In Vivo Study: Chemopreventive Effects of *Nigella sativa* (Black Cumin) Against Cyclophosphamide and Buthionine-SR-Sulfoximine Provoked Bladder Toxicity

Bechr Hamrita[1]*[#]**, Kamel Rouissi**[2][#]**, Sabrine Ben Hamed**[1]**, Ferdaws Hafi**[3]**, Rania Elayeb**[4] **and Sami Achour**[4]

[1]*Virology & Antiviral Strategies Research Unit UR17ES30 (ViroBiotech), University of Monastir, Tunisia*

[2]*Laboratory of Genetics, Immunology, and Human Pathology, University of El Manar I, Tunisia*

[3]*Department of Anesthesiology and Surgical Intensive Care Unit, Fattouma Bourguiba University Hospital, Tunisia*

[4]*Higher Institute of Biotechnology of Monastir, University of Monastir, Tunisia*

[#]*These authors contributed equally to this work*

Abstract

Aims: Bladder toxicity is one of the major toxicity effects associated with cancer chemotherapeutic treatments with Cyclophosphamide (CP) and Buthionine-SR-Sulfoximine (BSO). In the current study, we utilized a Swiss albino mouse model to investigate the effects of *Nigella sativa* seeds (NSE) pre-treatment on the urotoxicity produced by acute concentrations of CP and Buthionine-SR-Sulfoximine (BSO).

Methods: To evaluate the toxicity modulation, we carried out several measurements such as Lipid Peroxidation (LPO), Peroxide Hydrogen production (H_2O_2), and antioxidants in the urinary bladder of the mice.

Results: The findings revealed that NSE pre-treatment induced a protective effect in terms of LPO, H_2O_2 and enzymatic antioxidant activities. The levels of Glutathione S-Transferase (GST), Glutathione Reductase (GR), Glutathione Peroxidase (GP), and Catalase (CAT) in mice exposed to CP were significantly lower than in controls. The data obtained also show a decrease in Glutathione (GSH) levels and an increase in LPO activity in the CP-treated mice group. BSO treatment has shown an additive toxic impact in CP-treated mice. Our findings emphasize NSE's ability to restore enzymatic activity and hence provide protection against CP and BSO.

Conclusion: From the outcome of our investigation, it is possible to conclude that NSE has a powerful preventive effect against CP and BSO urotoxcicity. As a pre-treatment, NSE restores GSH which might contribute to the CP-induced apoptosis reversal and the free radical-mediated LPO.

Keywords: Cyclophosphamide; BSO; Urotoxicity; *Nigella sativa*; Mice

Introduction

Cancer is a leading cause of death worldwide and represents a troublesome burden on patients and their relatives as well as on societies and health care systems. Different cures and therapies, such as surgery, radiation, and chemotherapy, have been suggested to counteract this fatal disease. In spite of the significant advances in the field of cancer treatment, each of these therapies is associated with a number of vexing side effects. Surgeries have often been blamed for causing bodily mutilations and dysfunctions; radiation and chemotherapeutic treatments have frequently been reproached for entailing traumatic side effects, such as nausea, vomiting, and diarrhea, in addition to losses of appetite, weight, and hair.

Cyclophosphamide (CP) and Buthionine-SR-Sulfoximine (BSO) represent the two most commonly recommended medications for cancer treatment. CP is the most widely utilized alkylating anticancer agent in anti-neoplastic to treat both malignant and non-malignant disorders, despite its hazardous immunotoxicity, hematotoxicity, and mutagenicity [1,2]. Various studies report that CP can be linked to cardiac damage, carcinogenicity, and even hemorrhagic cystitis [2]. It has been observed in the literature that CP often leads to extensive tissue damage and lung toxicity [3]. This alkylating agent has also been reported to cause urological cytotoxicity and hemorrhagic cystitis

in the bladder [3]. Several bladder epithelial damages have also been previously attributed to acrolein, products of CP hydroxylation in the liver [4,5]. The aggregation of CP toxic metabolites in the urine bladder has been related to the generation of Reactive Oxygen Species (ROS) that could affect the urinary bladder and several other vital organs [6].

CP therapy has been demonstrated to increase Glutathione (GSH) levels in the urine bladder, which represent among the most critical organic molecules within the cell that dictates its capacity to evacuate toxic materials like chemotherapeutic drug metabolite [7]. As GSH has a key role in tumor cell resistance to certain chemotherapeutic drugs, its reduction seems to be an interesting strategy for sensitizing malignant cells to some chemotherapy drugs. According to several studies, BSO can considerably lower GSH levels and may aid in the restoration of sensitivity to alkylating drugs in resistant tumors.

BSO, a strong inhibitor of r-glutamylcysteine synthetase was shown to improve the anticancer activity of several medications *in vitro*, notably CP, melphalan, adriamycin, daunomycin, and mitomycin C [8]. In experimental models, BSO has been seen to boost the cytotoxic impacts of multiple chemotherapeutic drugs and radiotherapy through the reduction of this crucial enzyme [9]. The amount of glutathione in normal tissues and malignant cells is affected by BSO and other medications that modulate glutathione levels [10]. BSO's anticancer function is considered to be related to its regulation of cytotoxicity via GSH depletion. However, the decrease of GSH after BSO treatment has been linked to many toxic effects, involving cardiotoxicity, hepatic damage, and respiratory issues, according to many investigations [11,12].

Based on the different toxic effects associated with CP care alone or combined with BSO, recent studies have focused on the investigation of tissue antioxidants that could assist to minimize or eliminate this damage. A wild range of natural and synthetic substances have been demonstrated to decrease CP toxicity *via* their antioxidant properties [7,13]. Recent investigations have found that CP-induced GSH depletion is mainly driven by the action of its reactive metabolite acrolein with GSH [4]. While acrolein reacts spontaneously with glutathione due to its high reactivity, glutathione S-transferases catalyze the production of Michael adducts between Glutathione and acrolein (GSTs). Other studies have found that CP therapy might rise the pro-inflammatory cytokines like tumor necrosis factor-α (TNF-α), Interleukin-1β (IL-1β), and type 2 cyclooxygenase in the bladder epithelium [14,15]. Other markers could assist with the CP-induced urotoxicity pathogenesis including cytokines, Cyclooxygenase-2 (COX-2), and ROS.

