Carnitine Deficiency: A Causative Clue or a Sequel in Carboplatin Myelosuppression

Hossam M.M. Arafa[1,*], Raed S. Ismail[2], Nesreen Nabil[3] and Adel M. Mostafa[4]

[1]Dept. of Pharmacology & Toxicology, Faculty of Pharmacy, MTI University; [2]Department of Pharmacology & Toxicology, Faculty of Pharmacy, Al-Azhar University; [3]Department of Biochemistry, Faculty of Pharmacy, MTI University; [4]Department of Pharmacology, Faculty of Pharmacy, Helwan University, Cairo-Egypt

Abstract: We have previously demonstrated that carnitine deficiency aggravated paracetamol-induced hepatopathy and carboplatin-induced nephropathy. As a continuum, we have addressed in the current study as to whether carboplatin-induced myelosuppression would be exacerbated by carnitine deficiency. Challenging male Wistar rats with a single dose of carboplatin (35 mg/kg, IP) induced bone marrow suppression manifested as anemia, leucopenia, thrombocytopenia as well as increased frequencies of the micronucleated bone marrow cells; MPCE and MNCE with notable reduction in the P/N ratio. The platinum drug also elevated serum TNF-α and reduced serum free and total carnitine levels. Besides, ATP levels in red and T cells were lowered. Likewise, the mitochondrial membrane potential in T lymphocytes was reduced following the use of the potentiometric dye; JC-1, and this was well correlated with cellular ATP production. Carnitine deficiency exacerbated carboplatin myelotoxicity as it exaggerated all biochemical, hematological and cytogenetic parameters. To address as to whether carnitine deficiency was a causative clue or merely a sequel of carboplatin myelotoxicity, L-carnitine was supplemented ahead of carboplatin challenege. Herein, L-carnitine mitigated all the biochemical, hematological and cytogenetic effects possibly *via* modulating the release of TNF-α, cellular ATP production and restoring the mitochondrial membrane potential. Irrespective of the mechanisms involved, the current results may afford the potential role for carnitine supplementation as add-on nutraceutical in carboplatin-based chemotherapy.

Keywords Carboplatin, Carnitine, Myelosuppression, Micronucleus Assay, TNF-α, ATP Mitochondrial membrane potential.

INTRODUCTION

Carboplatin is the first of the second generation platinum compounds to gain wide spread use in oncological practice. Since its clinical introduction in 1981, carboplatin has proven efficacy in a variety of human malignancies including small-cell lung [1], ovarian [2], and head and neck [3] cancer. However the clinical usefulness of the platinum compound has been hampered by detrimental side effects with the most important of which being myelosuppression [4].

Myelosuppression with its dual of anemia and neutropenia is responsible for chemotherapy delays, discontinuations and dose modifications [5]. The mechanism of platinum-induced myelosuppression is not fully explored. Carboplatin, however, was found to target the hematopoietic stem cell fraction [6] possibly via DNA cross-linking and generation of oxidative stress products [7].

Carnitine deficiency, on the other hand, has been implicated in the pathogenesis of an array of organ maladies including diabetes [8], epilepsy [9] and cardiovascular disorders [10]. Uremia-induced carnitine deficiency, which is magnified by dialysis, is also associated with anemia hyporesponsive to erythropoietin [11].

L-carnitine is a naturally occurring quaternary ammonium compound, which is endogenously synthesized in man and also found in the diet [12]. It is cofactor of enzymes for the transformation of middle- and long-chain fatty acids. It regulates carbohydrate metabolism, maintains cell membrane structure and cell viability and acts also as a scavenger of oxygen free radicals in mammalian tissues [13, 14]. Besides, it has been proved that L-carnitine and in particular its propionyl ester, stimulates erythropoiesis, which was expressed by a significant increase of erythroid progenitors in bone marrow and other hematopoietic organs [15]. Also, the observed relationship between carnitine serum level and osmotic erythrocyte fragility suggests indirectly its beneficial role in erythrocyte membrane stabilization [16].

Taken together, the main objective of the present work was to address the role of carnitine deficiency in carboplatin-induced myelosuppression. This study was further extended to elucidate if carnitine supplementation would mitigate the myelotoxic and hematotoxic effects of the cytotoxic drug in an attempt to unravel the riddle as to whether carnitine deficiency is a possible forerunner or a consequence of such myelotoxicity.

*Address correspondence to this author at the Department of Pharmacology and Toxicology, Faculty of Pharmacy, MTI University, Cairo, Egypt; Tel: +202-01001529178; Fax: +202-22014052; E-mail: hmmarafa@yahoo.com

ISSN: 1929-2260 / E-ISSN: 1929-2279/14

In this sense, carnitine deficiency model was induced in rats by repeated administration of D-carnitine which was used as an effective carnitine deficiency surrogate model for carnitine-deficient diet as it interferes with the normal absorption and disposition of the active L-form. Animals were challenged thereafter with a single IP dose of carboplatin. Myelosuppression was assessed hematologically, biochemically as well as by examination of bone marrow specimens. Herein, complete blood count (CBC) has been done with special emphasis on hemoglobin, neutrophils, and thrombocytes. Serum TNF-α level was assessed as a proinflammatory cytokine. Cytogenetic examination of bone marrow specimens encompassed the determination of the number of polychromatic erythrocytes (PCE) and normochromatic erythrocytes (NCE) as well as the PCE/NCE ratio. The frequencies of micronucleated PCEs and NCEs have been computed as a measure of chromosomal damage. Cellular ATP levels in both red and T cells were determined as a measure of energy status. Measurement of mitochondrial potential was done in T lymphocytes since mitochondria play the primary role in energy production and ATP generation.

MATERIALS AND METHODS

Drugs

Carboplatin was obtained as ampoules from Bristol-Myers-Squibb (Princeton, NJ, USA). D-carnitine and L-carnitine were purchased from Sigma-Aldrich (St. Louis, MO, USA) as white powder freely soluble in water.

Chemicals

Acetyl Coenzyme A (CoA) and carnitine acetyltransferase were supplied by Roche Diagnostics (Barcelona, Spain). Giemsa's and May-Gruenwald's solutions were purchased from Merck (Darmstadt, Germany). All other chemicals were of the finest analytical grade.

Animals

Male Wistar rats weighing 150-180 g were obtained from Theodor Bilharz Institute (Giza, Egypt). Animals were housed in the animal facility of the Pharmacy College, Al-Azhar University in Cairo. The animals were kept at temperature of 21 ºC and a relative humidity of 55% with a regular 12 h light/12 h dark cycle. The animals were fed standard chow (El-Nasr Co., Abou-Zaabal, Cairo, Egypt), and allowed free access to water. All animal experiments were conducted according to the regulations of the Committee on Bioethics for Animal Experiments of Al-Azhar University.

