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The Impact of High-dose Sodium Selenite Therapy on Bcl-2 Expression in Adult Non-Hodgkin's Lymphoma Patients: Correlation with Response and Survival

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Abstract The present study was undertaken to explore the effect of administration of high doses of sodium selenite on the expression of Bcl-2 in patients with non-Hodgkin's lymphoma (NHL). 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 kg^{-1} 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 ng/ml vs 3 6.9±7.9 ng/ml, $P<0.05$). Also, complete response reached 60% in group A-II compared to 40% in group A-I. Significant increase in CD4/CD8 ratio was noticed in group A-II compared to group A-I after therapy (1.45± 0.36 vs 1.10±0.28 p 0.04). Overall survival time in months was significantly longer in complete remission patients in group A-II (21.87±1.41) compared to group A-I (19.70± 1.95) ($p=0.01$). It is concluded that sodium selenite administration at the dosage and duration chosen acts as a downregulator of Bcl-2 and improves clinical outcome.

Keywords Sodium selenite · Bcl-2 · Non-Hodgkin's lymphoma · Overall survival

Introduction

Non-Hodgkin's lymphoma (NHL) ranks the fifth common malignancy and fifth leading cause of cancer deaths all over the world with a rising incidence [1,2]. Copious efforts have been spent to develop an effective and safe therapy for NHL.Variable modalities of therapy

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have been introduced in NHL targeting different pathways and stages of cellular proliferation [3,4].

The Bcl-2 protein families are intracellular signaling proteins, most of them having an antiapoptotic influence on the cells, rendering the cells immortal and resistant to the effect of chemotherapy [5,6].

Selenium (Se) is an essential micronutrient for the human body, whose importance was recognized 40 years ago [7]. Selenium is a potent antioxidant element, which has a role in preventing atherosclerotic heart disease [8], delaying progression of AIDS in HIV-infected patients [9], and slowing the aging process [10]. The antineoplastic role of Se has been elaborated by the higher incidence of malignancy in Se deficient areas [11,12]. Different mechanisms of action have been postulated, including downregulation of protooncogenes, upregulation of tumor suppressor genes, and influencing apoptosis [13].

Recently, Asfour et al. [14] concluded that sodium selenite administration acts as a cytoprotective agent, alleviating the immunosuppressive effects of cytotoxic chemotherapy.

This work aimed to explore the effect of high-dose sodium selenite on the expression of the antiapoptotic Bcl-2 oncoprotein in patients with NHL.

Patients and Methods

Fifty adult patients with newly diagnosed NHL stage IV of intermediate and high grade were selected for the study (groups A-I and A-II). The patients were treated at the Hematology Department, Ain Shams University Hospital, Cairo, Egypt. Twenty-five age- and sex-matched volunteer subjects were selected as control group (group B). Informed consent was obtained from all participants at the beginning of the study.

All patients were subjected to different clinical, laboratory, and radiological screening for proper diagnosis and staging. They were all subjected to quantification of Bcl-2 using enzyme-linked immunosorbent assay (ELIZA) test, CD4/CD8, and CD56 by flow cytometry during different stages of therapy.

The patients were randomly divided into two groups. Group A-I (n=25) was treated with chemotherapy alone and group A-II (n=25) was treated with chemotherapy and Se given in the form of sodium selenite.

Therapeutic Protocol

All patients received a combination chemotherapy (cytoxan, hydroxydaunomycin [Adriamycin], vincristine [Oncovin], and prednisone [CHOP]) used in the treatment of NHL. The treatment consists of 750 mg/m^2 cyclophosphamide given intravenously (IV) on the first day of treatment the cycle (D1), 50 mg/m^2 doxorubicin IV D1, 1.4 mg/m^2 vincristine IV D1, and 100 mg prednisone orally D1 to D5. Chemotherapy cycles were repeated every 28 days. Group A-II received, in addition to chemotherapy, sodium selenite (Table 1).

The dose of selenite administered to the patients was first determined on the basis of animal experiments and was gradually increased. The dose ultimately chosen (0.2 mg sodium selenite/kg body weight [50 kg average body weight]) was the highest possible level still tolerated without causing serious side effects.

