in colon cancer (16). Also, the over expression of HER-2/neu was found to be correlated with increased tumor aggressiveness (17) and targeted by soybean isoflavones in bladder cancer (18). The present work investigates, the gradual changes in the levels of some markers of oxidative stress and the protein levels of three cancer related genes (Human epidermal growth factor receptor, HER-2/neu, bcl-2 and p53) in liver tissue of rats administrated with DBN precursors in combination with SB or NS seeds.

MATERIALS and METHODS

Experimental animals

The present work included 192 young male Swiss Albino rats weighing about 30-40 g. Rats were housed in breeding cages and received a similar basic care, in compliance with international ethical standards, during the study period. Initially, before starting the experiment rats were fed with standard commercial diet (Egyptian Company of Oils and Soaps) and drink tap water ad libitum for 7 days.

Dosage and Administration

Nitrosamine precursors namely DBA and sodium nitrate (19) were given in the drinking water in a concentrations of 1000 part per million (ppm) for DBA (Sigma Diagnostics Inc, USA) and 2000 ppm for sodium nitrate (Sigma Diagnostics Inc, USA). Both SB (Egyptian Poultry Co.) Soybean and Nigella Sativa, however, were administared as a powder mixed with the diet in concentrations of 20 % and 10 % (weight/weight of control diet), respectively.

Animals grouping

According to the treatment of rats with the carcinogen precursors, SB or NS, rats were categorized into 4 groups (48 rats each). Group 1 included untreated rats (as normal healthy control group), fed standard diet which composed of (24 % proteins, 5.55 % fibers, 5.5 % ash) and drink tap water. Group 2 included rats fed on the carcinogen precursors. Group 3 included rats administrated with the carcinogen precursors in drinking water and SB in diet. Group 4 included rats co-administrated with a diet containing NS and water containing the carcinogen precursors. Rats in different groups were, serially sacrificed, where 8 rats from each group were killed every 2 months, the whole liver of tissues were stored at –80 ^{o}C until the use. A portion of liver was fixed in 10 % formalin, section in paraffin, stained with haematoxyline and eosine and histologically examined. For biochemical investigations, liver tissues were homogenized in phosphate buffer 100 mm, pH 7.4 and used to determine the parameters listed below.

Analytical procedures

1- Determination of oxidative stress markers

Reduced glutathion (GSH) concentration was determined in fresh deproteinized liver tissue homogenate using trichloroacetic acid as deproteinizing agent according to the methods described by Beutler and Kelley (20). The method based on the reduction of 5,5' dithiobis- (2 nitro-benzoic acid) (DTNB) with GSH to produce yellow color, which is spectrophotometrically measured at 412 nm. Nitric oxide (NO), on the other hand, was estimated using Nitric oxide colorimeteric assay Kit (Boehringer Mannheim, Cat No. 1 756 281) following the manufacturer instructions. The method based on reduction of nitrate to nitrite by reduced nicotinamide adenine dinucleotide phosphate (NADPH) in the presence of nitrate reductase. The nitrite formed reacts with sulphanilamide and N- (1-naphthyl) -ethylenediamine dihydrochloride to give a red-violet diazo dye, which is measured at 550 nm.

2-Determination of HER-2/neu, bcl-2 and p53 proteins

The Triton c-erbB-2 tissue Extract EIA kit (Triton-Ciba Corning Diagnostics, CA, USA) was used to estimate the level of HER-2/neu protein in rat liver tissue following the manufacturer instructions. Bcl-2 ELISA kit (Enzyme linked-immuno-sorbent Assay) (Oncogene, Research Products, MA, USA, Cat# QIA23) was used to estimate the bcl-2 protein. This method uses anti-bcl-2 monoclonal antibody immobilized on microwells and

standard bcl-2. According to the manufacturer protocol, samples and detector monoclonal antibody were incubated into the wells. After washing out the unbound bcl-2 protein, HRP-conjugated anti-FITC antibody was added. The absorbance of the developed yellow color was measured at 450 nm and applied to the standard curve (constructed in parallel to the unknown samples) to determine the concentration of bcl-2 protein.

Assay designs p53 titerzyme enzyme immunometric

Assay (EIA) kit (Assay designs, catalog No. 900-117, MI, USA) was used to estimate p53 protein following the manufacturer guidelines. The protocol based on binding of the p53 (from unknown samples or standards) with a monoclonal antibody specific for p53 coating the wells of the microtiter strips provided. A polyclonal antibody to p53 labeled with enzyme HRS was added followed by incubation and washing out the unbound labeled antibody, the reaction was stopped and the color generated was measured at 450 nm. The concentration corresponding to the absorbance of the test samples was determined using a standard curve, which was simultaneously constructed with the test samples.

