

High-Dose Sodium Selenite Can Induce Apoptosis of Lymphoma Cells in Adult Patients with Non-Hodgkin's Lymphoma

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Abstract The present study was undertaken to explore the effect of administration of high doses of sodium selenite on the apoptosis of lymphoma cells in patients with non-Hodgkin's lymphoma (NHL). Forty patients with newly diagnosed NHL were randomly divided into two groups. Group I received standard chemotherapy, whereas group II received adjuvant sodium selenite $0.2 \text{ mg kg}^{-1} \text{ day}^{-1}$ for 7 days in addition to chemotherapy. Flow cytometry was used for monitoring of lymphoma cells apoptosis at the time of diagnosis and after therapy in the two groups. Sodium selenite administration resulted in significant increase in percentage of apoptotic lymphoma cells after therapy in group II ($78.9 \pm 13.3\%$ versus $58.9 \pm 18.9\%$, $p < 0.05$). In addition, patients who received sodium selenite treatment demonstrated statistically significant increase in percentage of reduction of cervical and axillary lymphadenopathy, decrease in splenic size, and decreased percentage of bone marrow infiltration. Also, we found a statistically significant decrease in cardiac ejection fraction (CEF) in group I and no reduction in CEF in patients who received sodium selenite 'group II', denoting the cardioprotective effect of selenium. It is concluded that sodium selenite administration at the dosage and duration chosen has synergistic effect to chemotherapy in inducing apoptosis and, consequently, could improve clinical outcome.

Keywords Lymphoma · Non-Hodgkin's · Selenium · Sodium selenite · Chemotherapy · Apoptosis

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Introduction

Selenoproteins play a critical role in a number of biochemical pathways, notably in cellular antioxidant systems such as glutathione peroxidase and thioredoxin reductase [1, 2].

Many studies have reported an association between selenium intake at supranutritional doses or serum selenium concentration and cancer incidence [3] or overall age-adjusted cancer mortality [4].

Studies in animals have described the chemopreventive activity of selenium compounds [5], while clinical trials have also reported the protective effect of selenium supplementation in the development of prostate, lung, and colorectal cancer [6], with the benefit being clearest in prostate cancer [7].

Serum selenium concentration at diagnosis was recently reported to be independently predictive of both treatment response and long-term survival in patients with aggressive non-Hodgkin's lymphoma [8], and selenium administration with chemotherapy resulted in improved overall survival and was effective immunomodulator [9, 10].

Lu et al. [11] have shown that selenite treatment of mouse leukemic L 1210 cells induced DNA single-strand breaks followed by the activation of endonucleases that produced DNA double-strand breaks and the induction of cell death by apoptosis.

Se-induced apoptosis in cancer cells was related to its chemopreventive activity [12], and several groups have shown that selenocompounds induce apoptosis in cell culture systems [13, 14].

These and other findings [15, 16] encouraged researchers to add selenium plus other antioxidants as an adjuvant to the surgical and chemotherapeutic treatment of breast cancer in high-risk patients with promising results; selenium included in a mixture of antioxidants has been reported to induce *in vitro* alteration in surface phenotype of lymphoma cells. Therefore, it may be considered as a potential therapeutic agent in lymphoma [17].

Taking into consideration the clinical data above, *in vivo* study of the effects of high-dose sodium selenite administration on apoptosis of lymphoma cells in patients with non-Hodgkin's lymphoma has been undertaken.

Aim of the Work

The aim of this work was to explore the *in vivo* effect of high-dose sodium selenite administration on apoptosis in lymphoma cells in patients with non-Hodgkin's lymphoma.

Patients and Methods

Forty adult patients with newly diagnosed non-Hodgkin's lymphoma (NHL) of intermediate and high grade were selected for the study. The patients were treated at the Hematology Department, Ain-Shams University Hospital, Cairo. Informed consent was obtained from all participants at the beginning of the study.

All patients were subjected to different clinical, laboratory, and radiological investigations for proper diagnosis and staging. Patients were selected to have bone marrow infiltration at presentation.

They were all subjected to flow cytometric analysis of bone marrow lymphoma cells to assess the level of apoptosis during different stages of therapy.

The patients were randomly divided into two groups. Group I ($n = 20$) were treated by chemotherapy, and group II ($n = 20$) received chemotherapy and selenium given in the form of sodium selenite.