It's hypothesized that Nitric Oxide (NO) has an important role in the urinary tract damage related to CP treatment [14]. Another major source of tissue damage is oxidative stress, which is characterized by a sharp rise in malondialdehyde levels and an inhibition of the peroxidase and catalase enzymes [15]. Of particular interest, CP treatment is often associated with reductions in the GSH (reduced glutathione) levels in the urinary bladder [7]. In general, the reactive metabolites of CP seem to be subject to a variety of therapeutic and toxic activities [2,5]. In fact, despite its potent antitumor activity, CP-induced urotoxicity is considered as a limiting step against the absolute approval for its application in clinical practice [16].

As such, novel compositions for use as broad-spectrum chemoprotectants are needed to overcome the current troublesome side effects associated with CP and BSO treatments. These novel compositions have been used as an adjuvant to chemotherapies, protecting patients' normal cells from the toxicity associated with such treatments. In this regard, folk medicine appears to be gaining popularity among medical researchers. To put it another way, there is a new interest in recent studies toward the discovery of tumor-therapeutic agents from natural sources. In fact, despite the fact that neither its active ingredients nor the modes of action are properly known, folk medicine has frequently been used with tremendous success in the treatment of many diseases. Of particular interest to the aims of the present study, the black cumin (*Nigella sativa*) represents a reputed healing herbaceous spicy plant, whose seeds have long been used to maintain good health and cure various diseases, including bronchial asthma, headache, dysentery, infections, obesity, back pain, hypertension, gastrointestinal problems, and eczema [17]. The black cumin seed oil has frequently been reported to exhibit antioxidant, anti-inflammatory, and chemopreventive effects [18,19]. It has, for instance, been shown to suppress the proliferation of various tumor cells, including colorectal carcinoma, breast adenocarcinoma, osteosarcoma, ovarian carcinoma, myeloblastic leukemia, and pancreatic carcinoma, though being minimally toxic to normal cells [20,21].

The seeds of NS have been determined to contain this plant's active components. Traditionally, the black seed was being associated with medicinal properties in the Islamic culture [22]. For many decades, it has been used as a traditional medicine in the Middle and the Far East for a variety of diseases, such as bronchial asthma, dysentery, obesity, gastrointestinal problems [23]. The black seeds' extract has, however, been used only in a few clinical trials. Amino acids, proteins, carbohydrates, fixed and volatile oils, alkaloids, saponins, and several substances compose the chemical constitution of this seed. NS seeds have fixed oil content of 36% to 38%, proteins, alkaloids, saponin, and up to 2.5% of essential oil [24]. The volatile oil is made of 18.4% to 24% of Thymoquinone (TQ) and 46% of monoterpenes including p-cymene and α-piene [25]. Recent clinical and animal research indicates that the extract of black seeds has a wide range of significant therapeutic benefits, including immunomodulatory, antibacterial, hypotensive, hepatoprotective, and anti-diabetic properties [2].

Besides that, advanced toxicological studies have shown that seed's crude extracts and some of their active components such as volatile oil, Thymoquinone (TQ) may defend toward nephrotoxicity and hepatotoxicity caused by the disease or additives [24]. TQ is the chief component of NS and has been proven to have promising antioxidant capabilities [18]. It has been shown to partially protect the gastric mucosa against acute alcohol-induced mucosal damage, which is attributed to its radical scavenging function [18]. In the same context, Sayed-Ahmed et al. have investigated the possible protective effects of TQ against Gentamicin (GM) -induced nephrotoxicity [26]. The supplement of TQ was described to bring about a significant decrease in reduced Glutathione (GSH), a marked increase in Glutathione Peroxidase (GPx) and catalase. Compared to the control data, GM-induced a rise in blood urea nitrogen, creatinine, Thiobarbituric Acid-Reactive Substances (TBARS), and total nitrate/nitrite (NOx) and a significant drop in GSH, GPx, CAT, and ATP [18].

Accordingly, the current study was conducted to explore the effects of oral administration of a NS extract on antioxidant, Lipid Peroxidation (LPO), and H_2O_2 generation, in the urinary bladders of CP and BSO-treated mice that had been predisposed or

simultaneously exposed to a GSH decreasing drug in the case of an infection or antibiotic administration.

Materials and Methods

Nigella seeds extract (NSE)

The current investigation utilized a full aqueous semisolid extract of black cumin (*Nigella sativa*) seeds obtained from the plant extract division of the local Central Pharmacy in Tunisia. The moisture and ash levels of the seeds extract were 12% and 8%, correspondingly. The pH of 10% of the aqueous extract solution indicated 4.6. The extract's purity has been validated by the manufacturer's expert taxonomist.

Chemicals

Cyclophosphamide monohydrate [2-[bis-(2-chloroethyl) amino] tetrahydro- 2H-1,3,2-oxazaphosphorine 2-oxide monohydrate]; CAS 6055-19-2 and BSO [L-buthionine-SR-sulfoximine]; CAS 5072-26-4 were purchased from Sigma–Aldrich Co., St. Louis, MO, USA.

Animals

The current study's experimental investigations were carried out using male Swiss albino mice (252 g) furnished by the animal service of the Pasteur Institute of Tunis. Animal care and handling practices were carried out in compliance with the Institutional Animal Ethics Committee's recommendations (IAEC). The animals were bred and kept in a conventional lab environment (temperature $25 \pm 2°C$; photoperiod of 12 h). A standard (industrial) pellet diet and sufficient water were provided ad libitum.