Statistical analysis:

Data at different time points were expressed as mean ±standard deviation. Mean values were compared using variance non-parametric analysis of variance (ANOVA) followed by Duncan's multiple range test. P values less than 0.05 was considered significant. All statistical analysis was performed using GraphPad software (GraphPad Software, Inc. CA, USA).

RESULTS

Effect of soybean and Nigella sativa on the markers of oxidative stress

In normal control group, GSH levels were almost stable over 12 months with a mean value of 33.19 ± 1.42 mmol (Table 1). Treatment of rats with the nitrosamine precursors (DBA+$NaNO_3$), significantly decreased the GSH levels over the different periods of treatment (2, 4, 6, 8, 10 and 12 months) compared to the normal healthy rats ($P < 0.001$). In rats which received the nitrosamine precursors (group II), GSH showed its lowest level (17.11 ± 1.90 mmol) at the end of study (12 months). This value was significantly lower than the first measurement at 2 months ($P < 0.05$). Compared with the corresponding period of treatment of group II, both SB (group III) and NS (group IV), significantly, elevated the level of GSH to 24.88 ± 2.65 mmol after 2 months and to 24.38 ± 1.98 mmol after 4 months, respectively. SB and NS were able to, completely restore the normal levels of GSH after 10 months, where GSH after 10 and 12 months were more or less within normal level.

In normal healthy control rats (group I) insignificant changes were observed in the level of NO during the study, where the average NO concentration was 17.83 ± 1.90 mM. In the group of animals received nitrosamine precursors (group II), NO levels were, significantly increased during the different periods of treatment compared to the control rats ($P < 0.001$ after 2, 4, 6, 8, 10 and

Table 1. The level of reduced glutathione (GSH) and nitric oxide (NO) in the liver tissue homogenate of different groups after different periods of treatment

parameter	Group	periods of treatment (Months) 2	4	6	8	10	12
GSH (mmol)	I	32.85 ± 1.55	34.76 ± 1.48	33.66 ± 1.40	33.98 ± 1.28	31.98 ± 1.36	31.88 ± 1.42
	II	20.69 ± 1.38 c	18.87 ± 1.82 c	20.05 ± 1.36 c	19.78 ± 1.55 c	18.91 ± 1.17 c	17.11 ± 1.90 a, c
	III	24.88 ± 2.65 b,c	26.14 ± 2.06 b,c	27.38 ± 2.86 b,c	28.61 ± 2.08 a b,c	28.88 ± 2.10 a,b	30.00 ± 1.88 a b
	IV	23.61 ± 2.00 c	24.38 ± 1.89 b,c	24.82 ± 1.70 b,c	28.58 ± 1.76 a, b,c	29.18 ± 1.96 a, b	29.66 ± 2.69 a, b
NO (μM)	I	17.19 ± 2.36	15.95 ± 1.83	17.18 ± 1.37	18.10 ± 1.52	19.34 ± 2.22	18.22 ± 2.08
	II	62.61 ± 6.88 c	66.36 ± 4.69 c	68.54 ± 3.96 c	77.38 ± 6.84 a c	80.44 ± 6.35 a,c	83.73 ± 5.36 a c
	III	46.36 ± 2.45 b,c	45.32 ± 4.06 a b,c	31.81 ± 4.23 a b,c	27.38 ± 2.50 a b,c	22.73 ± 2.73 a,b	20.22 ± 3.71 a,b
	IV	48.34 ± 4.88 b,c	44.96 ± 3.80 b,c	42.62 ± 4.91 a b,c	38.50 ± 4.19 a b,c	28.87 ± 3.72 a b,c	20.56 ± 2.46 a,b

Small letters refer to statistically significant difference between the indicated groups versus:

(a) the 2-month measurement in the same group

(b) the time matched point of the carcinogen group (II)

(c) the time matched point of the normal control group (I)

Group: I (normal rats), II (rats fed with DBA/$NaNO_3$), III (rats fed with DBA+ $NaNO_3$ +SB) and IV (rats fed with DBA+ $NaNO_3$+NS).

12 months), where the level increased from 17.19 ± 2.36 mM at 2 months (in group I) to 62.61 ± 6.88 mM (in group II) (P <0.001) with a 3 fold increase. Within group II the NO level increased, progressively to 83.73 ± 5.36 mM after 12 months. Compared with all the corresponding periods of treatment of group II, both SB (group III) and NS (group IV) were able to inhibit the effect of nitrosamine precursors, where a significant reduction in NO level was observed starting 2 month (P <0.001) (Table 1). In group III, the level of NO decreased from 46.36 ± 2.45 mM (at 2 months) to 20.22 ± 3.71 mM (after 12 months). Compared with the corresponding rats in group I, the normalization of NO level by SB was more rapid than NS, where a marked decrease in NO level started after 10 months of treatment compared to 12 months in group IV.