Therapeutic Protocol

All patients received combination chemotherapy composed of cyclophosphamide doxorubicin, vincristine, and prednisone (CHOP) used in treatment of intermediate and high-grade NHL. The treatment consisted of 750 mg/m² on the first day of the treatment cycle (D1) intravenously (IV) cyclophosphamide, 50 mg/m² IV D1 doxorubicin, 1.4 mg/m² IV D1 vincristine, and 100 mg po D1 to D5 prednisone. Chemotherapy cycles were repeated every 28 days.

It has been proposed that sodium selenite antagonizes the apoptotic action of doxorubicin [18]. The administration of sodium selenite was postponed until plasma clearance of doxorubicin ($t_{1/2} = 18\text{--}30$ h) was achieved [18].

Therefore, sodium selenite was administered on days3–7 of the chemotherapy. Quantification of lymphoma cells apoptosis was done by flow cytometric analysis performed on bone marrow aspirate samples collected on day 0, and eight in patients receiving sodium selenite and on d 0, and eight in patients receiving chemotherapy alone (Table 1).

Sodium selenite anhydrous with purity of about 99% (Sigma Chemical, St. Louis, MO, USA) in a dose of 0.2 mg kg⁻¹ day⁻¹ was dissolved in water and given orally once daily for 5 days [19].

Patients were kept under close observation for appearance of adverse effects such as epigastric pain, vomiting, diarrhea, garlic taste, or any other symptoms and/or signs.

Follow-up with laboratory investigations was done. Sodium selenite was administered to group II patients from days3 to 7 in one cycle only.

The dose and duration of sodium selenite has been a matter of debate. It was determined by means of animal model studies and then escalated to human intake levels without toxic effects. Sodium selenite (Sigma Chemical) was given in an oral daily dose of 0.2 mg Se kg⁻¹ day⁻¹ for 5 days. To avoid possible selenite-induced vomiting, an antiemetic drug was also given to the patients in group II.

During the course of the study, all the patients were subjected to thorough clinical screening, including a complete blood, serum, liver, cerebrospinal, bone marrow, biopsy, immunophenotyping, and histopathological, electrocardiogram, radiological, and echocardiography studies.

In addition, quantification of in vitro apoptosis of lymphoma cells by flow cytometry was conducted.

All of the studies were carried out on bone marrow aspirate before and after chemotherapy in group I patients and after chemotherapy and selenium in group II patients.

Table 1 Study Design

Group I	Group II
D0: sample (1) collection	D0: sample (1) collection
D1: Chemotherapy	D1: chemotherapy
	D ₃ : start of selenium
D8: sample (2) collection	D ₈ : sample (2) collection

Sample Collection

Fresh bone marrow (2 ml) aspirate samples were collected in sterile tubes on heparin for flow cytometric immunophenotyping and analysis of apoptosis.

Mononuclear Cell Separation

Mononuclear cells were separated using Ficoll-hypaque density gradient centrifugation.

Bone marrow aspirates were diluted with phosphate buffer saline (PBS, one part blood to three parts PBS).

Ficoll-hypaque (2 ml) is dispensed in conical centrifuge tubes (Sigma Chemicals). Then, 4 ml of diluted blood was carefully layered on the surface, and the tubes were centrifuged at 1,500 rpm for 20 min at room temperature.

The cells were collected from the PBS/ficoll-hypaque interface and washed twice in PBS by centrifugation at 300 rpm for 10 min at 4°C. The cells were counted and adjusted to 2×10^6 /ml.

Flow Cytometric Analysis of Apoptosis Using Propidium Iodide Staining

After the second wash, the cells were suspended in 1 ml complete RPMI media (10 ml RPMI 1640 medium + 1 ml fetal calf serum +100 μ l antibiotics, streptomycin and penicillin) in a sterile tube.

The cell suspension was poured in a sterile tissue culture plate and then was incubated for 48 h at 37°C in 5% CO₂ incubator.

The cell suspension in tissue culture plates was transferred to a tube, and 100 μ l pepsin (0.5%, pH 1.5) was added to each tube and gently mixed and then was incubated at 37°C for 20 min. After incubation, the cells were washed twice using PBS and then the cell pellet resuspended in 0.5 ml PBS.

