GENOTOXICITY AND CARCINOGENICITY

Diphenyl diselenide supplementation delays the development of *N*-nitroso-*N*-methylurea-induced mammary tumors

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Abstract The effect of dietary diphenyl diselenide
(1 ppm) on *N*-nitroso-*N*-methylurea (NMU)-induced mam-
mary carcinogenesis was examined in female Wistar rats.
Beginning at 5 weeks of age, the animals were fed with
either control or diphenyl-diselenide-supplied diets until
the end of the study (210 days). At 50 days of age, mam-
mary tumor was induced by the administration of three
doses of NMU (50 mg/kg body wt, intraperitoneally) once
a week for 3 weeks. In experimental trials, latency to tumor
onset was extended in rats fed with diet supplemented with
diphenyl diselenide ($P < 0.05$). The incidence and frequency
of tumors were significantly small in animals supplemented
with diphenyl diselenide. However, the multiplicity of
tumors was not altered by dietary diphenyl diselenide.
Diphenyl diselenide supplementation also restored super-
oxide dismutase (SOD) activity and vitamin C levels
altered in the NMU group ($P < 0.05$). Our results suggest
that diphenyl diselenide can be considered a chemopreven-
tive agent, even when supplemented at a relatively low
concentration.

Introduction

Breast cancer is the second most frequent cause of cancer-
related deaths in women (Schairer et al. 2004); however, its
etiology remains obscure and primary prevention strategies
are yet not available. Moreover, advances in therapy are
limited and, consequently, alternatives need to be devel-
oped for breast cancer control. Thus, the search for syn-
thetic or natural chemical agents that inhibit and/or delay
the preneoplasic events has received an increasing attention
in cancer therapy.

Selenium intake at low concentrations is recognized as
essential in animal and human nutrition (Navarro-Alarcón
and Lopes-Martinez 2000). In the form of selenocysteine,
selenium is a component of a number of antioxidant
enzymes, e.g. glutathione peroxidase and thioredoxin
reductase (Rotruck et al. 1973; Arner and Holmgren 2000).
Conversely, high doses of selenium can be cytotoxic via its
ability to catalyze the oxidation of thiols and to generate
free radicals (Barbosa et al. 1998; Nogueira et al. 2004). Of
particular importance, this element has received consider-
able attention for its possible role as an effective, naturally
occurring, anticarcinogenic agent, when used in both physi-
ological and/or supranutritional concentrations (Fleming
et al. 2001; Spallholz 2001; Letavayová et al. 2006). Thus,
the mechanism involved in the anticarcinogenic activity of
selenium seems to be, at least in part, associated with its
antioxidant and pro-oxidative effects (Das et al. 2004;
Spallholz et al. 2004; Valko et al. 2006).

Human epidemiological studies have clearly indicated
that low selenium status is invariably associated with

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increased cancer risk and that selenium supplementation is associated with reduction in the incidence of several cancers (Clark et al. 1996; Nayini et al. 1991; Mukherjee et al. 2001; Das et al. 2004), including breast cancer (Nayini et al. 1989; Hunter et al. 1990; El-Bayoumy 1994).

In the in vitro and in vivo experimental models, organic and inorganic selenium supplementation have been shown to suppress carcinogenesis in initiation and/or post-initiation phases in different types of cancer by multiple mechanisms (Spallholz 2001; Spallholz et al. 2004; Das and Bhattacharya 2004; Waters et al. 2005). Similarly, seleno-methionine and/or sodium selenite, at the doses well above the dietary requirement, inhibit mammary carcinogenesis but can induce severe liver necrosis (El-Bayoumy 1994; Thompson et al. 1994). Consequently, in the last few years, attempts have being made to develop or search for chemicals with high efficacy against cancer, but low toxicity to mammals. Of particular importance, a number of novel synthetic organoselenium compounds inhibit tumor development caused by a variety of chemical carcinogens (El-Bayoumy 1994; Thompson et al. 1994). However, the majority of the studies have used synthetic organoselenium compounds at concentrations considerably greater than the nutritional requirement of selenium (Nayini et al. 1991).