Design of Work

A total of sixty male Wistar rats were allotted in six groups; ten animals each. Treatment regimens were assigned as such: (1) A group was given distilled water (0.2 ml/100 g bd. wt., IP) once daily for 10 consecutive days. (2) A second group was administered D-carnitine (250 mg/kg bd. wt., IP) once daily for ten consecutive days [17]. (3) A third group was challenged with a single dose of carboplatin (35 mg/kg bd. wt., IP). (4) A fourth group was given D-carnitine as before followed thereafter by carboplatin in the same dose regimen. (5) A fifth group received L-carnitine (500 mg/kg, IP) for 10 consecutive days [18]. (6) A sixth group was administered L-carnitine and then challenged with carboplatin as before.

Retro-orbital blood samples were withdrawn under light ether anesthesia using heparinized micro-capillaries (Optilab, Berlin, Germany) 6 days after carboplatin administration. Thereafter, animals were euthanized by cervical dislocation and terminally bled. Heparinized blood was used for assessment of hematological parameters. Serum was separated following centrifugation at 500 g for 10 min at 4◦C and used for carnitine and TNF-α analysis. Rest of blood was used for separation of RBCs and T cells. Bone marrow was separated from femurs and processed as in methodology prior to micronucleus assay.

Assessment of Carnitine Levels

Total and free carnitine concentrations were determined in serum according to the method reported by Prieto *et al.* [19]. In brief, carnitine reacts with acetyl-CoA catalyzed by carnitine acetyltransferase. The coenzyme A liberated combines with 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB) and forms thiophenolate ion, whose generation is proportional to the amount of carnitine and can be measured spectrophotometrically at 412 nm. For total carnitine assay, the method requires precipitation of proteins with 0.6 M perchloric acid, followed by alkaline hydrolysis of acylcarnitines. Carnitine level was computed from a calibration curve for carnitine hydrochloride.

Determination of raTNF-α Concentration in Rat Serum

Serum raTNF-α was assessed using a commercial kit purchased from Biosource International (California,

USA). It is a solid phase sandwich enzyme-linked immunosorbent assay (ELISA). The assay method was preformed utilizing the principle of immuno-sorbent assay previously described by Wolters *et al.* [20]. The color intensity measured at 450 nm is directly proportional to the concentration of raTNF-α present in the original specimen.

Micronucleus Assay

The micronucleus test in erythrocytes of rat bone marrow was proposed as a screening test by Von Ledbur and Schmid [21]. The main purpose of the test is to determine the number of micronucleated PCEs. The frequency of micronuclei can be most easily evaluated in young erythrocytes shortly after the main nucleus is expelled. These young erythrocytes are termed polychromatic (PCE) and are distinguished from the mature normochromatic (NCE) ones by their different staining properties. With a combination of Giemsa and May-Gruenwald staining [22] the PCEs stain bluish to purple due to their high content of RNA in the cytoplasm. In contrast, the NCEs stain reddish to yellow and are also slightly smaller than PCEs. The ratio of NCE to PCE is determined by counting a total of about 2000 erythrocytes per slide including both NCEs and PCEs. From each slide, PCEs and NCE cells were examined for MPCEs and MNCE under 1000x magnification using an Olympus microscope (PA, USA). In addition, the numbers of PCEs and NCEs per slide were recorded to evaluate bone marrow toxicity; PCE (P): NCE (N) ratio was calculated as % PCE = [PCE/ (PCE + NCE)] x 100 to determine a reduction of erythroblast proliferation.

Luciferease-Luminescence ATP Assay

The level of ATP in T cells and RBCs was determined according to Mikirova *et al*. [23] using a commercial CellTiter-GloLuminescent cell Viability Assay kit (Promega, USA). The assay generates a glow type signal produced by luciferase reaction, which is proportional to the amount of ATP present in cells. In this assay, the signal half-life was greater than one hour. For photon count, 50 microL of reaction mixture was mixed with 50 microL of cells and the count was measured by luminometer (BD Biosciences, USA). RBCs were separated from other cells and plasma by centrifugation at 500 g for 10 min. The supernatant and "buffy coat" were removed by aspiration. Packed cells were diluted by PBS with 10 mM glucose and 1mg/ml BSA (bovine serum albumin) to preserve the normal shape of RBCs. The RBCs were then washed two times by PBS and centrifuged at 200 g for 2-3 min. The final dilution of cells by PBS was greater than 1:3000. Before ATP measurements, cells were counted by hemo-cytometer (Neubauer, Berlin, Germany). T cells were separated by "PosetteSep" procedure from Stem Cell Technology (USA). In this procedure, 50 microL of RosetteSep cocktail was added to each mL of blood and mixed well. Cells were incubated 20 min at room temperature. After incubation, cells were diluted with an equal volume of PBS containing 2% FBS (fetal bovine serum), mixed well and layered on the top of Ficoll-Paque (Amersham Bioscience). After 20 min of centrifugation at 1200 g, enriched cells were separated, washed in PBS with FBS, counted, and used for analysis. For determination of the standard curve, ATP (Sigma-Aldrich, USA) was dissolved in PBS to make concentrations of 50-500 nM. ATP was measured in nM/1 x 10^6 cells.

Assessment of Mitochondrial Membrane Potential

Mitochondrial potential was assessed by using the fluorescent potentiometric dye, JC-1 (5,52,6,62,-tetrachloro-1,12,3,32 tetraethylbenzimidazolylcarbo-cyanine iodide) as described earlier by Cossarizza *et al.* [24]. JC-1 is able to selectively enter the mitochondria of intact cells and form aggregates that emit at 585 nm. If the mitochondrial potential is reduced, JC-1 disaggregates to monomers that fluoresce at 527 nm. The ratio between both signals is indicative of the mitochondrial potential. Emission was measured by fluorescence plate reader (SPEX Instruments, IL, USA) at excitation 510 nm in the scan range of 520-600 nm.

Hematological Parameters

Complete blood count (CBC) was assessed to determine the numbers or proportions of white and red blood cells in the body. Cell counting was achieved by aperture impedance method (Coulter- Counter technique) that was earlier described by Coulter [25]. Blood samples were mixed with 10 microL EDTA and then placed in Swelab's fully automatic auto- counter, model AC920EO+ (Cape Town, South Africa). Up to 15 parameters were simultaneously counted.

