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PROTECTIVE EFFECTS OF B-GLUCAN AND G-CSF ON THE PHYSIOLOGICAL ALTERATIONS INDUCED BY CYCLOPHOSPHAMIDE IN MICE

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Abstract:-
Cyclophosphamide (CTX) is a DNA alkylating chemotherapeutic agent, causing hepatocytes injury. Granulocyte colony stimulating factor (G-CSF) promotes granulocyte counts and antioxidants to increase. This study aimed to investigate the effect of certain natural products such as β- glucan from medicinal fungi as adjuvant systems to reduce toxicity of chemotherapy, enhance the effects of G-CSF to mobilize stem cells and improve the quality of hepatocytes functions. Naïve mice were injected with CTX, G-CSF and β-glucan for five days then physiological parameters were measured. The results showed that CTX administration decreased the parameters of red blood cell distribution width (RDW) counts and total white blood cells)WBC(count. Furthermore, CTX administration has hepatocyte oxidative stresseffectwhich is characterized by increasing prooxidants xanthine oxidase (OX), thiobarbituric acid-reactive substances (TBARS) and decreasing of antioxidants glutathione peroxidase (GPx).As a result, hepatocytes injury, characterized by elevating of serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities occurred. The treatment of mice with G-CSF and β-glucansuccess fully normalized the physiological parameters by returning WBCs, RDW and lymphocytes counts to normal levels, hepatocytes oxidative stress characterized by decreasing Prooxidants OX,TBARS and increasing antioxidants GPx reflected by significant decrease in the serum activities of AST and ALT activities.

Keywords:-*Cyclophosphamide; G-CSF; β-glucan; antioxidants;hepatocytes.*

INTRODUCTION

Cyclophosphamide is an alkylating chemotherapeutic agent with immunosuppressive activity [1]. It is effective against a wide spectrum of malignancies, such leukemia, lymphoma, breast, lung, prostrate, and ovarian cancers [2, 3]. The parent compound is inactive *in vitro* and *in vivo*, exerts its biological activity through metabolites mainly phosphoramide mustard by hepatic microsomal enzymes [4].

This study aimed to investigate whether the administration of β-glucan in combination with G-CSF in mice can delay the leucopenia induced by treatment with high dose of the anti-cancer drug CTX and to test whether its effect is correlated with correction of the alteration in the biochemical readouts [5]. The rebound phase is accompanied by growth factors release and proliferation of bone marrow precursors, and as consequence associates with the mobilization of bone marrow precursors for transplantation [6].

Granulocyte colony-stimulating factor (G-CSF) is commonly used to mobilize stem cells into the blood and as growth factor to promote granulocyte counts in immunocompromised patients. As such, it is frequently used as an adjunctive agent in tumor chemotherapy owing to its regulatory effect on the maturation, proliferation and differentiation of leukocyte precursor cells. It can effectively antagonize myelosuppression induced by chemotherapy agents through raising the leukocyte count of peripheral blood [7].

MATERIALSAND METHODS

Experimental Animals:

Adult female Swiss albino mice (CD1 strain) weighting 20 ± 2 g were purchased from National Research Center (NRC, Cairo, Egypt). Animals were housed (5animals per cage) at the animal facility at Zoology Department, Faculty of Science (Tanta University**,** Egypt) in clean and dry plastic cages, at a 12 h/12 h dark/light cycle under laboratory condition of temperature and humidity. The mice were fed with rodent pellets and tap water *ad libitum*. This study was performed in accordance to the guidelines for the use of experimental animals in research at Zoology Department, Faculty of Science, and Tanta University, Egypt.

Evaluation of Hematological Parameters:

Blood samples with anti-coagulant EDTA were analyzed for hematological parameters red blood cell distribution width (RDW) counts, White Blood Cell (WBC) counts and total number of Lymphocytes according to [8].

Treatment with CTX and G-CSF and β-glucan:

For induction of leukopenia, mice (n=5-6/group) were treated once with intraperitoneal (i.p.) injection of 200mg/Kg (4mg/mouse) CTX. For correction of leukopenia, mice were treated with subcutaneous (s.c.) injection of 5µg/mouse G-CSF (Neupogen®) daily for 5 consecutive days. To test the impact of β-glucan on leukopenia, mice were treated for 5 consecutive days with i.p. injection of 100µg; each injection was administered after G-CSF injection.

Serum Biochemical Analysis:

Serum total protein and activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were determined colorimetrically using kits obtained from Diamond Diagnostic, Egypt according to the methods of [9-10] respectively. The level of thiobarbituric acid reactive substances (TBARS) and xanthine oxidase (XO) as prooxidants indicator were measured according to Tappel and Zalkin [11] and Litwack *et al.* [12], respectively. The level of liver TBARS was calculated with the following equation (nmol/ml) = (At/0.156) × 10, where At is the absorbance of the test sample and ε = 0.156 is the extinction coefficient. The liver XO activity (nmol/min/ml) was estimated as follows: (C) ×10/ (0.284 × xanthine M. Wt), where 0.284 is a constant and C is the concentration in the test sample. The activity of the antioxidant enzyme glutathione peroxidase (GPx) was measured according to Paglia and Valentine [13]. The enzyme activity was calculated by using the following equation; GPx activity (nmol/min/ml) = (At × 6.2 × 10 × 10)/ (13.1 × 0.05 × 10), where ε1 = 6.2 and ε2 = 13.1 are extinction coefficients for H2O2 and DTNB (5, 5´dithiobis-(2-nitrobenzoic acid).