RNase solution (0.5 ml, Sigma Chemicals; 1 mg/ml in RBS) was added with gentle mixing followed by addition of 1 ml propidium iodide solution (100 μ g/ml in PBS), and the mixture was incubated for 15 min at room temperature in the dark then kept at 4°C until measured using flow cytometry (Coulter Epics Profile II). The following parameters were measured in 104 cells: log-forward scatter (LFS), integrated fluorescence 3 (FL3), and fluorescence 3 peak (FL3P); the data were displayed on three histograms:

Histogram 1: FL3 versus LFS;

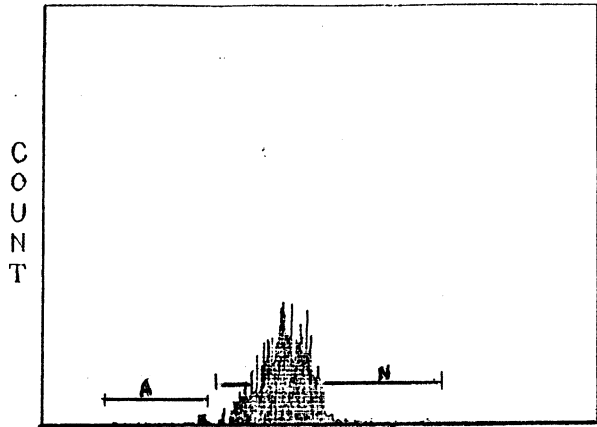
Histogram 2: FL3 versus FL3P;

Histogram 3: FL3.

An electronic gate (Bitmap 1) was drawn on histogram 2 to allow doublet discrimination. The DNA content was assessed as the mean channel number of FL3 on histogram 3.

The red fluorescence photomultiplier voltage was adjusted so that normal control fluoresces at mean channel number 200. Because of the fragmentation of chromatin during apoptosis, the nuclei of apoptotic cells take up less PI than the nuclei of normal cells, and the nuclei were scored apoptotic if they demonstrated a decrease in PI fluorescence relative to the nuclei of healthy non-apoptotic cells (Figs. 1 and 2) [20].

Fig. 1 Coulter cytometry test results pre-therapy (non-apoptotic population 92% apoptotic population 6.5%)



Statistical Analysis

Statistical analysis was performed with the Statistical Package for the Social Sciences software for Windows (version 10; SPSS, Chicago, IL, USA). The results were reported as the mean \pm SD. The statistical and correlation analysis were carried out with the Mann–Whitney and Spearman's rank correlation test coefficient, respectively. Statistical significance was set at $p < 0.05$.

Results

The study population consisted of 40 adult patients with newly diagnosed non-Hodgkin's lymphoma of intermediate and high grade. There were 25 men and 15 women. These patients were randomized into two groups according to the therapy received: group I included 20 patients who were treated with conventional chemotherapy, and group II included 20 patients who received conventional chemotherapy in addition to selenium therapy in the form of sodium selenite.

The patients' characteristics are summarized in Table 2.

Fig. 2 Coulter cytometry test results pre-therapy (non-apoptotic population 31.1% apoptotic population 65.6%)

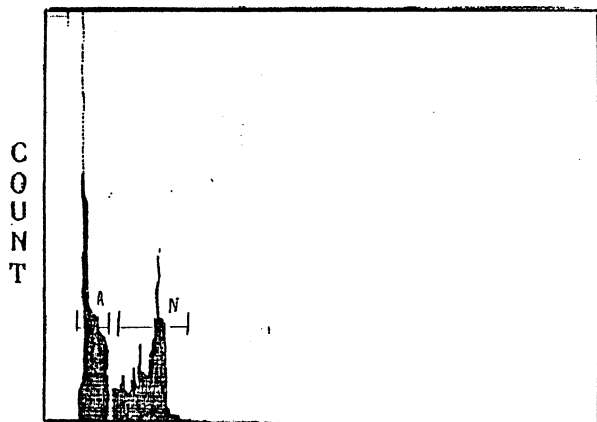


Table 2 Patients' Characteristic

	Group I	Group II
Age	49.9±8.5	46.7±7.3
Sex		
Male	13 (65%)	12 (60%)
Female	7 (35%)	8 (40%)
B symptoms	40%	75%
Splenomegaly	100% (4.5±2.1)	5.3±1.6 (100%)
Hepatomegaly	75% (2.1±1.8)	(2.5±1.7 (80%)
Bone marrow infiltration	63.6±12.3%	69.4±14.7%
Hemoglobin (g/day)	8.61±0.77	9.26±2.43
WBC	18.45±17.7	16.22±14.79
PLT	130.7±58.5	142.24±106.6
ESR	115.93±11.83	98.00±41.24
LDH	775.00±260.86	992.00±393.08
Uric acid	6.88±1.95	6.15±1.76

Efficacy

There was a statistically significant increase in the percentage of apoptotic lymphoma cells following chemotherapy in both studied groups. However, sodium selenite administration was effective in increasing the percentage of apoptotic lymphoma cells at day 8 following chemotherapy compared to the percentage of apoptotic lymphoma cell in group I ($78.9 \pm 13.3\%$ versus $58.9 \pm 18.9\%$, $p < 0.05$, respectively; Table 3).