Diphenyl diselenide is a synthetic organoselenium compound that possesses glutathione peroxidase-like activity and exhibits antioxidant, anti-hyperglycemic, and anti-inflammatory properties (Nogueira et al. 2004; Barbosa et al. 2006, 2007). Of interest, diphenyl diselenide has low toxicity to rodents, when used in pharmacological doses (Perottoni et al. 2005; Fachinetto et al. 2006). In contrast, diphenyl diselenide at high doses or concentrations has pro-oxidative and toxic effects in yeast, bacteria and rodents (Maciel et al. 2000; Rosa et al. 2003, 2005). Recently, studies from our group have indicated the protective role of diphenyl diselenide against acute liver damage induced by 2-nitropropane (Borges et al. 2005, 2006). However, data about the possible anticarcinogenic action of diphenyl diselenide on chemically induced cancer models are lacking in the literature.

In the current study, we investigated the effect of dietary diphenyl diselenide on *N*-nitroso-*N*-methylurea (NMU)-induced mammary carcinogenesis in order to delineate the possible potential of this compound as a chemopreventive agent when used at low concentrations in the diet.

Materials and methods

Chemicals

NMU (*N*-nitroso-*N*-methylurea) was obtained from Sigma. 2′,7′-Dichlorofluorescein diacetate (DCHF-DA) and dichlorofluorescein (DCF) were obtained from Sigma Chemical. Low melting point (LMP) agarose and normal agarose (electrophoresis grade) were obtained from Gibco-BRL (Grand Island, NY).

Diphenyl diselenide was synthesized by the method described by Paulmier (1986). Analysis of the ^{1}H NMR and ^{13}C NMR spectra showed analytical and spectroscopic data in full agreement with its assigned structure. The chemical purity of diphenyl diselenide (99.9%) was determined by GC/HPLC. All other chemicals were of analytical grade and obtained from standard commercial suppliers.

Animals and diets

Sixty virgin female Wistar rats (150–200 g), aged 30 days, were obtained from our own breeding colony. Rats were housed in plastic cages and maitained under the following conditions: water and food ad libitum, temperature at 22–23°C, humidity at roughly 56%, and 12 h light cycle. The animals were used according to guidelines of the Committee on Care and use of Experimental Animal Resources, Federal University of Santa Maria, Brazil. Rats were randomly divided into four experimental groups of 20 rats each: (1) Control (without NMU and selenium supplementation); (2) Diphenyl diselenide-supplemented (Selenium); (3) NMU and (4) NMU-diphenyl diselenide supplemented (NMU/Se).

Beginning at the age of 5 weeks, groups 1 and 3 were fed with the control diet, whereas groups 2 and 4 were fed the diet containing 1 ppm of diphenyl diselenide. The selenium compound was dissolved in soybean oil and mixed in the diet in a food mixer to insure uniform distribution. Diets were prepared weekly and stored at 4°C and continued until the end of the study. Table 1 outlines the composition of control- and selenium-supplemented diets.

Tumor induction

At 50 days-of-age, rats in groups 3 and 4 were given injections of NMU (50 mg/kg body weight) once a week for 3 weeks (at 50, 57, 64 days of age). NMU was dissolved in 0.9% NaCl solution pH 4.0 (acidified with acetic acid) and administered by intraperitoneal injection within 30 min after preparation. Groups 1 and 2 received an equal volume of vehicle.