Effects of soybean and Nigella sativa on cancer related genes

Another objective of this study was to follow the protein levels of three cancer related genes (HER-2/neu, bcl-2 and p53) in rats treated with the nitrosamine precursors in combination with SB or NS. The results indicated that administration of the nitrosamine precursors alone in group II, insignificantly affected the HER-2/neu level after 2 months compared with the corresponding of normal control rats. After 4 months the levels of HER-2/neu protein of the carcinogen treated group were significantly higher than the corresponding normal control group (P <0.001 at the time points from 4 to 12). Also, in group II, HER-2/neu level increased progressively after 4, 6, 8, 10 and 12 months (2.52 ± 0.06 ng/ml, 2.88 ± 0.12 ng/ml, 3.03 ± 0.12 ng/ml, 3.25 ± 0.07 ng/ml and 3.23 ± 0.08 ng/ml, respectively) (Table 2). In comparison with group II, a significant reduction in HER-2/neu level was observed after 6 months (1.75 ± 0.13 ng/ml) and after 4 months (1.88 ± 0.41 ng/ml) in rats received SB (group III) and NS (group IV), respectively. Both SB and NS maintained the levels of HER-2/neu proteins in a similar level of the normal control rats.

In the rats receiving nitrosamine precursors (group II) the levels of bcl-2 protein significantly increased over all the periods of treatment compared with the corresponding level of normal control rats fed with standard diet (group I) (P <0.001). The levels progressively increased from 4.64 ± 0.23 at 2 months to 6.66 ± 0.24 at 12 months (Table 2). Compared with rats of group II, SB or NS (groups III and IV, respectively) led to a significant decrease in the bcl-2 levels after 2 months (P <0.01). The normalization of bcl-2 levels by both SB and NS significantly noticed after 6 and 10 months, respectively.

The level of p53 protein in nitrosamine precursors-treated rats (group II) decreased over all the period of treatment compared with the normal healthy control rats (group I). The protein showed its lowest level (26.52 ± 2.66 U/ml) at the end of the treatment period. The rats received SB plus nitrosamine precursors plus (group III) showed increased level of p53 protein particularly at the end of treatment period (12 months) (Table 2). Rats received NS in addition to the nitrosamine precursors (IV) showed similar increase of the p53 protein particularly after 8 months, where the level at this period (35.46 ± 2.86 U/ml) was significantly higher compared to group II. (P <0.001), but still significantly lower than the corresponding normal control rats (P <0.001). In parallel with the oxidative stress and cancer related genes, the histopathological observation of liver tissue was performed at the end of the treatment for all groups. Figure 1 illustrates, a prominent improvements in the liver morphology were observed in rats received SB or NS compared with that received nitrosamine precursors alone.

DISCUSSION

The role of oxidative stress in liver hepatocarcinogenesis is well reported (15). This work focused on the changes in the pattern of the oxidative stress markers and the cancer related genes (HER-2/neu, bcl-2 and p53) in the livers of rats received SB and NS during hepatocarcinogenesis using nitrosamine precursors namely dibutylamine and nitrate. In liver disease, defect in the transulfuration pathway, may reduce the availability of cystein required for GSH production, and hence the GSH biosynthesis is reduced (21). This may explain the partial depletion of GSH in rats fed with the nitrosamine precursors. The