In addition, statistically significant higher percentage of patients in group II (who received sodium selenite administration) demonstrated statistically significant reduction in cervical and axillary lymphadenopathy, decrease in splenic size, as well as decreased percentage of bone marrow infiltration by lymphoma cells.

Sodium selenite administration was found to be cardioprotective in group II patients as evidenced by a non-statistically significant change in CEF compared to statistically significant reduction in CEF in group I patients (Table 4).

Safety

Sodium selenite administration was associated with garlicky breath odor in 90% of patients and with gastrointestinal upset with nausea and occasional vomiting that was controlled with antiemetics. Other side effects were abated spontaneously except for side effects during continued therapy for 3 months. Side effects are summarized in Table 5.

Table 3 Comparison Between Group I and Group II as Regard Percentage of Apoptotic Lymphoma Cells among Patients Pre-therapy and Post-therapy

Apoptosis (%)	Group I (mean±SD)	Group II (mean±SD)	<i>P</i> value	Significance
Pre-therapy	29.2±24.8	29.7±24.5	0.9	N.S.
Post-therapy	58.9±18.9	78.9±13.3	<0.001	H.S.
<i>P</i> value	<0.001	<0.001		
Significant	(H.S.)	(H.S.)		

Table 4 Comparative Statistics Between both Studied Groups

		Group I		Group II		P value	Significance
		Number	Percentage	Number	Percentage		
Tumor volume	Pre	12	60	16	80	0.2	N.S
	Post	6	30	1	5	0.04	Sign.
P value		<0.05		0.001			
Significance		(Sign.)		(H.S.)			
Axillary LNs	Pre	13	65	15	75	0.5	N.S.
	Post	7	35	2	10	0.04	Sign.
P value		<0.05		<0.001			
Significance		(Sig.)		(H.S.)			
Splenic size (in fingers)							
Pre-therapy		4.5+2.1		5.3+1.6		0.2	N.S.
Post-therapy		2.2+1.9		1.2+1.3		0.05	Significant
P value		0.001		<0.001			
Significance		(H.S.)		(H.S.)			
Bone marrow infiltration							
Pre-therapy		Group I (mean+SD) 63.6+12.3		Group II (mean+SD) 69.4+14.7		0.2	N.S.
Post-therapy		26.9+12.8		10.9+6.0		0.001	H.S.
P value		0.001		<0.001			
Significance		(H.S.)		(H.S.)			
CE.F (%)	Pre	60.4±11.3		63.2+10.3		0.4	N.S.
	Post	55.8±11.2		63±10.0		0.04	Significant

Discussion

Non-Hodgkin's lymphoma, although a potentially curable disease, has a rising incidence throughout the world [21, 22].

Developing a safe and effective therapy for malignancy is the target of many researchers. Selenium is an essential micronutrient in human diet that has a promising role in cancer therapy [23].

Epidemiological and experimental studies have suggested that Se possesses anticarcinogenic properties [24–26].

In this study, the impact of Se administration in mega doses ($0.2 \text{ mg kg}^{-1} \text{ day}^{-1}$ for 5 days) on lymphoma cells apoptosis was evaluated. Forty adult patients with newly diagnosed

Table 5 Side Effects Associated with Sodium Selenite Therapy

	Acute (at initial therapy)	Delayed
General	Mild fever (40%)	None
GIT	Vomiting (85%)	Anorexia (30%)
	Loose motion (30%)	None
	Garlicky breath odor (90%)	None
Skin, nail and hair	Pruritis (20%)	Redness and blister formation 5%, brittle nail with white spots 20%, dry hair 30%
Respiratory	None	None
Cardiovascular	None	None
Nervous	None	Peripheral anesthesia 5%
Liver function	Elevated ALT (upper normal in 10% of patients)	None

NHL were randomly selected and subdivided into two groups. Group I ($n = 20$) were treated by chemotherapy, and group II ($n = 20$) received chemotherapy and selenium given in the form of sodium selenite.