Statistical Analysis

Statistical analysis was accomplished using GraphPad InStat (version 3.10, Philadelphia, USA, 2009). Data were computed as mean ± SE. Multiple comparisons of parametric data were achieved using

Table 1: Total and Free Carnitine Levels in Serum of Male Wistar Rats following Treatment with D-Carnitine and/or Carboplatin

Treatment	Total serum carnitine (nmol/l)	Free serum carnitine (nmol/l)
Control	83.1 ± 7.5	53.4 ± 4.1
D-Carnitine	30.5[a] ± 4.8	22.3[a] ± 2.1
Carboplatin	60.2[a,b] ± 4.5	36.4[a,b] ± 3.2
D-Carnitine + Carboplatin	11.6[a,b,c] ± 2.5	7.8[a,b,c] ± 1.3

Data were presented as mean ± SE, n = 10.
[a]Significantly different from control.
[b]Significantly different from D-carnitine.
[c]Significantly different from carboplatin.
[1]Control animals received saline (0.2 ml/100 g bd. wt., IP).
[2]D-carnitine (250 mg/kg, IP) was administered for 10 consecutive days.
[3]Carboplatin (35 mg/kg, IP) was given as a single dose.
[4]Animals were challenged with carboplatin as before after repeated D-carnitine administration.
-Multiple comparisons were accomplished using one way ANOVA followed by Tukey as a post-hoc test.

one way ANOVA followed by Tukey as post-hoc test. Correlation analysis was done by calculating Pearson *r*. The criterion for significance was chosen to be $p < 0.05$.

RESULTS

Serum Carnitine Level

Total and free serum carnitine levels are compiled in Table **1**. D-carnitine reduced total and free carnitine levels by 63 and 58% respectively, compared to control values. Carboplatin reduced total and free carnitine levels by 28 and 32% respectively, compared to control group. Prior administration of D-carnitine ahead of carboplatin challenge resulted in serum nadir of total and free carnitines amounted to 86 and 85 % respectively, compared to control levels. The combination modality also reduced the serum total and free carnitine levels by 81 and 79%, respectively compared to carboplatin-treated animals, and by 62 and 65 % compared to D-carnitine-treated rats.

Serum raTNF-α Concentration

D-carnitine increased serum TNF-α concentration by 32 % compared to control rats (Figure **1a**). Administration of carboplatin resulted in significant increase in serum TNF-α by 153 % compared to control animals (Figure **1a**). Prior administration of D-carnitine before carboplatin caused substantial increase in serum TNF-α amounted to about 206 % compared to control group (Figure **1a**). Also, the combination modality increased the serum cytokine level by about 21 and 132% respectively, compared to carboplatin- and D-carnitine-treated animals, respectively. L-carnitine had no effect on serum TNF-α concentration compared to control level (Figure **1b**). Pretreatment with L-carnitine before carboplatin, however, resulted in marked decrease in serum TNF-α concentration by 58 % compared to animals treated with carboplatin alone.

Assessment of the Cytogenetic Effects

Effects of carnitine isomers and/or carboplatin on the frequencies of of PCE and NCE and their micronucleated erythroblasts as well as the P/N ratio in the bone marrow of male Wistar rats are shown in Tables **2-3**. D-carnitine had no effect on the frequencies of either PCE or NCE cells let for the significant occurrence of micronucleated erythroblasts. Carboplatin, however, significantly reduced the number of PCE by about 14% and increased the count of NCE by about 13% compared to control values. Besides, the P/N ratio was reduced by about 23 % compared to control ratio (Table **2**). The number of micronucleated cells; MPCE and MNCE, was increased by 139 and 138 % respectively, compared to control group (Table **2**). Prior administration of D-carnitine before carboplatin resulted in significant decrease in P/N ratio by 32% compared to control value, and 12% compared to carboplatin-challenged animals (Table **2**). The combination modality also increased the incidence of MPCE and MNCE by 239 and 217%, respectively compared to control values and by 42 and 33% compared to carboplatin-treated rats (Table **2**). L-carnitine had no effect on the number of either PCE or NCE let for the P/N ratio compared to control group (Table **3**). Further, it did not affect the incidence of micronucleated erythroblasts compared to control values (Table **3**). Pretreatment with L-carnitine before carboplatin resulted in a significant increase in the P/N

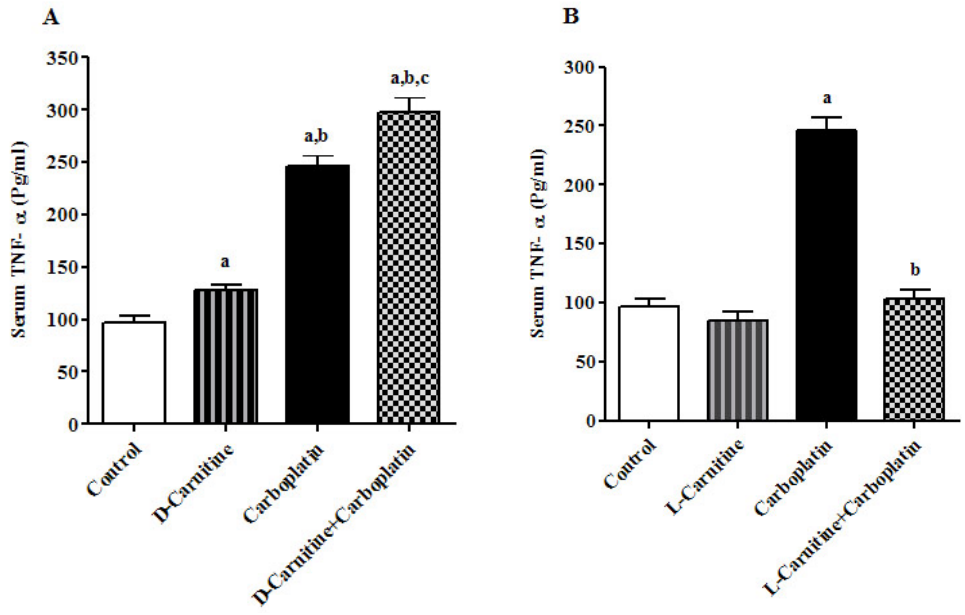

Figure 1: a: Effects of D-carnitine and/or carboplatin on serum TNF-α in male Wistar rats.
b: Effects of L-carnitine and/or carboplatin on serum TNF-α in male Wistar rats.