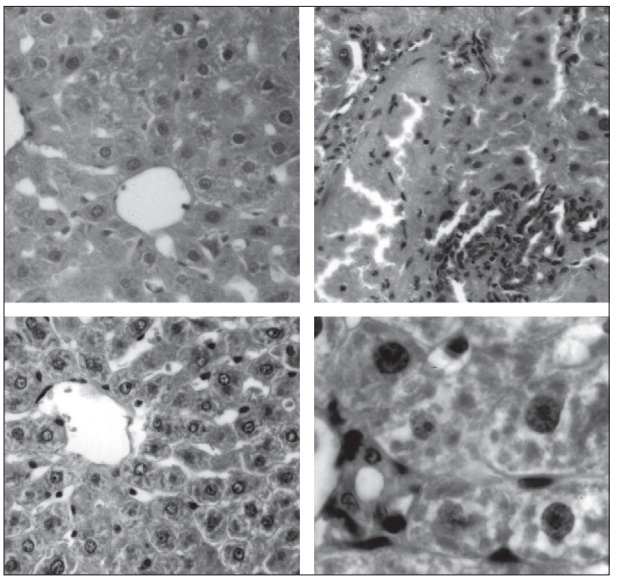

Figure 1. Histopathological sections (stained with Hx & E) of livers from rats in different groups after 12 months of treatment. (A) section of liver from normal rat (group I) showing a normal pattern of hepatic lobule with normal blood sinusoid (X 200), (B) section of liver from rat received the carcinogen precursors (group II) showing a destruction of the portal tract, which is surrounded by inflamatory cells (X 200), (C) section of liver from rat received both the carcinogen precursors + SB (group III) showing a marked recovery of hepatic cells with well defined activated nuclei (X 200) and (D) section of liver from rat received both the carcinogen precursors + NS (group IV) showing many cells in mitotic division as a sign of regeneration of binucleated cells, normal cytoplasm and membrans indicating a marked recovery of the histologic pattern (X 1000).

Table 2: The level of HER-2/neu, bcl-2 and p53 in the liver tissue homogenate of different groups after different periods of treatment .

Parameter	Group	periods of treatment (Months) 2	4	6	8	10	12
HER-2/neu (ng/ml)	I	2.02 ± ± 0.20	1.88 ± 0.12	1.90 ± 0.15	02.0 ± 0.09	1.97 ± 0.10	1.93 ± 0.11
	II	2.11 ± 0.11	2.52 ± 0.06 a,b,c	2.88 ± 0.12 a,b,c	3.03 ± 0.12 a,b,c	3.25 ± 0.07 a,b,c	3.23 ± 0.08 a,b,c
	III	1.93 ± 0.16	1.99 ± 0.21	1.75 ± 0.13 b	01.74 ± 0.2 b	1.93 ± 0.30 b	1.83 ± 0.12 b
	IV	2.05 ± 0.21	1.88 ± 0.11 b	1.78 ± 0.19 b	1.89 ± 0.07 b	1.78 ± 0.12 b	2.00 ± 0.13 b
Bcl-2 (ng/ml)	I	3.14 ± 0.17	3.00 ± 0.20	3.07 ± 0.18	2.82 ± 0.19	2.90 ± 0.20	2.84 ± 0.16
	II	4.64 ± 0.23 c	4.96 ± 0.30 a,c	5.10 ± 0.30 a,c	5.93 ± 0.24 a,c	6.02 ± 0.27 a,c	6.66 ± 0.24 a,c
	III	4.1 ± 0.18 b,c	3.49 ± 0.19 b,c	3.34 ± 0.21 b	3.00 ± 0.20 b	2.94 ± 0.21 b	2.92 ± 0.17 b
	IV	4.17 ± 0.26 b,c	3.84 ± 0.29 b,c	3.72 ± 0.43 b,c	3.66 ± 0.27 b,c	3.31 ± 0.25 b	3.19 ± 0.35 b
P53 (U/ml)	I	44.33 ± 2.93	43.55 ± 1.38	44.56 ± 1.28	44.36 ± 1.03	43.99 ± 1.61	43.82 ± 1.66
	II	33.69 ± 1.38 c	31.52 ± 1.52 c	31.63 ± 3.06 c	27.52 ± 2.36 a,c	28.00 ± 2.30 a,c	26.52 ± 2.66 a,c
	III	36.76 ± 1.24 c	35.92 ± 0.99 b,c	34.79 ± 2.68 b,c	36.4 ± 2.46 b,c	37.06 ± 2.46 b,c	41.58 ± 3.02 a,b
	IV	32.68 ± 1.43 c	33.53 ± 2.07 c	34.45 ± 1.50 c	35.46 ± 2.86 b,c	36.02 ± 2.96 b,c	39.27 ± 2.66 a b,c

Small letters refer to statistically significant difference between the indicated groups versus:

(a) the 2-month measurement in the same group

(b) the time matched point of the carcinogen group (II)

(c) the time matched point of the normal control group (I)

Group: I (normal rats), II (rats fed with DBA+$NaNO_3$), III (rats fed with DBA+ $NaNO_3$ +SB) and IV (rats fed with DBA+ $NaNO_3$ + NS).