Flow cytometry was used for the monitoring of lymphoma cells apoptosis at the time of diagnosis and after therapy in both groups.

Sodium selenite administration resulted in significant increase in percentage of apoptotic lymphoma cells after therapy in group II ($78.9 \pm 13.3\%$ versus $58.9 \pm 18.9\%$, $p < 0.05$). In addition, patients who received sodium selenite treatment demonstrated a statistically significant increase in the percentage of reduction of cervical and axillary lymphadenopathy, decrease in splenic size, and decreased percentage of bone marrow infiltration. In addition, we found a statistically significant decrease in CEF in group I and no reduction in CEF in patients who received sodium selenite 'group II', denoting the cardioprotective effect of selenium.

The mechanisms by which Se exerts its chemopreventive activity is unknown; however, several plausible explanations have been put forward including the role of Se in inducing cell cycle arrest [27], DNA strand breaks [11, 16], and apoptosis [11, 28].

The control of cell cycle progression plays a key role in cellular growth and differentiation [29–30]. Se induces cell cycle arrest at different phases depending on the chemical form of Se and the cell type [31, 32].

The inhibitory effect on cell proliferation, with a preference for tumor cells versus non-transformed cells, is considered to be a mechanism for the anticarcinogenic capability of Se [33]. Se-induced apoptosis in cancer cells was related to its chemopreventive activity [12], and several groups have shown that selenocompounds induce apoptosis in cell culture systems [11, 13, 14].

Apoptosis is a suicide process essential for development, maintenance of tissue homeostasis, and elimination of unwanted or damaged cells [34] with characteristic morphological features that include nuclear membrane breakdown, chromatin condensation and fragmentation, cell membrane blebbing, and the formation of apoptotic bodies [35].

Se modulates cellular activities presumably by acting on the functions of many intracellular proteins important for signal transduction [1, 36, 37].

However, the modulatory functions of selenite and other Se compounds on intracellular signaling pathways are not fully characterized.

NF- κ B is constitutively upregulated in many cancers, including lymphomas, and targeting NF- κ B has shown clinical activity in this setting [38]. NF- κ B is also involved in the cellular response to oxidative stress and plays a role in chemosensitivity [39, 40]. A decrease in NF- κ B activity is reported to overcome chemoresistance in many malignancies [41].

In a recent study by Juliger et al. [42] using a panel of human B cell lymphoma cell lines, the cytotoxic effects of chemotherapeutic agents (e.g., doxorubicin, etoposide, 4-hydroperoxycyclophosphamide, melphalan, and 1-B-D-arabino-furanosylcytosine) were increased by up to 2.5-fold when combined with minimally toxic concentrations (EC_{5-10}) of the organic selenium compound, methylseleninic acid (MSA). DNA strand breaks were identified using comet assays, but the measured genotoxic activity of the combinations did not explain the observed synergistic effects in cell death. However, minimally toxic (EC_{10}) concentrations of MSA induced a 50% decrease in nuclear factor- κ B (NF- κ B) activity after an exposure of 5 h, similar to that obtained with the specific NF- κ B inhibitor, BAY 11-7082. Combinations of BAY 11-7082 with these cytotoxic drugs also resulted in synergism, suggesting that the chemosensitizing activity of MSA is mediated, at least in part, by its effects on NF κ B [42].

Gopee et al. [43] determined the role sodium selenite plays on intracellular signaling, including protein kinase C (PKC), nuclear factor- κ B (NF- κ B), and inhibitor of

apoptosis protein (IAP) in murine B lymphoma (A20) cells. In vitro supplementation of A20 cells with low concentrations of sodium selenite (0.005–5 μM) caused a significant increase in cellular proliferation exclusively at 72 h. Proliferation and cell viability were decreased in response to selenium concentrations of $>25 \mu\text{M}$ and $>5 \mu\text{M}$ at 72 and 96 h, respectively. Flow cytometric analysis of A20 cells exposed to 5 μM Se at 72 and 96 h indicated G2-M phase arrest and increased cell death at higher concentrations.

Se-induced cytotoxicity was associated with apoptosis indicated by nuclear fragmentation and DNA laddering. Se concentrations, which induced cell cycle arrest and apoptosis, were associated with inhibition of cytosol to membrane translocation of PKC- δ and PKC activity at 72 h. Co-incubation of cultures with 0.5- μM phorbol 12-myristate 13-acetate (PMA) and Se (5 and 25 μM) reversed the Se-induced cell death at 72 h. The nuclear NF- κB translocation and NF- κB DNA binding were inhibited by increasing concentrations of Se (5 and 25 μM) at 72 h. After 72 h exposure to 5 and 25 μM Se, cIAP-2 concentration was decreased.