Table 2: Effects of D-Carnitine and/or Carboplatin on the Frequencies of MPCE and MNCE as well as the P/N Ratio in the Bone Marrow of Male Wistar Rats

Treatment	PCE	NCE	MPCE	MNCE	PCE/NCE (P/N) Ratio
Control[1]	947.10 ± 10.2	1047 ± 8.32	2.8± 0.38	2.4 ± 0.37	0.90 ± 0.01
D-carnitine [2]	922.80[b] ± 8.6	1077[b] ± 8.64	2.2[b] ± 0.25	2.9[b] ± 0.35	0.86[b] ± 0.02
Carboplatin[3]	816.10[a,c] ± 9.3	1184[a,c] ± 9.36	6.7[a,c] ± 0.70	5.7[a,c] ± 0.26	0.69[a,c] ± 0.01
D-carnitine+ Carboplatin[4]	765.10[a,b,c] ± 10.2	1235[a,b,c] ± 10.24	9.5[a,b,c] ± 1.17	7.6[a,b,c] ±0.85	0.61[a,b,c] ± 0.02

Data are presented as means ± SEM, n = 10.
[1]Control animals received distilled water in a dose of 0.2 ml/100 g, IP.
[2]D-carnitine (250 mg/kg body weight, IP) was given for 10 consecutive days.
[3]Carboplatin (35 mg/kg body weight, IP) was administered once.
[4]D-carnitine was given as before followed thereafter by carboplatin in the same dose regimen.
[a]Significantly different from control at $p < 0.05$.
[b]Significantly different from carboplatin at $p < 0.05$.
[c]Significantly different from D-carnitine at $p < 0.05$.
- Multiple comparisons were accomplished using one way ANOVA followed by Tukey as a post-hoc test.

ratio amounted to 13% compared to rats treated with carboplatin alone. The combined regimen also reduced the frequencies of MPCE and MNCE by 39 and 37%, respectively compared to carboplatin-treated animals (Table **3**).

ATP Levels in Red and T Cells

D-carnitine lowered ATP levels in red cells and T cells by 50 and 44 %, respectively compared to control values (Figure **2a**). ATP levels in red cells and T cells were reduced by 39 and 27 %, respectively compared to control animals following carboplatin challenge. D-carnitine followed thereafter by carboplatin notably reduced cellular ATP levels by about 71 and 64 % compared to control group; and 49 and 50 %, respectively compared to carboplatin-treated rats (Figure **2a**). L-carnitine increased cellular ATP levels by 54 and 33% compared to control values (Figure **2b**). Prior administration of L-carnitine ahead of carboplatin injection significantly increased cellular ATP levels in both cell types by 36 and 32 % respectively, compared to carboplatin-treated group (Figure **2b**).

Mitochondrial Membrane Potential in T Lymphocytes

D-carnitine lowered the mitochondrial membrane potential in T cells by about 37.5 % compared to control value (Figure **3**). Carboplatin reduced the

Table 3: Effects of L-Carnitine and/or Carboplatin on the Frequencies of MPCE and MNCE as well as the P/N Ratio in the Bone Marrow of Male Wistar Rats

Treatment	PCE	NCE	MPCE	MNCE	PCE/NCE (P/N) Ratio
Control[1]	947.1 ± 10.2	1047 ± 8.32	2.8± 0.38	2.4 ± 0.37	0.90 ± 0.014
L-carnitine[2]	936.9 ± 10.3	1065 ± 9.64	1.5 ± 0.71	1.7 ± 0.21	0.88 ± 0.017
Carboplatin[3]	816.1[a] ± 9.3	1184[a] ± 9.36	6.7[a] ± 0.70	5.7[a] ± 0.26	0.69[a,] ± 0.013
L-carnitine+ Carboplatin[4]	878[a,b] ± 12.4	1115[a,b] ± 16.4	4.1[a,b] ± 0.35	3.6[a,b] ± 0.16	0.78[a,b] ± 0.027

Data are presented as means ± SEM, n = 10.
[1]Control animals received distilled water in a dose of 0.2 ml/100 g, IP.
[2]L-carnitine (500 mg/kg body weight, IP) was given for 10 consecutive days ahead of carboplatin challenge.
[3]Carboplatin (35 mg/kg body weight, IP) was administered once.
[4]L-carnitine was given as before followed thereafter by carboplatin in the same dose regimen.
[a]Significantly different from control at $p < 0.05$.
[b]Significantly different from carboplatin at $p < 0.05$.
- Multiple comparisons were accomplished using one way ANOVA followed by Tukey as a post-hoc test.

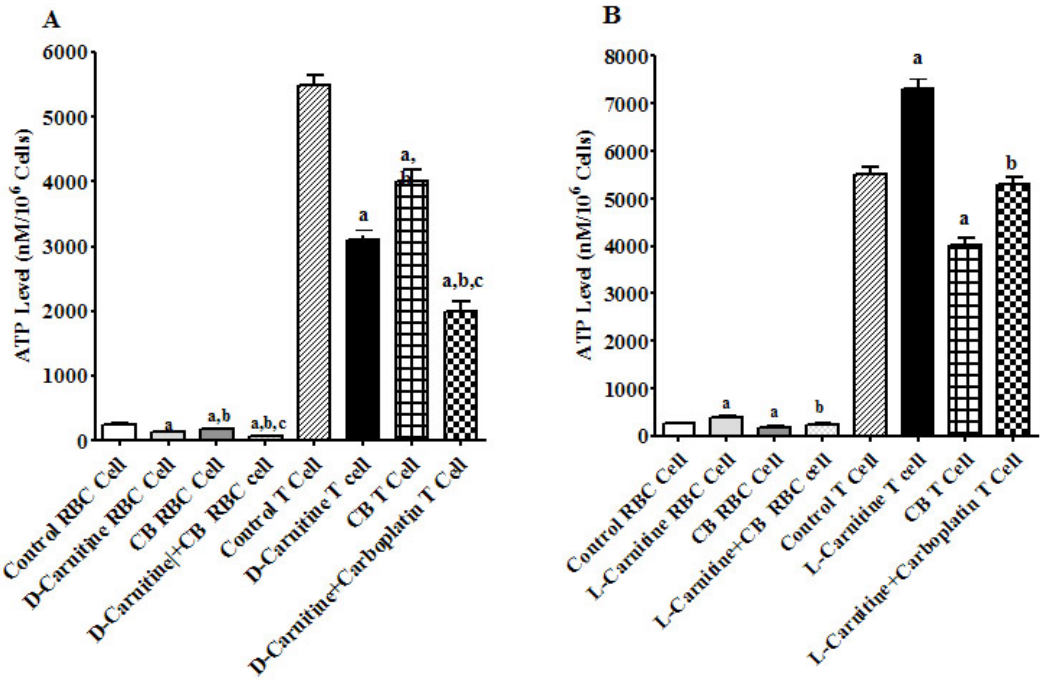

Figure 2: a: Effects of D-carnitine and/or carboplatin on ATP levels in red and T cells of male Wistar rats.