Dosage and experimental groups

Normal saline solution was used to maintain BSO, CP, and NSE extracts. The methodology entails dividing mice into seven groups, Groups I to VII, each with 6 mice. Group I represent the control where mice received a normal saline p.o. during 10 days and a single i.p. injection on the 10th day. Group II (BSO) referred to the mice receiving BSO (500 mg/kg body wt) and a single i.p. injection on the 10th day. The mice in Group III (CP) received CP (50 mg/kg body wt.) and a single i.p. dose on the 10th day of therapy. Mice in Group IV (BSO + CP) were given BSO i.p. 5 h prior to CP administration. Mice in Group V (CP + NSE) were given seeds extract for 10 days and a single i.p. injection of CP on the 10th day. Mice in Group VI (BSO + NSE) received seeds extract treatment (100 mg/kg body wt.) p.o. for 10 days and a single i.p. injection of BSO on the tenth day. The animals in Group VII (BSO + CP + NSE) were given seeds extracted for 10 days and then CP and BSO on the 10th day.

Dosing was conducted in such a manner that all mice can be sacrificed on the same day (day 11). The BSO and CP doses were chosen based on pilot studies that included the assay of a wide range of doses, as well as data from previously published reports [27].

Biochemical investigations

Once the treatment was complete, mice were sacrificed under moderate anesthesia, and their bladders were collected. A potter homogenizer was used to homogenize the bladder tissue in chilled phosphate buffer (0.1 M, pH 7.4), and another portion of the tissue was saved for histological investigation. The homogenate was centrifuged at 10,500 g for 30 min at 4°C to get Post-Mitochondrial Supernatant (PMS), which was then used for the biochemical assays outlined below.

Cell culture and peroxide hydrogen production:

Cell culture: For its ability to respond to appropriate stimuli, the urothelial cell line T24 was used as an *in vitro* model of the human bladder. this cell line was offered from the Pasteur Institute of Tunis, Tunisia, and was commonly grown in 75 cm² flasks (Nunc, Denmark) and maintained in Minimum Essential Medium (MEM) (Invitrogen, Glasgow, UK) augmented with 10% fetal bovine serum (FBS, Hyclone, Logan, UT), 100 units/ml penicillin and 100l g/ml streptomycin. Cultures were kept at 37°C in a humidified environment with 5% CO_2. Cell dissociation was accomplished using 0.05% of trypsin and 0.02% of EDTA.

Cells were seeded on 24-well culture plates in the medium at a density of about 105 cells/cm², and after 24 h of stabilization, bladder cells line T24 were co-cultured with a medium comprising different concentrations of CP and BSO (200 and 800 M) and NSE (10, 50, and 100 M) for 24 h.

The CP concentration was chosen based on prior cytotoxic values in cultivated cells [28].

For the stock solution, CP and BSO were dissolved in MilliQ plus sterilized water at an 800 mM concentration, and NSE was dissolved in Dimethylsulfoxide (DMSO) to get 100 mM concentration. The trial concentrations used in the experiments were freshly made in the basal medium with a final DMSO concentration of 0.15.

Measurement of H_2O_2 production: The Ferrous ion Oxidation Xylenol orange (FOX1) technique was used to determine H_2O_2 levels [29]. FOX1 reagent included 25 mM sulphuric acid, 250lM ferrous ammonium sulfate, 100 mM xylenol orange, and 0.1 M sorbitol. In brief, 100 μl of culture media were mixed with 900l of FOX1 reagent, vortexed, and incubated at room temperature for 30 min. After centrifuging the solutions at 12000 g for 10 min, the amount of H_2O_2 in the supernatants was measured using a spectrophotometer set at 560 nm.

Lipid peroxidation: LPO was determined by using Uchiyama and Mihara method [30]. The test mixture contained 0.67% of thiobarbituric acid; TBA (Sigma-Aldrich), 10 mM butylated hydroxy toluene, BHT (Sigma-Aldrich), 1 percent ortho-phosphoric acid (Sigma-Aldrich), and tissue homogenate in a volume of 3 ml: The activity of LPO was represented as nmol of TBA Reactive Substances (TBARS) formed/h/g of tissue that used a molecular extinction coefficient epsilon (ϵ) of 1.56×10^5 M^{-1}.

Measurement of GSH: The GSH level in the PMS of the urine bladder was determined using the method reported previously [13]. 1 ml of PMS was precipitated with 1 ml of 4% sulfosalicylic acid (Sigma-Aldrich). The plates were stored for 1 h at 4°C before being centrifuged at 1200 g for 15 min at 4°C. In a total amount of 3 ml, the test mixture contained 0.2 ml of filtered aliquot, 2.6 ml of sodium phosphate buffer (0.1 M, pH 7.4), and 0.2 ml 100 mM DTNB (dithio-bis-2-nitrobenzoic acid, Sigma-Aldrich). At 412 nm, the absorbance of the reaction product was quantified, and data were represented as nmol GSH/g tissue.

Antioxidant enzyme measurements: The activity of Glutathione-S-Transferase (GST) was measured using the technique described by Haque et al. [29]. In a total volume of 2 ml, the reaction mixture contained 1.675 ml sodium phosphate buffer, 0.2 ml of 1 mM GSH (Sigma-Aldrich), 0.025 ml of 1 mM CDNB (1-chloro-2,4-dinitrobenzene, Sigma-Aldrich), and 0.1 ml of PMS. The absorbance change was measured at 340 nm, and the enzymatic activity was calculated as nmol CDNB conjugates formed/min/mg protein

utilizing ϵ of 9.6×10^3 M^{-1} cm^{-1}.

The activity of GR (glutathione reductase) was measured using Sharma et al. [30] methods. In a total volume of 2 ml, the test mixture contained 1.6 ml sodium phosphate buffer, 0.1 ml 1 mM ethylenediamine tetraacetic acid disodium salt (EDTA, Sigma-Aldrich), 0.1 ml NADPH (nicotinamide adenine dinucleotide phosphate reduced, Sigma-Aldrich), 0.1 ml oxidized glutathione (Sigma-Aldrich), and PMS (0.1 ml). Enzymatic activity was determined at 340 nm as nmol NADPH oxidized/min/mg protein utilizing epsilon (ϵ) of 6.22×10^3 M^{-1} cm^{-1}. Glutathione Peroxidase (GP) activity was measured using the approach of Sharma et al. [30].