Tumor detection

After the first NMU injection, all animals were weighed weekly and palpated up to 210 days for tumor detection. To evaluate mammary tumor development, the following parameters were recorded: (a) Latency period (the number of days between the first NMU injection and the appearance of the first tumor in each rat), (b) tumor incidence (% of

Table 1 Composition of the control and diphenyl diselenide-supplemented diets (weight dry) (g/Kg)

Ingredients	Quantity/kg of diet	Protein	Carbohydrate	Lipid
Wheat meat	300	20.27	154.00	1.80
Corn Starch	66	4.50	34.54	0.36
Soybean meal	133	43.24	13.51	0.90
Sucrose	86	–	58.55	–
Soybean oil	33	–	–	23.52
Lard	33	–	–	22.52
Eggs	266	17.70	–	12.28
Mineral mixture[a]	80	a	a	a
Vitamin mixture[b]	6	b	b	b

[a] The salt mixture has the following composition (g/Kg): NaCl, 152; KCl, 96.3; $MgSO_4$, 56.7; $ZnCl_2 \cdot 7H_2O$, 0.4; $CuSO_4 \cdot 5H_2O$, 0.7; $MnSO_4$, 1.2 and $FeSO_4 \cdot 7H_2O$, 2.0

[b] The vitamin mixture (mg/IU g) was composed of Vitamin A, 5000 UI; Vitamin D 400 UI; thiamin,1.5 mg; riboflavin, 1.7 mg; pyridoxine, 2 mg; ascorbic acid, 60 mg; Vitamin E, 30 UI; Vitamin K1, 0.025 mg; nicotinamide, 0.02 mg; folic acid, 0.4 mg; calcium D-panthotenate, 10 mg; biotin, 0.03 mg, metiocolinB_{12}. Selenium supplemented diet containing 1 ppm of diphenyl diselenide $(PhSe)_2$.

animals that develop at least one tumor), (c) tumor multiplicity (average number of tumor per animal), and (d) tumor frequency (number of tumors per group).

At the end of the experimental period (dated from the first NMU injection), animals were sacrificed under ether anesthesia, and mammary tumors were excised, fixed in 10% formaldehyde, and prepared for histological analysis. The samples of tissues (uterus and spleen) with macroscopic alterations were also harvested for histological examination. The weight gain of all animals was measured weekly until the end of the experimental period.

Tissue preparation

After the treatment period, rats were killed by decapitation. The samples of tissues were quickly removed, placed on ice, and homogenized in cold 50 mM Tris–HCl pH 7.4. The homogenate was centrifuged at $4,000 \times g$ for 10 min to yield the low-speed supernatant fraction that was used for biochemical assays. Whole blood was collected by cardiac puncture into heparinized tubes for biochemical analysis and ascorbic acid determination.

To evaluate the short-time effect of NMU the animals were divided into 4 groups: CT (control short-time); Se (selenium short-time); NMU (NMU short-time) and NMU/Se (NMU and selenium short-time) and submitted to an identical treatment to that described above for cancer study. Twenty-four hours after the last NMU administration, animals were killed for in vitro assays (hepatic and renal biomarkers; superoxide dismutase activity; vitamin C; lipid peroxidation and RS measurements). In this experimental protocol, a group of five to ten animals was usually tested in each experiment.

Biochemical analysis

Protein measurement

The protein content was determined by the method of Bradford ([1976]) using bovine serum albumin as the standard.

Hepatic and renal biomarkes

AST (aspartate aminotransferase), ALT (alanine aminotransferase), LDH (lactate dehydrogenase), γ-GGT (γ-glutamyl transferase) enzymes, as well as urea and creatinine levels were determined in plasma by using commercial Kits (Labtest, Minas Gerais, Brazil).

Superoxide dismutase (SOD) activity

The measurement of SOD activity in liver was determined by the capacity of enzyme to inhibit the epinephrine autooxidation at alkaline pH at 480 nm as described by Misra and Fridovich ([1973]).