b: Effects of L-carnitine and/or carboplatin on ATP levels in red and T cells of male Wistar rats.

membrane potential by about 25 % compared to control group. Challenging animals with carboplatin following D-carnitine administration resulted in reduced membrane potential amounted to about 63, 50 and 40% compared to control-, carboplatin-, and D-carnitine-treated animals, respectively. Pretreatment with L-carnitine before the platinum drug increased the membrane potential by 22% compared to carboplatin group. There was also a positive correlation between ATP level in T cells and the mitochondrial membrane potential with a Pearson value (*r*) of 0.844 (Figure **4**).

Effects on Hematological Parameters

Hematological parameters following different treatment regimens that incorporated carboplatin with either D-carnitine or L-carnitine are compiled in Tables **4**,**5**. D-carnitine had no effect on CBC compared to control values. Carboplatin, however, altered all the blood elements tested. Carboplatin provoked notable nadirs in hemoglobin content (Hb), RBCs, PLT and WBC counts by about 21%, 31%, 44% and 35%, respectively compared to control rats. Prior administration of D-carnitine before carboplatin

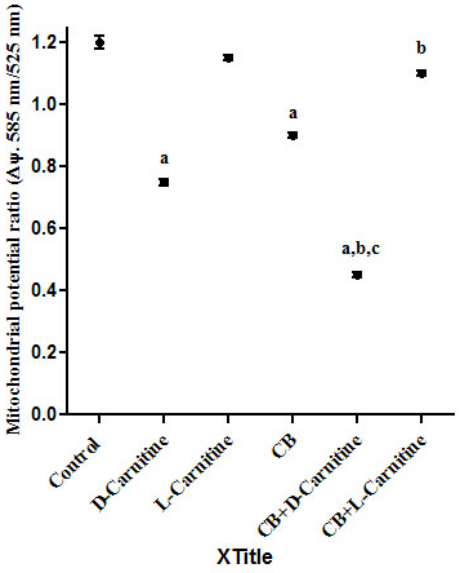

Figure 3: Effects of either D- or L-carnitine and/or carboplatin on mitochondrial membrane potential in T lymphocytes of male Wistar rats.

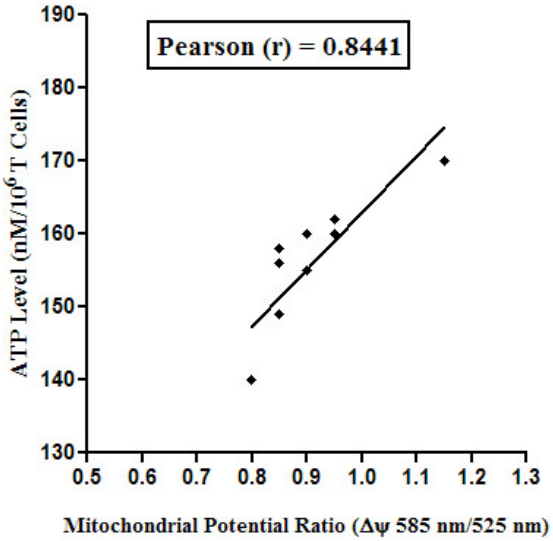

Figure 4: Correlation between ATP concentration in T lymphocytes and mitochondrial membrane potential.

produced marked decreases particularly in Hb content, RBCs, PLT and WBC counts amounted to about 36%, 40%, 52% and 43%, respectively compared to control animals, 34%, 38%, 49% and 37%, respectively compared to rats given D-carnitine alone, and, 18%, 13%, 15% and 12%, respectively compared to carboplatin-treated rats. L-carnitine had no effect on CBC compared to control rats. Pretreatment with L-carnitine before carboplatin increased most of the blood elements levels particularly Hb content, RBCs, PLT and WBC counts by about 20 %, 15%, 12% and 12%, respectively compared to animals treated with carboplatin alone.

Table 4: Effects of D-Carnitine and/or Carboplatin on Hematological Parameters in male Wistar Rats.

Parameter	Control[1]	DC[2]	CB[3]	DC+CB[4]
RBCs ($10e^6$/µl)	7.5 ± 0.08	7.35 ± 0 .08[b]	5.2 ± 0.17[a,c]	4.5 ± 0.11[a,b,c]
HCT (%)	38.6 ± 0.15	37.8 ± 0.16[b]	32.4 ± 0.13[a,c]	29.9 ± 0.16[a,b,c]
MCV (um^3)	86.7 ± 0.45	85.1 ± 0.27[b]	75.0 ± 0.10[a,c]	72.6 ± 0.21[a,b,c]
RDW (%)	13.30 ± 0.51	13.20 ± 0.59[b]	8.10 ± 0.23[a,c]	6 ± 0.25[a,b,c]
HGB (g/dl)	14.26 ± 0.11	14.10 ± 0.18[b]	11.20 ± 0.22[a,c]	9.28 ± 0.22[a,b,c]
MCH (pg)	28.30 ± 0.32	27.17 ± 0.36[b]	23.21 ± 0.34[a,c]	21.25 ±0.27[a,b,c]
MCHC (g/dl)	33.0 ± 0.17	32.2 ± 0.24[b]	27.8 ± 0.29[a,c]	25.5 ± 0.15[a,b,c]
PLT ($10e^3$/µl)	366.1 ± 2.6	346.2 ± 1.19[b]	206.2 ± 1.15[a,c]	175.8 ± 1.5[a,b,c]
PCT (%)	3.10 ± 0.07	2.94 ± 0.07[b]	1.77 ± 0.06[a,c]	0.84 ± 0.05[a,b,c]
MPV (μm^3)	9.09 ± 0.07	8.75 ± 0.13[b]	6.52 ± 0.17[a,c]	5.64 ± 0.05[a,b,c]
PDW (μm^3)	10.59 ± 0.06	10.35 ± 0.09[b]	8.40 ± 0.07[a,c]	7.16 ± 0.09[a,b,c]
WBC ($10e^3$/µl)	13.5 ± 0.30	12.36 ± 0.37[b]	8.76 ± 0.35[a,c]	7.69 ± 0.17[a,b,c]
LYMPH($10e^3$/µl)	2.71 ± 0.06	2.58 ± 0.07[b]	1.23 ± 0.04[a,c]	0.67 ± 0.04[a,b,c]
MID ($10e^3$/µl)	2.59 ± 0.04	2.59 ± 0.04[b]	1.21 ± 0.03[a,c]	0.18 ± 0.03[a,b,c]
GRAN ($10e^3$/µl)	4.24 ± 0.08	4.38 ± 0.07[b]	3.13 ± 0.03[a,c]	2.37 ± 0.05[a,b,c]