In a total volume of 2 ml, the testing mixture contained 1.49 ml sodium phosphate buffer, 0.1 ml EDTA (1 mM), 0.1 ml sodium azide (1 mM, Central Pharmacy of Tunis, Tunisia), 0.1 ml 1 mM GSH (Sigma-Aldrich), 0.1 ml NADPH (0.02 mM), 0.01 ml of 0.25 mM hydrogen peroxide (H$_2$O$_2$, CDH Chemicals), and 0.1 ml PMS. The oxidation of NADPH was determined spectrophotometrically at 340 nm. Enzymatic activity was measured as nmol NADPH oxidized/min/mg of protein utilizing epsilon (ϵ) of 6.22×10^3 M^{-1} cm^{-1}. CAT (catalase) level was determined referring to Haque et al. [29] approach. In an entire amount of 3 ml, the testing mixture contained 1.95 ml phosphate buffer, 1 ml H$_2$O$_2$ (0.09 M), and 0.05 ml PMS. At 240 nm, the change in absorbance was measured kinetically. The level of H$_2$O$_2$ consumed/min/mg protein has been used to determine CAT activity.

Protein measurement

Protein was determined using the Lowry et al. procedure [31].

Histological studies

Bladder urothelium tissue isolated from control and treated mice was stained in 10% buffered formalin before paraffin sectioning. To examine under a light microscope, sections of around 5 m thickness were stained with hematoxylin and eosin.

Statistical analysis

To assess if there were significant variations in data from different groups, a single factor one-way analysis of variance (ANOVA) was used. P values <0.05 represent a statistical significance level. The significance of the different treatment groups was then analyzed and compared using the Student-Newman-Keuls test. The results were presented as mean ± SE.

Results

During the treatment, no mice died and no significant changes in body weight were observed in the various groups of animals.

Lipid peroxidation

The analysis indicates that BSO treatment resulted in a significant rise (P<0.01) in LPO levels in the bladder when compared to the control values (Figure 1). The administration of CP was also found to significantly increase LPO.

Similarly, the combined impact of BSO+CP led to a significant rise in LPO as compared to the control group (Group I): When compared to the CP group (Group III) separately, the BSO+CP group (Group IV) had a significant (P<0.01) rise in LPO levels (Figure 1). Animals pre-treated with NSE and then exposed to BSO (BSO+NSE) had a significant (P<0.01) decrease in LPO in regards to the LPO levels in the bladder. When the data obtained for the CP+NSE group (Group V) were compared with those obtained for the CP group
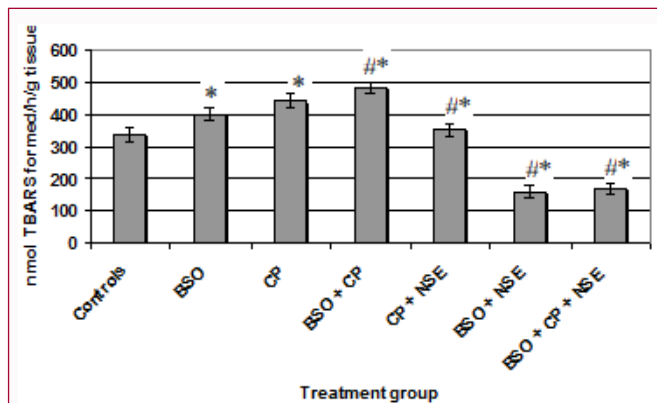

Figure 1: Effects of Nigella Seeds Extract (NSE), BSO, and CP on the lipid peroxidation in the urinary bladder of mice. Significant differences are indicated by *P<0.01 and *P<0.01 in Group II (BSO) and Group III (CP) of treated animals, respectively; and *#P<0.01 in Group IV (BSO+CP), when compared with control animals (Group I). *P<0.01 indicates significant levels for the BSO+CP group when compared to the CP group. *#P<0.01 and *#P<0.05 indicate significant difference of data related to Group V (CP+NSE) and Group VI (BSO+NSE) when compared with Group III and Group II, respectively. *#P<0.01 when data related to Group IV were compared with those of Group VII. Values are means ± SE (n=6).
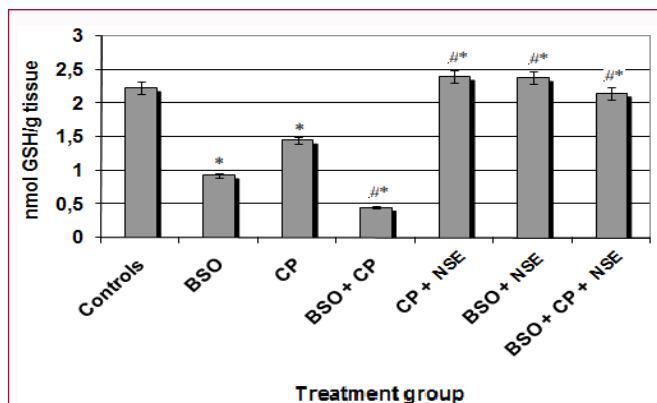

Figure 2: Effects of Nigella Seeds Extract (NSE), BSO, and CP on GSH content in the urinary bladder of mice. Significant differences are indicated by *P<0.01 and *P<0.01 in Group II (BSO) and Group III (CP) of animals, respectively; and *#P<0.01 in Group IV (BSO+CP) when compared with control animals (Group I). *#P<0.01 indicates significant change in the BSO+CP group when compared with the CP group. *#P<0.01 and *#P<0.01 indicate significant differences in observations in Group IV (CP+NSE) and Group VI (BSO+NSE) when compared with data related to the GSH of Groups III and II, respectively. *#P<0.01 indicates significant differences when data related to the GSH of Group IV were compared with Group VII. Values are means ± SE (n=6).