Sodium selenite anhydrous with purity of about 99% (Sigma Chemical Co., St. Louis, MO, USA) in a dose of 0.2 mg kg/day was dissolved in water and given once daily for 30 days [15]. Patients were kept under close observation for appearance of adverse effects

Table 1 Therapeutic Protocol

	Group A-I	Group A-II
Cyclophosphamide (750 mg/m^2)	D1	D1
Vincristine (1.4 mg/m^2)	D1	D1
Adriamycin (50 mg/m^2)	D1	D1
Prednisone (100 mg)	D1–5	D1–5
Sodium selenite (0.2 mg/kg)		D1–30

such as epigastric pain, vomiting, diarrhea, garlic taste, or any other symptoms and/or signs. Follow-up with laboratory investigations was done.

During the course of the study all patients were subjected to a thorough clinical screening, including a complete blood, serum renal, hepatic, cerebrospinal, bone marrow (BM) biopsy, immunophenotyping, ECG, histopathological, radiological, and echocardiography studies.

Quantification of human Bcl-2 by ELIZA (human Bcl-2 BMS244/2 kit, MedSystems Diagnostics GmbH, Rennweg 95b, A-1030, Vienna, Austria) was carried out on BM aspiration samples before and after three cycles of chemotherapy in group A-I and A-II [16].

Sodium selenite was administered to group A-II patients from days 1 to 30 in one cycle only.

Sample Collection

From each patient and control subject 2 ml of whole BM was collected under sterile conditions. Bone marrow samples were centrifuged at 3,000 rpm for 20 min to allow serum to separate from cells then the serum was stored at −70°C until required for analysis.

Quantitative Determination of Human Bcl-2 by Enzyme-linked Immunosorbent Assay

All reagents were mixed thoroughly without foaming. Before use, each sample, standard, blank, and optional control samples, was assayed in duplicates in the microwell strips after washing the microwell strips twice with 300 ml wash buffer (content 50 ml of the wash buffer concentrate was brought to a final volume of 1,000 ml with deionized water, then mixed gently to avoid foaming). The pH of the final solution was adjusted to 7.4. Wells were thoroughly aspirated and emptied between washes without allowing the wells to dry. One hundred milliliters of sample diluent were added to each standard and blank well. Standard dilutions were prepared by repipetting 100 ml from well to well five times, creating two rows of Bcl-2 standard dilutions ranging from 32 to 0.5 ng/ml; and 80 ml was added in duplicate to the sample wells and 20 ml of each sample was added in duplicate to the designated wells.

Fifty milliliters of biotin-conjugate diluted 1:100 with assay buffer (5 ml of assay buffer concentrate was added to 95 ml deionized water and mixed gently to avoid foaming) was added to all wells, including blank wells.

The microtitre plate (MTP) was covered and incubated at room temperature for 2 h in a shaker set at 100 rpm. Then the microtitre plate cover was removed and the well emptied then washed three times as before.

One hundred milliliters of diluent streptavidin-HRP (the concentrated streptavidin-HRP solution was diluted in 1:100 dilutions with assay buffer) was added to wells, including the blank wells. Then the MTP was covered and incubated at room temperature for 1 h in a shaker set at 100 rpm.

One hundred milliliters of freshly prepared tetramethylbenzidine substrate solution was added to each wells, including blank wells, after washing the microwell strips three times as before. Then MTP was incubated at room temperature for 10–20 min on a shaker set at 100 rpm.

Adding 100 ml of stop solution into each well, including the blank wells, quickly stopped the enzyme reaction and results were read within 1 h at 2–8°C in the dark. Absorbance of the wells was read on a spectrophotometer using 450 nm as the primary wavelength and 620 nm as the reference wavelength.

The average absorbance values were calculated for each set of duplicate standards and samples. A standard curve was created through plotting the mean absorbance for each standard concentration on the ordinate against the Bcl-2 concentration on the abscissa. The best-fit curve was drawn through the points of the graph (Fig. 1).

The concentration of the circulating Bcl-2 for each sample was determined through finding the mean absorbance value on the ordinate and extending a horizontal line to the standard curve where a vertical line extending from the point intersection to the abscissa to plot a corresponding Bcl-2 concentration in nanograms per milliliter.

CD4/CD8 Ratio

The peripheral blood lymphocytes were studied for CD4/CD8 ratio among $CD3^+$ lymphocytes using whole blood lysis technique and tricolor flow cytometric analysis Epics XL flow cytometer using system II software.