Data are presented as means ± SEM, n = 10.
[1]Control Animals received distilled water in a dose of 0.2 ml/100 g, IP.
[2]D-carnitine (250 mg/kg body weight, IP) was given for 10 consecutive days.
[3]Carboplatin (35 mg/kg body weight, IP) was administered once and blood samples were withdrawn after 6 days.
[4]D-carnitine was given as before followed thereafter by carboplatin in the same dose regimen and blood samples were withdrawn after 6 days.
[a]Significantly different from control at $p < 0.05$.
[b]Significantly different from Carboplatin at $p < 0.05$.
[c]Significantly different from D-carnitine at $p < 0.05$.
- Multiple comparisons were accomplished using one way ANOVA followed by Tukey as a post-hoc test.

Table 5: Effects of L-Carnitine and/or Carboplatin on Hematological Parameters in male Wistar Rats

Parameter	Treatment Regimens			
RBCs ($10e^6$/µl)	**Control[1]**	**LC[2]**	**CP[3]**	**LC+CP[4]**
HCT (%)	7.5 ± 0.08	7.3 ± 0.07	5.2 ± 0.17[a]	6.0 ± 0.06[a,b]
MCV (um^3)	38.6 ± 0.15	38.1 ± 0.20	32.4 ± 0.13[a]	34.6 ± 0.14[a,b]
RDW (%)	86.7 ± 0.45	86.8 ± 0.28	75.0 ± 0.10[a]	77.0 ± 0.09[a,b]
HGB (g/dl)	13.30 ± 0.51	12.5 ± 0.26	8.10 ± 0.23[a]	10.50 ± 0.30[a,b]
MCH (pg)	14.26 ± 0.11	14.89± 0.24	11.20 ± 0.22[a]	13.59 ± 0.13[a,b]
MCHC (g/dl)	28.30 ± 0.32	28.09± 0.25	23.21 ± 0.34[a]	23.8 ± 0.14[a,b]
PLT ($10e^3$/µl)	33.0 ± 0.17	33.2± 0.21	27.8 ± 0.29[a]	29.9 ± 0.18[a,b]
PCT (%)	366.1 ± 2.6	364.2 ± 2.08	206.2 ± 1.15[a]	231.6 ± 0.99[a,b]
MPV (μm^3)	3.10 ± 0.07	3.41 ± 0.04	1.77 ± 0.06 [a]	2.54 ± 0.04[a,b]
PDW (μm^3)	9.09 ± 0.07	9.46 ± 0.07	6.52 ± 0.17[a]	7.96 ± 0.05[a,b]
WBC ($10e^3$/µl)	10.59 ± 0.06	10.85 ± 0.07	8.40 ± 0.07[a]	9.36 ± 0.07[a,b]
LYMPH($10e^3$/µl)	13.5 ± 0.30	12.5 ± 0.39	8.76 ± 0.35[a]	10.26 ± 0.28[a,b]
MID ($10e^3$/µl)	2.71 ± 0.06	2.57 ± 0.05	1.23 ± 0.04[a]	1.95 ± 0.05[a,b]
GRAN ($10e^3$/µl)	2.59 ± 0.04	2.61 ± 0.04	1.21 ± 0.03[a]	1.83 ± 0.04[a,b]

Data are presented as means ± SEM, n = 10.
[1]Control Animals received distilled water in a dose of 0.2 ml/100 g, IP.
[2]L-carnitine (500 mg/kg body weight, IP) was given for 10 consecutive days ahead of carboplatin challenge.
[3]Carboplatin (35 mg/kg body weight, IP) was administered once and blood samples were withdrawn after 6 days.
[4]L-carnitine was given as before followed thereafter by carboplatin in the same dose regimen and blood samples were withdrawn after 6 days.
[a]Significantly different from control at $p < 0.05$.
[b]Significantly different from carboplatin at $p < 0.05$.
- Multiple comparisons were accomplished using one way ANOVA followed by Tukey as post hoc test.

DISCUSSION

Carboplatin is one of the most active cytotoxic agents in current use. However, the clinical utility of the platinum compound has been precluded by untoward effects with the most important of which being myelosuppression [4].

Challenging animals with a single dose of carboplatin induced myelotoxicity that was characterized biochemically, hematologically as well as by cytogenetic analysis of bone marrow samples.

Carboplatin provoked marked reductions in almost all blood elements leading ultimately to anemia, thrombocytopenia, leucopenia and neutropenia. This is coping with many earlier reports that documented such bone marrow suppression [26, 27]. The exact mechanism whereby the platinum compound would exert such an effect is not fully explored. However, the finding in the current study that carboplatin notably increased serum TNF-α level may explain such myelotoxicity. Earlier study by Ulich *et al*. demonstrated that TNF-alpha was a potent inhibitor of erythropoiesis, and the cytokine has been shown *in vitro* to inhibit the growth of erythroid and myeloid progenitors [28]. TNF alpha and its soluble receptors may be involved in the pathogenesis of drug-induced myelosuppression [29]. The apparent reduction in cellular ATP levels in red and T cells and the decreased mitochondrial membrane potential in T cells following carboplatin challenge, which was correlated well with the ATP nadir observed in lymphocytes may provide an explanation for the myelotoxic, hematotoxic and genotoxic effects of carboplatin. This may be explained in light of the deterioration in membrane potential that led to mitochondrial disruption, which is a distinctive early feature of cellular apoptosis.

D-Carnitine increased serum TNF-α and reduced both cellular ATP content and mitochondrial membrane potential. We have reported similar elevations in serum levels of the proinflammatory cytokine in carnitine-deficient rats [17, 30]. Cellular deterioration of ATP may be due to the deficiency in carnitine; the fatty acids transport carrier that carries fatty acids from cytosol into mitochondria to be metabolized by beta oxidation to release ATP through citric acid cycle.