(Group II), NSE treatment was shown to significantly (P<0.05) lower LPO levels in the bladder. As shown in Figure 1, the comparison of the LPO values obtained for the BSO+CP group (Group IV) and those acquired for the BSO+CP+NSE group clearly demonstrates a significant reduction (P<0.01) in the latter (Group VII).

Reduced glutathione

When compared to the control values (2.3 nmol GSH/g tissue), the BSO, CP, and BSO+CP-treated groups had significant (P<0.01) reductions of 0.94, 1.45, and 0.45 nmol GSH/g tissue in GSH, accordingly (results produced for Cellular GSH of urine bladder are presented in Figure 2). Similarly, as compared to the CP group (Group III), the BSO+CP group (Group IV) had a significant (P<0.01) reduction in GSH levels.

Table 1: Activities of anti-oxidant enzymes in the urinary bladder of mice in different treatment groups.

Group	Activity of anti-oxidant enzyme			
	GST	GR	GP	CAT
I (Controls)	100 ± 3	99 ± 4	111 ± 5	60 ± 3
II (BSO)	56 ± 2 *	48 ± 4 *	78 ± 3 *	53 ± 2 *
III (CP)	71 ± 3 *	78 ± 5 *	95 ± 4 **	40 ± 3 *
IV (BSO+CP)	35 ± 3 *#	42 ± 2 *#	74 ± 3 *#	38 ± 3 *#
V (CP+NSE)	109 ± 5 #	108 ± 5 #	118 ± 2#	63 ± 5 #
VI (BSO+NSE)	103 ± 3 #	105 ± 6 #	112 ± 4#	69 ± 7 #
VII (BSO+CP+NSE)	101 ± 4 ##	104 ± 6 ##	110 ± 4##	60 ± 5 ##

CP: Cyclophosphamide; BSO: Buthionine Sulfoximine; NSE: Nigella Seeds Extract

Values are means ± SE (n=6). GST expressed as nmol CDNB conjugates/min/mg protein, GR as nmol NADPH oxidized/min/mg of protein, and GP as nmol NADPH oxidized/min/mg protein. CAT activity is expressed as nmol H_2O_2 consumed/min/mg protein. Significant differences are indicated by *$P<0.01$, **$P<0.001$, *$P<0.05$ when compared with control animals. (Group I) #$P<0.01$ when compared with Group II, ##$P<0.01$ when compared with CP-treated animals (Group III) and *#$P<0.01$ when compared with Group IV.

When the BSO and BSO+NSE groups were compared, the GSH level of the latter was found to be significantly higher ($P<0.01$). Likewise, the GSH levels in the bladder of the CP+NSE group (Group V) increased significantly ($P<0.01$) when compared to the group given only CP (Group III). When the GSH levels obtained for the BSO+CP (Group IV) group were compared with the results obtained for the BSO+CP+NSE group (Group VII), a significant ($P<0.01$) recovery in GSH was shown (Figure 2).

Antioxidant enzymes

When compared to the control group, BSO and CP treatments were shown to create significant ($P<0.01$) reductions in GST, GR, GP, and CAT levels in the bladder (Table 1). When compared to the CP or control group, the BSO+CP group demonstrated an additive significant ($P<0.01$) reduction in GST, GR, and GP levels. Nevertheless, no significant difference in CAT activity was found between both the animals in groups IV (BSO+CP) and III (CP).

As compared to their respective controls, the BSO (Group II) and CP (Group III), the activity of those antioxidant enzymes grew significantly ($P<0.01$) in both the BSO+NSE (Group VI) and CP+NSE (Group V) groups (Table 1). When mice were treated with NSE and then exposed to BSO+CP (Group VII, BSO+CP+NSE treatment), the activity of all antioxidant enzymes increased significantly ($P<0.01$) when compared to the BSO+CP group (Group IV).

Hydrogen peroxide (H_2O_2) production *in vitro*

The impacts of CP and NSE on H_2O_2 generation in bladder urothelium cell line T24 were shown in Figure 3. Cells were incubated for 24 h with CP (200 and 800 M) with various doses of NSE (10, 50, and 100 M) to see if the combination of CP and NSE had any benefic effect. H_2O_2's levels produced in cell medium were significantly ($P<0.05$) raised by 159 and 241% when compared to controls after exposure to CP (200 and 800 M), respectively, and were significantly ($P<0.05$) reduced by (27% t, 41%, and 20%) cells co-cultured with NSE (10, 50, and 100 M) and CP at dose (200 M) and by (40%, 49%, and 25%) at the dose of 800 μM when compared with CP separately (200 and 800 μM) respectively.

Histological examination of bladder urethelium

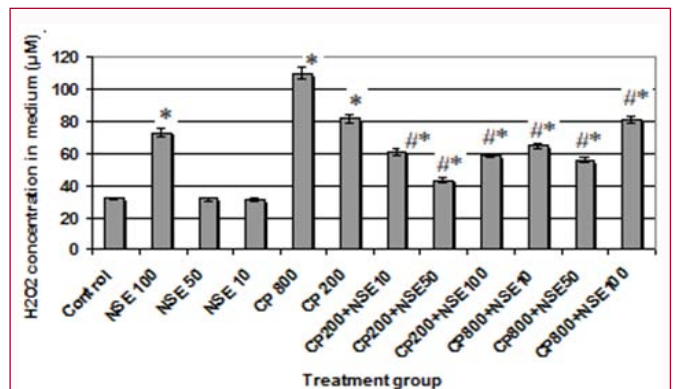The histological observations of bladder urothelium tissue in