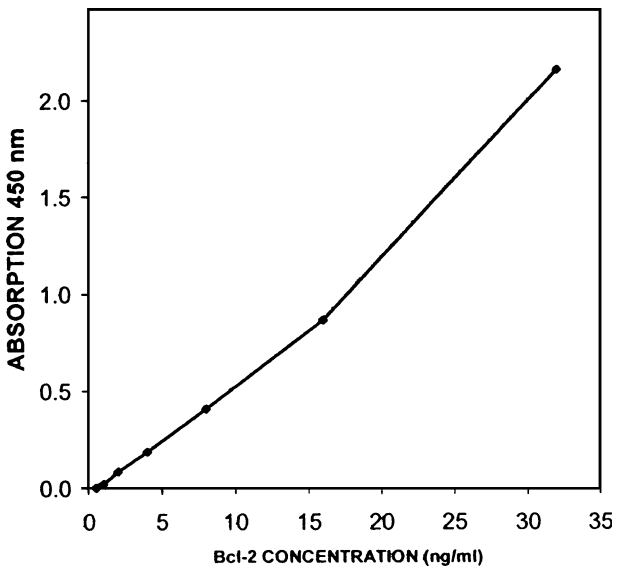

Fig. 1 The standard curve for Bcl-2 ELIZA prepared within this assay

Quantitation of CD56 Expression by Flow Cytometry

The cell pellet remaining after removal of serum of BM sample, as previously described, was then used to evaluate the expression of CD56 by natural killer T cells using the erythrocyte lysis technique. An Epics XL flow cytometer (Coulter Electronics, Hialeah, FL, USA).

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±SD. The statistics and survival analysis were carried out with the Mann–Whitney test and the Kaplan–Meier analysis, respectively. Statistical significance was set at $P<0.05$.

Results

The current study encompasses 50 patients with newly diagnosed NHL of intermediate and high grade. They were randomized into two groups:

Group A-I included 25 patients who received CHOP.
Group A-II included 25 patients who received Se in the form of sodium selenite (0.2 mg kg/day) for 30 days in addition to CHOP in the first cycle.

Twenty-five volunteer control subjects were included in group B. The patients' characteristics at presentation are summarized in (Table 2).

Efficacy

Sodium selenite administration resulted in a significant increase in CD4/CD8 ratio but a nonsignificant decrease in Bcl-2 level; however, CD56 expression increased but is statistically nonsignificant (Table 3).

Sodium selenite administration was effective in significantly reducing the level of Bcl-2, but increasing CD4/CD8 ratio as well as CD56 was not significant compared to pretreatment levels in group A-II (Table 4). Also, Se administration resulted in higher complete response and lower relapse rate in group A-II compared to group A-I (60 vs 40% and 20 vs 12%, respectively).

Event-free survival was longer in patients with intermediate risk (IPI-B) (mean=12, range 10–15) than those with high risk (IPI-C) (mean=5, range 4–7). The low-risk patients (IPI-A) were all free of event (Fig. 1); log rank=7.27, $P=0.02$ (Fig. 2).

The overall survival (OS) time was longer in group A-II (21.8±1.4 months vs 19.7± 1.9 months in group A-I, with a $p=0.01$. (Fig. 3).

Selenium Toxicity in A-II group

Sodium selenite toxicity noticed in group A-II included five patients who developed garlic taste, gastritis, abdominal pain, and diarrhea; five patients who suffered from garlic taste and gastritis; five patients who developed garlic taste only; and 10 patients who had sore throat, sneezing, and garlic taste, but no signs or symptoms of severe toxicity were detected (CNS manifestation, hair, nail, and skin changes). Mild elevation of liver enzymes in patients treated with sodium selenite was noted; however, the elevation was statistically nonsignificant.