Carnitine deficiency exacerbated carboplatin-induced anemia, leucopenia and thrombocytopenia. Herein, it was reported that nutritional carnitine deficit

would alter erythropoiesis [31]. Blocking by D-carnitine of the crucial role of endogenous L-carnitine in the transport of free fatty acids to the mitochondrial metabolic machinery in bone marrow cells, as energy source for erythropoiesis, cannot be excluded as a possible mechanism.

Carnitine deficiency prior to carboplatin challenge notably increased the number of micronucleated cells in both PCE and NCE cells. It also decreased the P/N ratio. This again confirms the potentiative effects of carnitine deficiency on carboplatin myelotoxicity.

The finding that L-carnitine has been shown to prolong RBC lifespan and to abrogate anemia in animal and human studies including hemodialysis patients beside its beneficial effects on erythropoiesis [32] warranted our attention to address in the current study the postulate as to whether carnitine deficiency would possibly exacerbate carboplatin myelosuppression. The study was further extended to unravel the presumable protective role of carnitine replenishment in this toxicity paradigm in an attempt to clarify the conundrum as to whether carnitine deficiency is a causative clue or a sequela for carboplatin myelotoxicity.

Carnitine replenishment improved myelotoxicity as manifested by correction of anemia, leucopenia and thrombocytopenia. It also apparently reduced serum TNF-alpha level and reduced the percentage of micronucleated cells in both PCE and NEC populations when administered ahead of carboplatin challenge. The P/N ratio was also significantly reduced. This finding is in line with previous reports that demonstrated anti-clastogenic effects of carnitine [33, 34]. Also, Abd-Allah *et al*. [35] have also demonstrated a protective role of carnitine in bone marrow cells following exposure to carboplatin.

Based on the broad observations in this study, one could conclude that carnitine deficiency exacerbated carboplatin myelosuppression and consequently hematotoxicity presumably *via* increasing TNF-α production, altering mitochondrial membrane potential alongside with reduced ATP production. By doing that, it would possibly pose a further burden on subjects undergoing carboplatin-based chemotherapy. The notion that carnitine replenishment was able to effectively mitigate such myelotoxicity and correct the hematotoxic effects of the platinum drug when given ahead of carboplatin challenge would warrant our attention to the interesting possibility of using such nutraceutical as adjunctive add-on agent in chemotherapeutic armamentarium.

CONFLICT OF INTEREST

They authors herewith declare that they have no conflict of interest.

REFERENCES

[1] Tsubata Y, Okimoto T, Miura K, Karino F, Iwamoto S, Tada M, Honda T, Hamaguchi S, Ohe M, Sutani A, Kuraki T, Hamada A, Isobe T. Phase I Clinical and Pharmacokinetic Study of Bi-weekly Carboplatin/Paclitaxel Chemotherapy in Elderly Patients with Advanced Non-small Cell Lung Cancer. Anticancer Res 2013; 33(1): 261-266. http://www.ncbi.nlm.nih.gov/pubmed/23267154

[2] Glaze S, Teitelbaum L, Chu P, Ghatage P, Nation J, Nelson G. Dose-dense Paclitaxel with Carboplatin for advanced ovarian cancer: a feasible treatment alternative. J Obstet Gynaecol Can 2013; 35(1): 61-67.

[3] Fury MG, Sherman E, Ho A, Katabi N, Sima C, Kelly KW, Nwankwo O, Haque S, Pfister DG. A phase I study of temsirolimus plus carboplatin plus paclitaxel for patients with recurrent or metastatic (R/M) head and neck squamous cell cancer (HNSCC). Cancer Chemother Pharmacol 2012; 70(1): 121-128. http://dx.doi.org/10.1007/s00280-012-1894-y

[4] Soffietti R, Nobile M, Ruda R, Borgognone M, Costanza A, Laguzzi E, Mutani R. Second-line treatment with carboplatin for recurrent or progressive oligodendroglial tumors after PCV (procarbazine, lomustine, and vincristine) chemotherapy: a phase II study. Cancer 2004; 100: 807-813. http://dx.doi.org/10.1002/cncr.20042

[5] Weycker D, Malin J, Edelsberg J, Glass A, Gokhale M, Oster G. Cost of neutropenic complications of chemotherapy. Ann Oncol 2008; 19: 454-460. http://dx.doi.org/10.1093/annonc/mdm525

[6] Su WC, Chang SL, Chen TY, Chen JS, Tsao CJ. Comparison of *in vitro* growth-inhibitory activity of carboplatin and cisplatin on leukemic cells and hematopoietic progenitors: the myelosuppressive activity of carboplatin may be greater than its antileukemic effect. Jpn J Clin Oncol 2000; 30: 562-567. http://dx.doi.org/10.1093/jjco/hyd137

[7] Weijl NI, Elsendoorn TJ, Lentjes EG, Hopman GD, Wipkink-Bakker A, Zwinderman AH, Cleton FJ, Osanto S. Supplementation with antioxidant micronutrients and chemotherapy-induced toxicity in cancer patients treated with cisplatin-based chemotherapy: a randomised, double-blind, placebo-controlled study. Eur J Cancer 2004; 40: 1713-1723 http://dx.doi.org/10.1016/j.ejca.2004.02.029

[8] Evangeliou A, Vlassopoulos D. Carnitine metabolism and deficit--when supplementation is necessary? Curr Pharm Biotechnol 2003; 4: 211-219. http://dx.doi.org/10.2174/1389201033489829

[9] Stanley CA. Carnitine deficiency disorders in children. Ann N Y Acad Sci 2004; 1033: 42-51. http://dx.doi.org/10.1196/annals.1320.004

[10] Broderick TL. Hypocarnitinaemia induced by sodium pivalate in the rat is associated with left ventricular dysfunction and impaired energy metabolism. Drugs R D 2006; 7: 153–161 rugs R D 2006; 7: 153–161 http://dx.doi.org/10.2165/00126839-200607030-00002

[11] Calò LA, Vertolli U, Davis PA, Savica V. L-carnitine in hemodialysis patients. Hemodial Int 2012; 16(3): 428-434. http://dx.doi.org/10.1111/j.1542-4758.2012.00679.x

[12] Goa KL, Brogden RN. L-Carnitine. A preliminary review of its pharmacokinetics, and its therapeutic use in ischaemic cardiac disease and primary and secondary carnitine deficiencies in relationship to its role in fatty acid metabolism. Drugs 1987; 34: 1-24. http://dx.doi.org/10.2165/00003495-198734010-00001

[13] Athanassakis I, Mouratidou M, Sakka P, Evangeliou A, Spilioti M, Vassiliadis S. L-carnitine modifies the humoral immune response in mice after *in vitro* or *in vivo* treatment. Int Immunopharmacol 2001; 1: 1813-1822. http://dx.doi.org/10.1016/S1567-5769(01)00105-9

[14] Izgut-Uysal VN, Agac A, Derin N. Effect of carnitine on stress-induced lipid peroxidation in rat gastric mucosa. J Gastroenterol 2001; 36: 231-236. http://dx.doi.org/10.1007/s005350170108

[15] Kitamura Y, Satoh K, Satoh T, Takita M, Matsuura A. Effect of L-carnitine on erythroid colony formation in mouse bone marrow cells. Nephrol Dial Transplant 2005; 20: 981–984 http://dx.doi.org/10.1093/ndt/gfh758

[16] Trovato GM, Iannetti E, Murgo AM, Carpinteri G, Catalano D. Body composition and long-term levo-carnitine supplementation. Clin Ter 1998; 149: 209-214.