Figure 3: Effect of Nigella Seeds Extract (NSE) on H_2O_2 production in the medium culture of bladder urothelium cells exposed to CP. The cells were exposed to CP or co-exposed to CP (200 and 800 lM) and NSE (10, 50 and 100 lM) for 24 h. Data represent the mean ± SD from four independent experiments. *$P<0.05$ *vs.* control, and #$P<0.05$ *vs.* CP exposed cells.
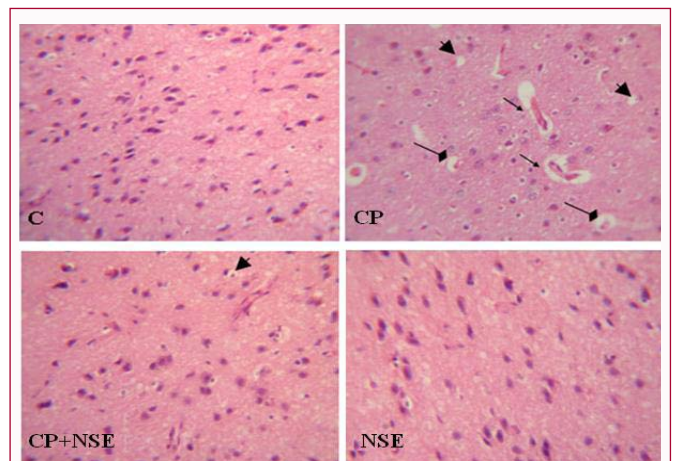
Figure 4: Photographs showing histopathological changes in bladder urothelium tissue in different groups, Control group (C), CP treated group (CP), Nigella Seeds Extract treated group (NSE), Cyclophosphamide + Nigella Seeds Extract treated group (CP+NSE) (hematoxylin and eosin staining, 400x).

treated mice are shown in Figure 4. The urothelium tissue underwent histopathological analysis, which indicated that CP treatment led to an aberrant cellular arrangement with few pyknotic nuclei, vacuolated spaces, and hemorrhagic state. Co-administration of NSE at 100 mg/kg body weight, on the other hand, prevented these alterations and preserved normal architecture with fewer pyknotic nuclei and architecture that was almost identical to that of the untreated control. When positive controls treated with NSE alone were compared to negative controls, there were no histological differences in the bladder urothelium.

Discussion

Cancer is a major cause of illness and death throughout the world and constitutes a troublesome burden on patients and their relatives as well as on societies and health care systems. Throughout the years, several anti-cancer treatments and therapies have been proposed, among which chemotherapy prevails. In spite of the significant advances in the field of cancer treatment, however, most of these treatments are associated with vexing toxic side effects.

Cyclophosphamide (CP) and Buthionine-SR-Sulfoximine (BSO) are two of the most commonly given cancer medicines. Despite their undisputed efficiencies in the treatment of cancer, their application is often reported to entail a number of troublesome side effects. Thus, recent research has focused on the discovery of new chemoprotectants that might be used as adjuvants to chemotherapies to protect patients' normal cells from the toxicity related to CP and BSO treatments. The current study was conducted to investigate the impacts of oral administration of an NS seeds extract on Lipid Peroxidation (LPO), H_2O_2 generation, and antioxidant activities in the urinary bladder of CP and BSO treated mice that were predisposed or concurrently exposed to a GSH reduction agent in the manner of either an infection or antibiotic utilize.

The data indicated that no mortalities were reported for the various experimental groups of animals during the treatment. When data of GSH decrease were examined, it was shown that BSO had a more significant impact than CP. 42% difference was seen between the two compounds, confirming that BSO is a more powerful GSH depletion than CP, as previously described in the literature. The interaction of its reactive metabolite, acrolein, with GSH P is largely responsible for CP-induced GSH depletion [4].

Acrolein interacts not just with GSH but rather with cysteine, which is one of GSH's component amino acids [4]. Several studies have found that substances containing free sulfhydryl groups may protect against the urotoxic effects of cyclophosphamide. Several Sulfhydryl (-SH) substances, including cysteine, have been shown to protect experimental mice from the toxicity of CP [32].

The injection of CP intraperitoneally was found to significantly increase LPO in the bladder. Indeed, lipid peroxidation is commonly employed as an indication of oxidative stress and cell membrane disruption [33].

Free radicals, like superoxide anion and hydroxyl radical, act in a toxic manner on DNA, membrane proteins, and lipids. LPO caused by CP has been documented in various tissues of exposed mice [34]. The function of acrolein in CP-induced LPO has been hypothesized [35]. It has been proposed that acrolein, by binding to nucleophilic amino acids, may directly influence transcription as well as the regulation of this process via its capacity to deplete GSH [4].

In terms of BSO treatment, the data indicated that it resulted in GSH depletion and an increase in LPO in the urinary bladder. GSH deficiency has also been linked to an increase in the vulnerability of cells to apoptosis [36]. Depletion of intracellular GSH has been documented in a variety of different apoptotic contexts, with various researches indicating that GSH loss in apoptotic cells is the consequence of the rapid efflux rather than oxidative depletion [37]. Furthermore, when BSO and CP were combined, an additive impact was found in terms of GSH, LPO, and other parameters. The goal of utilizing BSO in combination with CP was to investigate a possible case in which the host is exposed to a mixture of GSH depleting agents, including pathogens, and to examine if NSE seed extract treatment had any modulatory impact on their commutative/additive actions. *Nigella sativa* (a member of the ranunculaceae family) is a very interesting medicinal plant with a rich historical and religious history and strong therapeutic potential [24].

In this work, NSE pre-treatment not only had a significant protective impact against CP urotoxicity, but it also performed an important protective function in mice treated with the CP+BSO combination (Group VII). The pre-treatment with Black Cumin seeds extract was shown to restore depleted GSH as well as other antioxidants while lowering LPO levels in the bladder.

Generally, the results show that the NSE reported in this study is a potential immunomodulatory herbal extract with strong GSH restoring properties. Thus, this extract may open up new avenues opportunities for reducing the side effects of CP and BSO cancer therapies.