Vitamin C determination

Vitamin C determination was performed as described by Jacques-Silva et al. ([2001]). Protein (liver, spleen and blood) was precipitated in ten volumes of a cold 4% trichloroacetic acid solution followed by centrifugation. An aliquot of the supernatants (300 μL; in a final volume of 1mL) were mixed with 2,4-dinitrophenyldrazine (4.5 mg/Kg), CuSO4 (0,075 mg/Kg) and trichloroacetic acid 13.3% (final volume 1 ml) and incubated at 37°C for 3 h. After that, 1 ml H_2SO_4 65% (v/v) was added to the medium. The content of ascorbic acid was calculated using a standard curve

Table 2 Body weight gain (g) and organ weight (g) of female rats

Groups	B W gain	Liver	Kidney	Uterus	Spleen
1	117	6.2 ± 0.25	1.5 ± 0.07	0.73 ± 0.02	0.54 ± 0.24
2	126	7.7 ± 0.80	1.7 ± 0.08	0.70 ± 0.05	0.58 ± 0.07
3	80*	7.0 ± 0.52	1.5 ± 0.19	$1.48 \pm 0.09*$	0.74 ± 0.13
4	79*	6.8 ± 0.38	1.4 ± 0.06	$2.00 \pm 0.35*$	0.92 ± 0.23

Data are expressed as means $\pm$ SEM of five to six animals

* $P < 0.05$ as compared to group 1 (without NMU and selenium supplementation) (ANOVA/Duncan)

and expressed as μmol ascorbic acid/ml of plasma or μmol/g tissue.

Lipid peroxidation

Lipid peroxidation was performed as described by Draper and Hadley (1990). Briefly, the samples were mixed with 1 ml of 10% TCA and 1 ml of 0.67% thiobarbituric acid and incubated at 95°C for 60 min. TBARS (thiobarbituric acid reactive species) were determined by the absorbance at 535 nm and were expressed as nmol malondialdehyde (MDA)/g tissue.

RS measurement

To estimate the level of total blood reactive species (RS) production, heparinized samples were diluted (1:10) in phosphate buffer-saline (pH 7.4) and incubated with 1.6 μM of 2′,7′-dichlorofluorescein diacetate (DCHF-DA) in the presence or the absence of either a pro-oxidant (10 mM sodium azide) or antioxidant (40 μM ebselen) agents. The oxidation of DCHF-DA to fluorescent dichlorofluorescein (DCF) was measured for the detection of DFC reactive species (DCF-RS). The DCF fluorescence intensity emission was recorded at 520 nm (with 480 nm excitation) 20 min after the addition of DCHF-DA to the medium.

Comet assay

The alkaline comet assay was performed as described by Singh et al. (1998). Briefly, 5 μl of blood sample were mixed with 100 μl 0.5% LMP (Agarose Low Melting Point), spread on normal agarose-precoated microscope slide and placed at 4°C for 5 min to allow for solidification. The cells were lysed in high salt and detergent, and placed in a horizontal electrophoresis box. Subsequently, the cells were exposed to alkali (300 mM NaOH/1 mM Na$_2$EDTA, pH 13) for 30 min at 4°C, to allow for DNA unwinding and expression of alkali-labile sites. Electrophoresis (0.86 V/cm) was carried out for 30 min at 4°C. Positive controls treated with H$_2$O$_2$ (10 μM) for 5 min on ice were included for each experiment. After electrophoresis the slides were neutralized, silver stained, and analyzed at 200× magnification. One hundred randomly selected cells per sample were scored visually according to tail intensity into five classes (from undamaged 0, to maximally damaged 4). Thus, the damage score for each sample can range from 0 (completely undamaged − 100 cells × 0) to 4 (maximum damaged − 100 cells × 4). Differences in the extent of DNA strand breakage between the controls and the treatments were tested for significance using ANOVA.

Selenium determination

Elemental selenium (Se^0) analysis in diet was determined using a Perkin–Elmer (Norwalk, CT, USA) model 3030 atomic absorption spectrometer equipped with a MHS-10 hydride generation system.