Differential inhibition of PKC- δ , NF- κB , and cIAP-2 by Se may represent important intracellular signaling processes through which Se induces apoptosis and subsequently exerts its anticarcinogenic potential [43].

In our previous work [9], fifty patients with newly diagnosed NHL were randomly divided into two groups. Group A-I received standard chemotherapy, whereas group A-II received adjuvant sodium selenite 0.2 mg $\text{kg}^{-1} \text{day}^{-1}$ for 30 days in addition to chemotherapy. Enzyme-linked immunosorbent assay was used to assess Bcl-2 at the time of diagnosis and after therapy in the two groups. Sodium selenite administration resulted in significant decline of Bcl-2 level after therapy in group A-II (8.6 ± 6.9 vs 6.9 ± 7.9 ng/ml, $p < 0.05$). Furthermore, complete response reached 60% in group A-II compared to 40% in group A-I. It is concluded that sodium selenite administration at the dosage and duration chosen acts as a downregulator of Bcl-2 and improves clinical outcome [9].

Philchenkov et al. [44] studied apoptosis and cell cycle distribution of MT-4 cells exposed to inorganic selenium compounds assessed by flow cytometry upon propidium iodide staining. Bax expression was analyzed by flow cytometry of cells labeled with anti-Bax monoclonals. Extent of DNA damage was assessed by Comet analysis. Sodium selenite induced apoptosis in dose-dependent mode starting from concentrations of 10 μM , while sodium selenate was much less toxic, inducing apoptotic cell death only at 300 μM . Sodium selenite, but not sodium selenate, caused a slight arrest in G2/M phase of cell cycle.

DNA damaging effects visualized in DNA Comet assay accompanied the cytotoxicity of sodium selenite. Nevertheless, the drastic increase in Bax flow cytometry intensity was evident only in selenite-induced apoptosis. So, increased Bax expression could be another mechanism by which sodium selenite induced apoptosis [44].

Last et al. [45] investigated the activity of two selenium species, MSA and selenodiglutathione (SDG), in a panel of human lymphoma cell lines and in a primary lymphoma culture system and concluded that both compounds demonstrated cytostatic and cytotoxic activity with EC_{50} values in the range 1.0–10.2 μM . Cell death was associated with an increase in the sub-G1 (apoptotic) fraction by flow cytometry and was not preceded by any obvious cell cycle arrest. SDG, but not MSA, resulted in marked increases in intracellular reactive oxygen species (ROS), particularly in CRL2261 and SUD4 cells in which the cytotoxic activity of SDG was partly, or completely, inhibited by *n*-acetyl cysteine, suggesting a dependence on ROS for activity in some cells. Both MSA and SDG showed a concentration-dependent reduction in percentage viability after a 2-day exposure in primary lymphoma cultures, with EC_{50} values in the range 39–300 and 9–28 μM , respectively. Therefore, the selenium compounds MSA and SDG induce cell death in

lymphoma cell lines and primary lymphoma cultures, which, with SDG, may be partly attributable to the generation of ROS [45].

Xiang et al. [46] in an in vitro study use LNCaP cells which were transduced with adenoviral constructs to overexpress four primary antioxidant enzymes: manganese superoxide dismutase (MnSOD), copper-zinc superoxide dismutase (CuZnSOD), catalase (CAT), or glutathione peroxidase 1 (GPx1).

The MTT assay, chemiluminescence, flow cytometry, Western blot analysis, and Hoechst 33342 staining following overexpression of these antioxidant enzymes analyzed cell viability, apoptosis, and superoxide production induced by sodium selenite and concluded that: (1) selenite induced cancer cell death and apoptosis by producing superoxide radicals; (2) selenite-induced superoxide production, cell death, and apoptosis were inhibited by overexpression of MnSOD, but not by CuZnSOD, CAT, or GPx1; and (3) selenite treatment resulted in a decrease in mitochondrial membrane potential, release of cytochrome c into the cytosol, and activation of caspases 9 and 3, events that were suppressed by overexpression of MnSOD [46].

Taken together, these data encourage the further development of selenium as a potential modulator of cytotoxic drug activity in non-Hodgkin's lymphomas.

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