Previously, the NSE has been shown to have immunomodulatory properties in mice [38]. However, several factors are possibly involved in bladder toxicity and Reactive Oxygen Species (ROS) may play a major role in the pathogenesis of reperfusion injuries. ROS may react with proteins, lipids, and nucleic acids, causing lipid peroxidation in biological membranes. This can affect enzymatic activities such as ion pump function and DNA damage; limiting transcription and DNA repair [39]. TQ has been demonstrated to possess strong antioxidant properties and has been shown to protect non-tumor tissues from chemotherapy-induced damage [40]. The data obtained are broadly consistent with prior research [40,41]. Moreover, the present findings suggest that NSE and its derivative TQ have a potent chemo-protective potential for the inhibition of toxicity processes by modulating lipid peroxidation, H_2O_2 and cellular antioxidant environments. These results are in agreement with other previous reports [40,41]. The current study's findings show that NSE pre-treatment restored CP urotoxicity caused primarily by LPO and reduced GSH by reversing those impacts.

In this investigation, we found that exposing urothelium cells line T24 to CP (200 and 800 M) significantly enhanced H_2O_2 production in the extracellular medium, demonstrating that ROS production is the main determinant for CP-induced toxicity. NSE's capacity to influence CP-induced cellular damage is consistent with its increased efficacy in lowering ROS (H_2O_2 production). Our findings are in line with those of Fu et al. [41], who discovered that plant extract might have antioxidative proprieties and free radical scavenging characteristics *in vitro*, and can scavenge a variety of oxidizing radicals including OH•, NO2•, O2•, and RNS•.

In addition, Histopathological analysis of the bladder urothelium tissue indicates that CP therapy induces aberrant cellular organization with few pyknotic nuclei, vacuolated gaps, and hemorrhaging. Co-treatment with NSE, on the other hand, inhibits these alterations and preserves normal architecture with very few pyknotic nuclei.

In summary, this is the first study in Tunisia to emphasize the biological activity and the bladder urotoxicity. The seed extract reported here had a significant antioxidant capacity in several *in vivo* tests. Our results confirmed the efficiency of *Nigella sativa* seeds extract from Tunisia as a natural antioxidant agent. Because of its broad availability in nature and its low toxicity, this NSE might be a promising candidate for future use as an adjuvant to cancer chemotherapies. As a result, additional researches are being conducted in our laboratories to further investigate this extract in terms of cell cycle regulatory and antioxidant activities, to further confirm its efficacy as an anti-tumor agent, and, especially, to make it fit for a possible pharmaceutical use as a treatment.

References

1. Sheweita SA, El-Hosseiny LS, Nashashibi MA. Protective effects of essential oils as natural antioxidants against hepatotoxicity induced by cyclophosphamide in mice. PLoS One. 2016;11(11):e0165667.

2. Bhatia K, Kaur M, Atif F, Ali M, Rehman H, Rahman S, et al. Aqueous extract of Trigonella foenum-graecum L. ameliorates additive urotoxicity of buthionine sulfoximine and cyclophosphamide in mice. Food Chem Toxicol. 2006;44(10):1744-50.

3. Saghir AM, Alharbi SA, Al-Garadi MA, Al-Gabri N, Rady HY, Olama NK, et al. Curcumin prevents cyclophosphamide-induced lung injury in rats by suppressing oxidative stress and apoptosis. Processes. 2020;8(2):127.

4. Dave NM. Anesthesia for pediatric cancers. In: Textbook of onco-anesthesiology. Springer: Singapore; 2021. p. 335-54.

5. Tsai HC, Tsou HH, Lin CC, Chen SC, Cheng HW, Liu TY, et al. Acrolein contributes to human colorectal tumorigenesis through the activation of RAS-MAPK pathway. Sci Rep. 2021;11(1):12590.

6. Ibrahim M, Bhyravabhotla K, Khalaf B, Meter KV, Saba NS, Safah H, et al. The utility of hyperbaric oxygen therapy in post-transplant cyclophosphamide-induced hemorrhagic cystitis: A case report and review of the literature. J Med Case Rep. 2021;15(1):1.

7. Hadi NA, Reyes-Castellanos G, Carrier A. Targeting redox metabolism in pancreatic cancer. Int J Mol Sci. 2021;22(4):1534.

8. Wu S, Wang P, Qin J, Pei Y, Wang Y. GSH - depleted nanozymes with dual - radicals enzyme activities for tumor synergic therapy. Adv Funct Mater. 2021:31(31):2102160.

9. Louie KG, Behrens BC, Kinsella TJ, Hamilton TC, Grotzinger KR, McKoy WM, et al. Radiation survival parameters of antineoplastic drug-sensitive and -resistant human ovarian cancer cell lines and their modification by buthionine sulfoximine. Cancer Res. 1985;45(5): 2110-5.

10. Yu K, Hai X, Yue S, Song W, Bi S. Glutathione-activated DNA-Au nanomachine as targeted drug delivery platform for imaging-guided combinational cancer therapy. Chem Eng J. 2021;419:129535.

11. Hamsa TP, Kuttan G. Tinospora cordifolia ameliorates urotoxic effect of cyclophosphamide by modulating GSH and cytokine levels. Exp Toxicol Pathol. 2012;64(4):307-14.

12. Alfieri AB, Cubeddu LX. Nitric oxide and NK1-tachykinin receptors in cyclophosphamide-induced cystitis, in rats. J Pharmacol Exp Ther. 2000;295(2):824-9.

13. Fraiser LH, Kehrer JP. Murine strain differences in metabolism and bladder toxicity of cyclophosphamide. Toxicology. 1992;75(3):257-72.

14. Sherif IO. Uroprotective mechanism of quercetin against cyclophosphamide - induced urotoxicity: Effect on oxidative stress and inflammatory markers. J Cell Biochem. 2018;119(9):7441-8.

15. Ahmad A, Husain, A, Mujeeb M, Khan SA, Najmi AK, Siddique NA, et al. A review on therapeutic potential of Nigella sativa: A miracle herb. Asian Pac J Trop Biomed. 2013;3(5):337-52.