Statistical analysis

Statistical analysis was performed using analysis of variance (ANOVA), followed by Duncan's multiple range test when appropriate. Tumor incidence and multiplicity were analyzed statistically by the χ^2 method and Fisher's exact test and tumor latency by Student's t test. Differences between groups were considered significant when $P < 0.05$.

Results

No significant differences in biochemical parameters analyzed were found among groups treated with NMU by short-time period and control groups (data not shown).

Body and organs weight

The body weight gain of animals fed experimental and control diets are shown in Table 2. NMU administration produced a significant decrease in the body weight gain compared to the control group (group 1) and diphenyl diselenide supplementation (NMU/Se group) did not prevent this effect. Diphenyl diselenide supplementation, given alone, did not affect the body weight gain of animals.

Table 3 Effect of diphenyl diselenide supplementation on biochemical parameters in NMU-treated rats

Groups	1	2	3	4
ALT[a]	51 ± 13.4	24 ± 4.60	46 ± 13.8	35 ± 13.8
AST[a]	196 ± 19.2	130 ± 9.80	271 ± 46.4	176 ± 11.1
γ-GGT[a]	3.8 ± 0.35	4.0 ± 0.44	4.5 ± 0.76	5.2 ± 0.35
LDH[a]	962 ± 25.0	940 ± 16.9	2054 ± 230*	1080 ± 210
Urea[b]	43 ± 0.93	37 ± 4.24	43 ± 1.60	30 ± 3.30
Creatinine[b]	0.29 ± 0.17	0.36 ± 0.22	0.26 ± 0.04	0.21 ± 3.9

Data are expressed as means ± SEM of five to six animals

[a] Data of enzyme activities are presented as U/l

[b] Data of renal markers levels are presented as mg/dl

* $P < 0.05$ as compared to group 1 (without NMU and selenium supplementation) (ANOVA/Duncan)

No significant difference in liver, kidney, and spleen weights was observed between NMU and control group (group 1). However, NMU administration caused an increase in the mean uterine weight of animals. Diphenyl diselenide supplementation (NMU/Se group) did not modify the weight of this organ when compared to the NMU group (Table 2).

Hepatic and renal biomarkes

There is no difference in AST, ALT and γ-GGT activities among experimental groups. NMU-treated rats exhibited a significant increase (two-fold higher than the control group) in plasma LDH activity and supplementation with diphenyl diselenide (NMU/Se group) significantly reduced the NMU-induced increase in plasma LDH activity (Table 3). The renal markers were not altered by NMU and/or diphenyl diselenide treatments (Table 3).

Tumor development parameters

Histopathological evaluation confirmed the presence of mammary carcinoma in each of the tumors, in accordance with previous studies using this model of tumor induction (Cocca et al. 1998).

The results presented in Table 4 show a marked increase in the incidence and frequency of mammary tumors in the NMU group when compared to the control group (group 1). Conversely, the NMU/Se group did not differ from the control group (group 1) when tumor incidence and total number of tumors were considered.

Tumor multiplicity and animal survival index in NMU and NMU/Se groups were not significantly different from the control group (group 1) (Table 4).

Tumor latency (expressed as the number of weeks for the first tumor detection) indicated that the latency period was extended in rats fed diet supplemented with diphenyl diselenide. Tumors began to develop in the NMU group at eighth week following NMU administration. In rats fed with diphenyl diselenide (NMU/Se), tumor development was delayed by 16 weeks compared to the NMU group (Fig. 1a). The mean latency for the appearance of tumor was 28 and 44 weeks in NMU and NMU/Se groups, respectively (Fig. 1b).

Morphological analysis of uterus of the animals revealed that NMU administration induced the development of uterine inflammatory process in both NMU and NMU/Se groups.

RS production and lipid peroxidation

TBARS levels and DFC-RS production are shown in Table 5. In the NMU group, TBARS levels of liver and spleen were reduced when compared to the control group (group 1). There was no difference in basal TBARS levels in liver and spleen between the NMU/Se and the control group (group 1).