Table 2 The Demographic and Descriptive Data of Patients at Presentation

	Group A-I	Group A-II
Number of patients	25	25
Age (years)	46.9±8.2	49.1±13.1
Sex		
Male	15	16
Female	10	9
B-symptoms		
Positive	13	14
Negative	12	11
Tumor volume[1]		
Liver	6.96±3.3	6.40±2.8
Spleen	7.32±4.91	7.92±4.05
Laboratory		
Hemoglobin (gm/dl)	8.70±1.92	9.18±2.2
WBC	20.45±17.7	15.22±14.79
Platelet count	130.7±58.5	176.24±106.6
ESR	63.5±29.77	73.24±34.82
LDH	631.1±295.3	767.76±420.5
Uric acid	6.68±1.78	7.08±2.58
CD4/CD8 ratio	1.14±0.25	1.03±0.23
BM		
Aspirate	28.72±17.3	30.12±22.8
Trephine	21/25 (84%)	24/25 (96%)
CD56	8.80±1.83	8.04±2.37
Bcl-2 (ng/ml)	9.79±9.67	8.56±6.87
Treatment received	CHOP (every 28 days)	CHOP plus Sodium selenite 0.2 mg/kg/day first cycle

[1] tumour volume liver and spleen size in cm below costal margin

Discussion

Non-Hodgkin's lymphoma, although a potentially curable disease, has a rising incidence throughout the world [1,17]. Developing a safe and effective therapy for malignancy is the

Table 3 Comparative Statistics Between Both Groups Regarding Bcl-2, CD4/CD8 Ratio, and CD56 After Therapy

Variables	Group A-I (n=25)		Group A-II (n=25)		Z value	P value	Significance/Not Significance
	Mean	SD	Mean	SD			
CD4/CD8	1.10	0.28	1.45	0.36	−1.08	0.04	Significant
CD56	8.24	1.92	8.48	2.24	−0.94	0.34	Not significant
Bcl-2 level	7.70	8.07	6.94	7.93	−0.47	0.63	Not significant

Table 4 Comparative Statistics in Group A-II Regarding Bcl-2, CD4/CD8 Ratio, and CD56 Before and After Treatment

Variables	Group A-II		Mann–Whitney Test		
	Before (Mean±SD)	After (Mean±SD)	*Z* value	*P* value	Significance/Not Significance
CD4/CD8	1.03±0.23	1.45±0.36	−0.73	0.46	Not significant
CD56	8.04±2.37	8.48±2.24	−1.53	0.12	Not significant
Bcl-2	8.56±6.87	6.94±7.93	−2.37	0.01	Significant

target of many researchers. Selenium is an essential micronutrient in human diet that has a promising role in cancer therapy [13].

In this study, the impact of Se administration in mega doses (0.2 mg kg/day for 30 days) on the expression of Bcl-2 oncoprotein was evaluated. Fifty adult patients with newly diagnosed NHL were randomly selected and subdivided into two groups:

Group A-I received standard combination chemotherapy (CHOP).
Group A-II received sodium selenite (0.2 mg mg kg/day) for 30 days once in addition to CHOP.

After three cycles the patients were reevaluated for assessment of their response to chemotherapy.

Twenty-five age- and sex-matched volunteer subjects were enrolled as control group (group B).

Bcl-2 level in BM was significantly higher in NHL patients compared to the control group (*P* value<0.001).

The Bcl-2 family is composed of several members most of them playing an antiapoptotic role, thus prolonging cell survival. Higher expression of this protooncogene has been related to (14;18) gene translocation in follicular NHL [18]. Immortalization of malignant cells in lymphomas and other malignancies is the result of the increased antiapoptotic properties,