antioxidant effects of SB and NS were reported in many pathological conditions (22,23). Similarly, the results of this work demonstrate that, feeding rats with SB or NS group III and IV improved the GSH level compared with that received nitrosamine precursors alone. Both SB and NS however were unable to normalize the GSH level before 8 months. Consistent with previous reports (24), the decrease of NO levels in groups III and IV may be due to the inhibition of nitric oxide synthase-activities (23). Like GSH, the NO normal level was restored after 10 and 12 months, respectively. Thus the complete recovery of oxidative stress and normaliza tion of markers required a long and sustainable oral administration of SB or NS grinded seeds mixed with the diet. The antioxidant effect of SB may be attributed to soy isoflavones (25) and saponins (26). For NS seeds, however much of the biological activity has been shown to be due to thymoquinone, the major component of the essential oil (27). The majority of these reports have used one or more of a pre-fractionated SB or NS seeds in vitro studies or through intrapretonal administration of active ingredients (28). This explains the accelerated improvement of the oxidative stress compared with the late normalization obtained in this work. Although the in vitro studies are predictive (and not a conclusive), the approach undertaken in this work, largely validates the traditional use of crude seeds in food. Oral administration of investigated extracts however is usually challenged by the degradation in gut or liver. Genistein and daidzein, the major isoflavone aglyctones, for example undergo extensive metabolism in the gut and liver. Their metabolites (equol, 8-hydroxydaidzein, O-desmethylangiolensin, and 1,3,5 trihydroxybenzene), however showed a potent free radical-scavenging activity (29). Consequently it was hypothesized that the antioxidant effects of crude soybean and NS may depend on the metabolic processing of their active ingredients.

The protein coded by the HER-2/neu gene was suggested to be a growth factor receptor potentially involved in the growth and progression of malignant cells (30). This protein was used as a prognostic marker and therapeutic target in some human tumors, especially breast cancer (17). In hepatocellular carcinoma (HCC),

the overexpression of HER-2/neu gene was uncommon and no relations were observed between HER-2/neu oncogene, tumor size, histopathological grading (31). This oncogene, however was correlated to age and AFP level (32). In the present work, although the Her-2/neu gene product was estimated by ELISA rather than the commonly used immunostaining or FISH techniques, the present finding revealed its overexpresion in nitrosamine precursors group and the subsequent amelioration after 4 and 6 months by SB and NS, co-administration, respectively, compared to the group of animal received nitrosamine precursors alone. This supports previous finding indicating that HER-2/neu is a target of soybean isoflavones (18).

The present finding indicates that both SB and NS were able to down regulate the production of the anti–apoptotic bcl-2 protein. The normal level however was restored after 6 and 10 months, respectively. Although the mechanism through which bcl-2 was affected by both SB and NS was not investigated, several reports have indicated the low expression of bcl2 mRNA. This decreases the chance of survival of the transformed cells, which ultimately inhibits the tumor growth. The effect of SB or NS may be exerted on the protein level, where some reports have evidenced a difference between the level of bcl-2 protein and the level of the coding mRNA (33). In such case it is predicted that the improvement in the level of circulating bcl-2 protein may be exclusively due to the direct effect of both dietary factors. It was not clear whether the normalization of bcl-2 level detected in animals treated with SB or NS was due to low initial expression of bcl-2 mRNA or due to a post-translational degradation of bcl-2.

The wild-type p53 protein has a short half-life and is expressed in very low amounts (34). Exposure of cells to a variety of stress factors, however, may result in an increased rate of synthesis of the mutated protein. Once the p53 gene has mutated, mRNA concentration progressively declines, suggesting that mutation may lead to inactivation of the p53 gene. The compansation of the reduced level of p53 by SB may be due to the genistein, a soybean-derived isoflavone (33). Also, increase in p53 in NS treated rats may be due to the thymoquinone, the most abundant constituent in black seed NS, which can induce G2/M cell-cycle arrest associated with an increase in the expression of the tumor suppressor protein p53 and the downstream p53 target gene p21WAF1 (14) Although the p53 expression during the study period was, significantly lower in the carcinogene plus protective agents compared to the normal control group, yet, the normal values were not achieved before 12 months. This modification of the p53 expression may be due to the delayed hepatocarcinogenesis caused by the protective agents SB or NS. Similarly, in other finding (36) revealed that the delayed hepatocarcinogenesis through the suppression of pre-neoplastic cell proliferation and that it may partially depend on P21 induction through a p53-independent pathway. The improved in the biochemical parameters by SB and NS was confirmed by a remarkable improvement of the histopathological finding of liver tissue of rats received SB or NS.

In conclusion, long term feeding of diets containing SB and NS helps in correcting the biochemical disturbance induced by the administration of nitrosamine precursors. The parameters used to evaluate this protective effect were GSH, NO, an oxidative stress markers and cancer related genes namely bcl2, p53 and HER-2/neu. Further studies are required at different directions to assure its application for human patients.

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