NMU administration did not change the levels of DFC reactive species in total blood when compared to the control group (group 1), even when evaluated with or without sodium azide or ebselen. Diphenyl diselenide supplementation (NMU/Se) did not modify DFC-RS production when compared to the control group (group 1) (Table 5).

Vitamin C levels and SOD activity

Data in Table 6 show that diphenyl diselenide supplementation, given alone, did not modify the levels of ascorbic acid

Table 4 Effect of diphenyl diselenide supplementation on NMU-induced mammary tumors in female rats

Groups	1	2	3	4
Tumor incidence (%)	0/20	0/20	71.0 (10/20)*	35.4 (5.3/20)
Tumor multiplicity	0/20	0/20	1.6	1.3
Tumor frequency	0/20	0/20	13.3*	5.3
Survival (%)	100 (20/20)	100 (20/20)	80 (16/20)	55 (11/20)

Tumorigenicity parameters are calculated as % from group 1 (100%) (without NMU and selenium supplementation)

* $P < 0.05$ as compared to group 1 (without NMU and selenium supplementation) (Fischer's exact probability test, $P < 0.05$). Differences among NMU and NMU/Se groups were not statistically significant. Means indicate the number of animals with mammary tumors

Table 5 Effect of diphenyl diselenide supplementation on oxidative stress parameters in NMU-treated rats

	TBARS[a] DFC-RS		Spectrofluorometric assay[b]		
Groups	Liver	Spleen	Blood	Blood + Eb	Blood + Az
1	13.0 ± 0.57	10.2 ± 0.49	84.3 ± 5.72	27.3 ± 2.48	161.2 ± 4.84
2	12.6 ± 0.43	11.4 ± 0.55	82.4 ± 3.66	24.1 ± 0.80	145.1 ± 4.72
3	10.3 ± 0.69*[#]	6.0 ± 1.25*[#]	89.2 ± 8.87	34.2 ± 7.51	143.8 ± 7.83
4	11.6 ± 0.28	9.3 ± 1.05	83.8 ± 7.25	31.4 ± 5.54	134.7 ± 13.60

Data are expressed as means $\pm$ SEM of five to six animals

[a] Data of TBARS levels are presented as nmol MDA/g tissue

[b] Data of reactive species (RS) levels are presented as fluorescence intensity emission (UAF)

* $P < 0.05$ as compared to group 1 (without NMU and selenium supplementation) (ANOVA/Duncan)

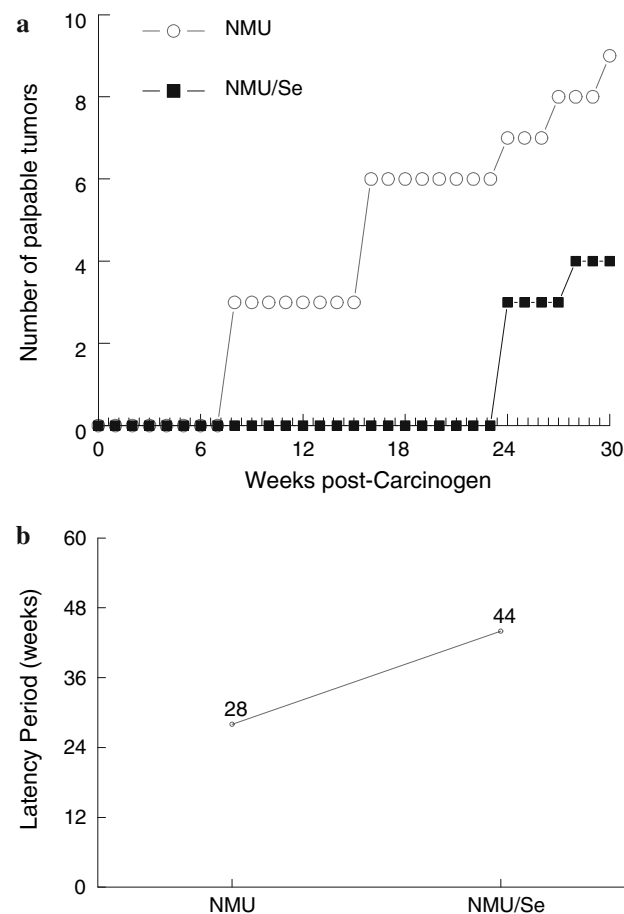[#] $P < 0.05$ as compared to group 2 (selenium supplementation) (ANOVA/Duncan, $P < 0.05$)