[17] Arafa HM. Carnitine deficiency: a possible risk factor in paracetamol hepatotoxicity. Arch Toxicol 2009; 83: 139-150. http://dx.doi.org/10.1007/s00204-008-0330-x

[18] Al Majed AA. Carnitine deficiency provokes cisplatin-induced hepatotoxicity in rats. Basic Clin. Pharmacol. Toxicol 2007; 100: 145-150. http://dx.doi.org/10.1111/j.1742-7843.2006.00024.x

[19] Prieto JA, Andrade F, Aldámiz-Echevarría L, Sanjurjo P. Determination of free and total carnitine in plasma by an enzymatic reaction and spectrophotometric quantitation spectrophotometric determination of carnitine. Clin Biochem 2006; 39(10): 1022-1027. http://dx.doi.org/10.1016/j.clinbiochem.2006.06.005

[20] Wolters G, Kuijpers LP, Kacaki J, Schuurs AH. Enzyme-linked immunosorbent assay for hepatitis B surface antigen. J Infect Dis 1977; 136: 311-317. 17. http://dx.doi.org/10.1093/infdis/136.Supplement_2.S311

[21] Von Ledebur M, Schmid W. The micronucleus test. Methodological aspects. Mutat Res 1973; 19: 109-117. http://dx.doi.org/10.1016/0027-5107(73)90118-8

[22] Adler ID, Kliesch U, van Hummelen P, Kirsch-Volders M. Mouse micronucleus tests with known and suspect spindle poisons: results from two laboratories. Mutagenesis 1991; 6: 47-53. http://dx.doi.org/10.1093/mutage/6.1.47

[23] Mirikova N, Riordan HD, Kirby RK, Klykov A, Jackson A. Monitoring of ATP Levels in Red Blood Cells and T Cells of Healthy and Ill Subjects and the Effects of Age on Mitochondrial Potential. J Orthomolec Med 2004; 20(1): 50-58.

[24] Cossarizza A, Baccarani-Contri M, Kalashnikova G, Franceschi C. A new method for the cytofluorimetric analysis of mitochondrial membrane potential using the J-aggregate forming lipophilic cation 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide (JC-1). Biochem Biophys Res Commun 1993; 197(1): 40-45 http://dx.doi.org/10.1006/bbrc.1993.2438

[25] Coulter PP. The public health nurse in the ANA. Am J Nurs 1953; 53: 1201-1202. http://dx.doi.org/10.1097/00000446-195310000-00017

[26] Karpova GV, Fomina TI, Voronova OL, Abramova EV, Loskutova OP. Early and delayed effects of carboplatin on the blood system. Bull Exp Biol Med 2001; 132: 1065-1069. http://dx.doi.org/10.1023/A:1017964407835

[27] Neuwelt EA, Pagel MA, Hasler BP, Deloughery TG, Muldoon L. Therapeutic Efficacy of Aortic Administration of N-Acetylcysteine as a Chemoprotectant against Bone Marrow Toxicity after Intracarotid Administration of Alkylators, with or without Glutathione Depletion in a Rat Model. Cancer Res 2001; 61: 7868–7874.

[28] Ulich TR, Shin SS, del Castillo J: Haematologic effects of TNF. Res Immunol 1993; 144: 347-354. http://dx.doi.org/10.1016/S0923-2494(93)80079-E

[29] Voog, E, Bienvenu J, Warzocha K, Moullet I, Dumontet C, Thieblemont C, Monneret G, Gutowski M, Coiffier B, Salles G. Factors That Predict Chemotherapy-Induced Myelosuppression in Lymphoma Patients: Role of the Tumor Necrosis Factor Ligand-Receptor System. J Clin Oncol 2000; 18: 325-331.

[30] Arafa HM. Carnitine deficiency aggravates carboplatin nephropathy through deterioration of energy status, oxidant/anti-oxidant balance, and inflammatory endocoids. Toxicology 2008; 254: 51-60. http://dx.doi.org/10.1016/j.tox.2008.09.010

[31] Jaureguy M, Choukroun G. Factors affecting the response to erythropoiesis-stimulating agents. Nephrol Ther 2006; 2: 274-282.

[32] Calò LA, Davis PA, Semplicini A, Pessina AC. L-Carnitine and erythropoiesis: relationship with haeme oxygenase-1. Nephrol Dial Transplant 2005; 20(8): 1769-1770. http://dx.doi.org/10.1093/ndt/gfh934

[33] Santoro A, Lioi MB, Monfregola J, Salzano S, Barbieri R, Ursini MV. L-Carnitine protects mammalian cells from chromosome aberrations but not from inhibition of cell proliferation induced by hydrogen peroxide. Mutat Res 2005; 587: 16-25. http://dx.doi.org/10.1016/j.mrgentox.2005.07.005

[34] Garcia CL, Filippi S, Mosesso P, Calvani M, Nicolai R, Mosconi L, Palitti F. The protective effect of L-carnitine in peripheral blood human lymphocytes exposed to oxidative agents. Mutagenesis 2006; 21: 21-27. http://dx.doi.org/10.1093/mutage/gei065

[35] Abd-Allah AR, Al Majed AA, Al Yahya AA, Fouda SI, Al Shabana OA. L-Carnitine halts apoptosis and myelosuppression induced by carboplatin in rat bone marrow cell cultures (BMC). Arch Toxicol 2005; 79: 406-413. http://dx.doi.org/10.1007/s00204-004-0643-3

Received on 13-11-2014 Accepted on 21-11-2014 Published on 04-12-2014

DOI: http://dx.doi.org/10.6000/1929-2279.2014.03.04.7