16. Yimer EM, Tuem KB, Karim A, Ur-Rehman N, Anwar F. Nigella sativa L. (Black Cumin): A promising natural remedy for wide range of illnesses. Evid Based Complement Alternat Med. 2019;2019:1-16.

17. Imran M, Rauf A, Khan IA, Shahbaz M, Qaisrani TB, Fatmawati S. Thymoquinone: A novel strategy to combat cancer: A review. Biomed Pharmacother. 2018;106:390-402.

18. Hangargekar CB, Quazi RS, Khan MSA, Joshi AA, Hangargekar PB. A review on kalonji (Nigella Sativa): A seed of blessing. Int J Res Anal Rev. 2020;7(2):590-6.

19. Ali BH, Blunden G. Pharmacological and toxicological properties of Nigella sativa. Phytother Res. 2003;17(4):299-305.

20. El-Kadi A. The black seed (Nigella sative) and immunity: Its effect on human T cell subset. Fed Proc. 1987;46:1222.

21. Hosseinzadeh H, Tavakkoli A, Mahdian V, Razavi BM. Review on clinical trials of black seed (Nigella sativa) and its active constituent, thymoquinone. J Pharmacopuncture. 2017;20(3):179-93.

22. Faruque A, Fakhruddin AA, Syed AA, Hisham HS, Shadma W, Mohammed IK, et al. An updated knowledge of black seed (Nigella sativa Linn.): Review of phytochemical constituents and pharmacological properties. J Herb Med. 2021;(25):100404.

23. Ostadpoor M, Gholami-Ahangaran M. A review on hepatoprotective effects of Nigella sativa L. J Med Herbs. 2021;12(1):49-54.

24. Mansour MA, Nagi MN, El-Khatib AS, Al-Bekairi AM. Effects of thymoquinone on antioxidant enzyme activities, lipid peroxidation and DT-diaphorase in different tissues of mice: A possible mechanism of action. Cell Biochem Funct. 2002;20(2):143-51.

25. Sagit M, Korkmaz F, Gürgen SG, Kaya M, Akcadag A, Ozcan I. The protective role of thymoquinone in the prevention of gentamicin ototoxicity. Am J Otolaryngol. 2014;35(5):603-9.

26. Dadarkar SS, Fonseca LC, Thakkar AD, Mishra PB, Rangasamy AK, Padigaru M. Effect of nephrotoxicants and hepatotoxicants on gene expression profile in human peripheral blood mononuclear cells. Biochem Biophys Res Commun. 2010;401(2):245-50.

27. Ou P, Wolff SP. A discontinuous method for catalase determination at 'near physiological' concentrations of H2O2 and its application to the study of H2O2 fluxes within cells. J Biochem Biophys Methods. 1996;31(1-2):59-67.

28. Uchiyama M, Mihara M. Determination of malonaldehyde precursor in tissues by thiobarbituric acid test. Anal Biochem. 1978;86(1):271-8.

29. Haque R, Bin-Hafeez B, Parvez S, Pandey S, Sayeed I, Ali M, et al. Aqueous extract of walnut (Juglans regia L.) protects mice against cyclophosphamide induced biochemical toxicity. Hum Exp Toxicol. 2003;22(9):473-80.

30. Sharma N, Trikha P, Athar M, Raisuddin S. Inhibition of benzo[a]pyrene- and cyclophoshamide-induced mutagenicity by Cinnamomum cassia. Mutat Res. 2001;480-1:179-88.

31. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem. 1951;193(1):265-75.

32. Gaweł S, Wardas M, Niedworok E, Wardas P. Malondialdehyde (MDA) as a lipid peroxidation marker. Wiad Lek. 2004;57(9-10):453-5.

33. Selvakumar E, Prahalathan C, Mythili Y, Varalakshmi P. Mitigation of oxidative stress in cyclophosphamide-challenged hepatic tissue by DL-α-lipoic acid. Mol Cell Biochem. 2005;272(1):179-85.

34. Habibi E, Shokrzadeh M, Chabra A, Naghshvar F, Keshavarz-Maleki R, Ahmadi A. Protective effects of Origanum vulgare ethanol extract against cyclophosphamide-induced liver toxicity in mice. Pharm Biol. 2015;53(1):10-5.

35. Armağan HH, Nazıroğlu M. Glutathione depletion induces oxidative injury and apoptosis via TRPM2 channel activation in renal collecting duct cells. Chem Biol Interact. 2021;334:109306.

36. Saadat S, Aslani MR, Ghorani V, Keyhanmanesh R, Boskabady MH. The effects of Nigella sativa on respiratory, allergic and immunologic disorders, evidence from experimental and clinical studies, a comprehensive and updated review. Phyther Res. 2021;35(6):2968-96.

37. Khan F, Garg VK, Singh AK, Kumar T. Role of free radicals and certain antioxidants in the management of huntington's disease: A review. J Anal Pharm Res. 2018;7(4).386-92.

38. Banerjee S, Padhye S, Azmi A, Wang Z, Philip PA, Kucuk O, et al. Review on molecular and therapeutic potential of thymoquinone in cancer. Nutr Cancer. 2010;62(7):938-46.

39. Badary OA. Thymoquinone attenuates ifosfamide-induced Fanconi syndrome in rats and enhances its antitumor activity in mice. J Ethnopharmacol. 1999;67(2):135-42.

40. Badary OA, Taha RA, Gamal El-Din AM, Abdel-Wahab MH. Thymoquinone is a potent superoxide anion scavenger. Drug Chem Toxicol. 2003;26(2):87-98.

41. Fu H, Lin M, Muroya Y, Hata K, Katsumura Y, Yokoya A, et al. Free radical scavenging reactions and antioxidant activities of silybin: Mechanistic aspects and pulse radiolytic studies. Free Radic Res. 2009;43(9):887-97.