Fig. 1 Effect of dietary diphenyl diselenide on mean latency period of NMU-induced mammary tumors in female rats (**a**). The mean latency to tumor is expressed as the time interval (in weeks) from NMU administration to the appearance of the first palpable tumor (**b**). The groups were significantly different ($P < 0.05$) by analysis of variance

Table 6 Effect of diphenyl diselenide supplementation on levels of antioxidant defenses in NMU-treated rats

	SOD	Vitamin C		
Groups	Liver	Spleen	Liver	Blood
1	15.8 ± 1.34	223 ± 3.26	379 ± 17.8	224 ± 3.81
2	18.6 ± 1.06	257 ± 16.3	448 ± 28.1	258 ± 15.9
3	22.7 ± 0.77*	199 ± 8.98[#]	355 ± 20.4[#]	195 ± 9.79[#]
4	16.9 ± 1.96	222 ± 27.3	418 ± 23.6	222 ± 24.0

Data are expressed as means $\pm$ SEM of five to six animals

[a] Data of SOD activity are presented as U/mg protein

[b] Data of vitamin C levels are presented as μmol/g tissue or μmol/ml plasma

* $P < 0.05$ as compared to group 1 (without NMU and selenium supplementation) (ANOVA/Duncan)

[#] $P < 0.05$ as compared to group 2 (selenium supplementation) (ANOVA/Duncan, $P < 0.05$)

this reduction was normalized by diphenyl diselenide supplementation (NMU/Se) (Table 6).

SOD activity in liver of the NMU group was significantly increased when compared to the control group (group 1). Diphenyl diselenide supplementation (NMU/Se) restored enzyme activity to the control levels (Table 6).

Comet assay

NMU administration did not increase the extent of DNA strand breakage at this experimental period. Furthermore, the supplementation with diphenyl diselenide did not change the comet assay, regardless of the NMU treatment (data not shown).

Discussion

It has been demonstrated that the dietary supplementation with organic or inorganic selenium compounds can

content in liver, spleen and blood of the animals. Vitamin C levels were significantly decreased in spleen, liver, and blood of the NMU group as compared to the levels found in the diphenyl diselenide group (group 2). On the other hand,

inhibit both initiation and post-initiation phases of spontaneous or chemically induced mammary tumorigenesis (El-Bayoumy et al. 1996). However, the antitumorigenic effect of selenium compounds has been consistently associated with supranutritional levels of exposure to this element, which produces toxic effects in animals (El-Bayoumy 1991; El-Bayoumy et al. 1995; Spallholz et al. 2001).

In the current study, focusing on tumorigenisis process, dietary diphenyl diselenide promoted pronounced increase in the latency to the onset of tumor development, as indicated by prolonged mean latency period in the diphenyl diselenide supplemented group when compared to the NMU group. The incidence and frequency of tumors were also small in animals supplemented with diphenyl diselenide. The results indicated that this compound presents a protective effect against the tumor development, even when supplemented at a relatively low concentration. This attribute may represent an advantage over the other selenium compounds, since most of them, when tested at low concentrations, have provided small protective effect on the development of mammary tumors. Accordingly, studies have shown that benzyl selenocyanate (BSC) and 1,4-phenylenebis (methylene) selenocyanate (p-XSC), structurally modified synthetic organoselenium compounds, were much more effective in inhibiting the development of DMBA-induced mammary tumors only when supplemented at levels above 5 ppm (Nayini et al. 1989; El-Bayoumy et al. 1996).