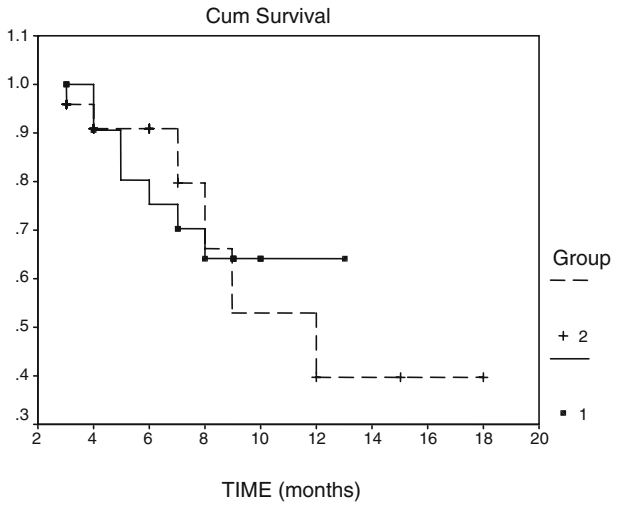

Fig. 2 Event-free survival time between groups A-I and A-II

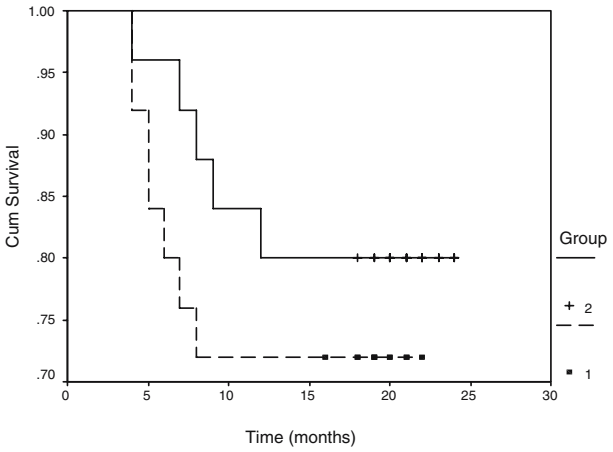

Fig. 3 Overall survival time between groups A-I and A-II

involving Bcl-2 overexpression [19]. It has been settled that Bcl-2 overexpression presents an adverse prognostic parameter and higher incidence of chemotherapy resistance [20,21].

Targeting and downregulating this oncoprotein is one of the hypothesized effects of Se as an adjuvant to cancer chemotherapy [22,23].

Group A-II patients demonstrated a more significant reduction in tumor volume after three cycles of therapy than group A-I, which is reflected by liver and spleen size. This supports the hypothesis that Se has an effective role in altering the behavior of malignant cells [24]. This action can be attributed to its antioxidant effect and its capability to promote tumor-cell apoptosis. In addition, Se has been proven to suppress preneoplastic potentials, therefore diminishing cancer incidence [13].

Patrick [11], in a retrospective study, demonstrated increased cancer incidence in patients with low serum Se. In an earlier study, Asfour et al. [25] elucidated that adult patients with NHL had lower serum Se levels than age- and sex-matched control subjects. This was followed by another study, which demonstrated the effective role of Se administration in enhancing apoptosis of NHL cells [26]. Other studies declared the role of antioxidants, including Se, in inducing apoptosis and suppressing angiogenesis in malignant cells, inviting for a potential role of antioxidants in cancer therapy [13].

These observations directed the study group to assess one of the hypothesized mechanisms of Se proapoptotic roles, which is downregulation of Bcl-2 antiapoptotic oncoprotein.

In this study, quantitative determination of Bcl-2 level by ELIZA in BM samples was performed before and after therapy for both group of patients. Selenium-supplemented patients demonstrated significant reduction of Bcl-2 after therapy, whereas group A-I did not show a significant reduction. This supports the postulated mechanism of Se-induced apoptosis by downregulating Bcl-2 expression.

Upregulation of the wild p53 and modulating caspases are additional mechanisms of the anticancer role of Se [27]. Interestingly, Se proapoptotic influence is selectively directed toward tumor cells, sparing normal cells. Husbeck et al. [28] demonstrated that Se has tumor-selective killing in patient matched pairs of normal and malignant prostate cells. Likewise, Asfour et al. [14] demonstrated downregulation of polymorphonuclear cells apoptosis by Se in NHL patients when receiving chemotherapy, thus reducing infectious episodes.

CD56, which is a surface marker natural killer cells in BM, exhibited a nonsignificant rise in Se-supplemented group, compared to a nonsignificant decline in group A-I patients after therapy.

CD4/CD8 ratio, reflecting immune status of the patients, was significantly higher in Se-supplemented patients than group A-II. These findings point to the role played by Se in supporting the immune system, and hinder the immunosuppressive effects of chemotherapy in NHL patients. In concordance, Mantovani et al. [29] elucidated the positive influence of Se on the immune status of cancer patients.

Sixty percent of group A-II achieved complete remission compared to 40% in group A-I. Among the completely remitted patients the OS was significantly longer in Se-supplemented (21.87 ± 1.41 vs 19.70 ± 1.95 months, respectively; p value = 0.01)..

It is concluded that adjuvant Se therapy in NHL patients improves the response to chemotherapy, supports the immune system, and has a positive influence on patients' survival.

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