Similarly, at the doses above the physiological requirement, inorganic selenium is an established chemopreventive agent. In fact, supplementing the diet or drinking water with inorganic selenium forms protects against cancer of mammary gland, colon, lung, pancreas, liver, and skin (El-Bayoumy 1991; Tanaka et al. 1995). However, chronic feeding of inorganic selenium compounds at levels $\geq$5 ppm is usually hepatotoxic to animals (El-Bayoumy 1991). Some naturally occurring selenium containing amino acids, such as selenomethionine and selenocysteine, were equally effective chemopreventive agents and had comparable toxicity to that of inorganic selenium (Thompson et al. 1994; Ip 1998).

In this study, diphenyl diselenide supplemented at relative low concentration (1 ppm) delayed the initiation stage of chemically induced mammary carcinogenesis and did not produce significant toxicity. In fact, diphenyl diselenide supplementation did not affect the body weight gain of animals and did not cause hepatotoxicity as indicated by the activities of plasma AST, ALT, and γ-GGT enzymes. Furthermore, dietary diphenyl diselenide protected against NMU-induced cytotoxicity by reducing the accentuated increase in plasma LDH activity caused by NMU administration.

However, the molecular mechanism(s) by which selenium delayed the initiation stage of chemically induced carcinogenesis remains to be elucidated. The protective effects of selenium, at the physiological dosage range, seem to be primarily associated with its presence in the glutathione peroxidases, which are known to protect DNA and other cellular components from damage by oxygen radicals (Letavayová et al. 2006; Valko et al. 2006). Selenium is also known to play a role in the control of cell division, in the oxygen metabolism, in detoxification processes, in apoptosis induction, and in the functioning of the immune system (Ramakrishnan et al. 1996; Fleming et al. 2001; Spallholz 2001; Spallholz et al. 2004). Thus, it is possible that selenium compound acts at several stages of the multi step carcinogenesis processes.

The participation of DNA damage in NMU-induced carcinogenesis was investigated by using the comet assay. Results indicated that NMU, diphenyl diselenide and their combination did not change comet assay. These results can be explained by the fact that the DNA-toxicity induced by NMU is transitory and occurs just after the cancer induction. In line with this, literature data indicate that the DNA damage caused by NMU is only seen shortly after NMU exposure (Buschfort et al. 1997; Uhl et al. 1999).

Even though DNA alkylation products induced by NMU were not observed in this experimental protocol, the risk of cells to accumulate potentially oncogenic mutations does not depend only on carcinogen exposure but also of the cellular capacity for protective DNA repair, such as the antioxidant defenses. Moreover, it has been shown that reactive oxygen species production in cellular processes results in the modulation of proto-oncogenes and/or the inactivation of some tumor-suppressor genes (Matés and Sánchez-Jiménez 2000), therefore antioxidants might prevent this damage and in turn protect against cancer (Tanaka et al. 1998, Kinnula and Crapo 2004; Waters et al. 2005; Valko et al. 2006).

The parameters related to oxidative stress such as reactive species production, was not altered in the NMU group. However, the SOD activity was increased in the NMU group and vitamin C tended to be decreased in the NMU-treated group. One unexpected finding of the present study was a small decrease in TBARS levels in liver and spleen of the NMU-treated animals. These results, such as the SOD activity, may be related to a compensatory response of the tissues to a previous insult. Thus, the hypothesis that the delay of the tumor development by diphenyl diselenide could be linked to its antioxidant properties cannot be discarded. In fact, diphenyl diselenide supplementation restored the activity of superoxide dismutase increased in the NMU group and elevated the levels of vitamin C decreased by NMU administration.

In conclusion, our findings suggest that diphenyl diselenide can retard cancer development and may be considered

a potential chemopreventive agent. However, a further dose-response study is needed to elucidate the efficacy of this compound as an anticancer agent.

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