Histological Method:

Small tissue specimens from liver of rats in different groups were collected and immediately fixed in 10% neutral buffered formalin. After proper fixation**, the** specimens were dehydrated in ethyl alcohol, cleared in xylol, embedded and casted in paraffin. Thin paraffin sections were prepared and stained with hematoxylin and eosin stain according to Drury and Wallington [14].

Statistical analysis:

Data obtained from each experiment were analyzed using Microsoft Excel (Seattle, WA) **.**The differences between the experimenta lgroups were assessed using the Student's t-test. $P > 0.05$ was considered to indicate statistical significance by using Graph Pad Prism version 4.0 software (Graph Pad).

RESULTS

Table 1 showed that CTX treatment significantly decreased the total numbers of white blood cells coincided with decreases in the numbers of lymphocytes and RDW when compared to normal group. The co-treatment with G-CSF returned the WBC and RDW to its normal coinciding with recovery of the relative numbers of lymphocytes as compared to control values (PBS group). Co-treatment with β-glucan increased the numbers of WBC and RDW count as compared

to PBS and CTX groups. Co-treatment with combination of G-CSF and β-glucan significantly decreased the numbers of WBC and RDW as compared to β-glucan plus CTX group which was nearly similar to PBS group counts.

Table.1: Effect of different treatments on blood cell counts.

Groups	WBCs ($\times 10^3$)	RDW($\times 10^4$)	Lymphocytes / cmm
PBS	5.40±0.32	50.21±0.59	6.23±39.89
CTX	1.71±0.30**	43.08±0.57**	952.25±16.88**
CTX+G-CSF	5.60±0.32ns	59.00±0.20**	2384.7±38.16ns
CTX+βGlucan	9.35±0.39**	61.48±1.23***	2282.2±37.40ns
CTX+βGlucan+G-CSF	4.61±1.48ns	54.98±0.63**	2268.9±27.64ns

CTX, cyclophosphamide; PBS, Phosphate buffer saline; WBCs, White blood cells; RDW, Red blood cell distribution width; and lymphocytes; ns, non-significant; **, *** signifiant différence compared to PBS group at P≤ 0.01and 0.001.

Table 2 showed The Co-administration of G-CSF with CTX decreased XO and increased GPx activities with no effect on the TBARS level as compared to the PBS group. However, treatment with β-glucan alone or with β-glucan plus G-CSF post CTX treatment decreased the pro-oxidant parameters (TBARS and XO) and increased the anti-oxidant ones to their normal level.

Table. 2: Effect of different treatments on hepatic prooxidant/antioxidant status:

Groups	GPx (U/g protein/g tissue)	Xanthine oxidase (IU)	TBARS (nmol/ g wet tissue
PBS	1.64±0.49	4.31±0.51	5±0.31
CTX	0.82 ±0.78**	5.69±0.97ns	2.06±0.48***
CTX+G-CSF	1.54 ±0.90ns	3.12±0.64ns	1.46±0.35ns
CTX+βGlucan	1.42±0.55ns	2.33±0.56**	5±0.25ns
CTX+βGlucan+G-CSF	1.38 ±0.51ns	3.64±0.90ns	1±0.58ns

CTX, Cyclophosphamide; PBS, Phosphate buffer saline; G-CSF, Granulocyte colony stimulating factor; TBARS, Thiobarbituricacid-reactive substances; and Glutathione Peroxidase (GPx); ns, nonsignificant;**, ***signifiant différence compare to PBS group atP≤ 0.01and 0.001.

Effect of different treatment on serum liver function parameters:

Figure 1and 2 represent changes in the concentrations of ALT and AST during the treatment of mice with CTX alone. The results showed that ALT and AST concentration significantly increased in serum of mice treated with CTX ($p \leq 0.001$) in comparison with control. The treatment with CTX with β Glucan was very effective in the prevention of oxidative damage induced by β Glucan and G-CSF which resulted in significantly lower ALT and ATS concentration. While β Glucan alone treatment reversed this change to control values

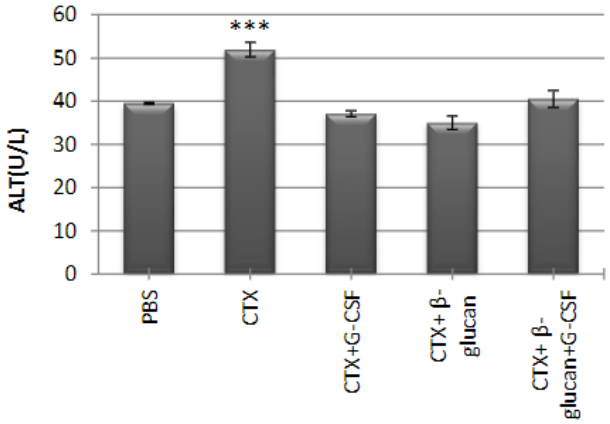

Figure 1: Changes in liver ALT activity after differen ttreatments.

*****p≤ 0.001 CTX treated group compared to PBS group.**

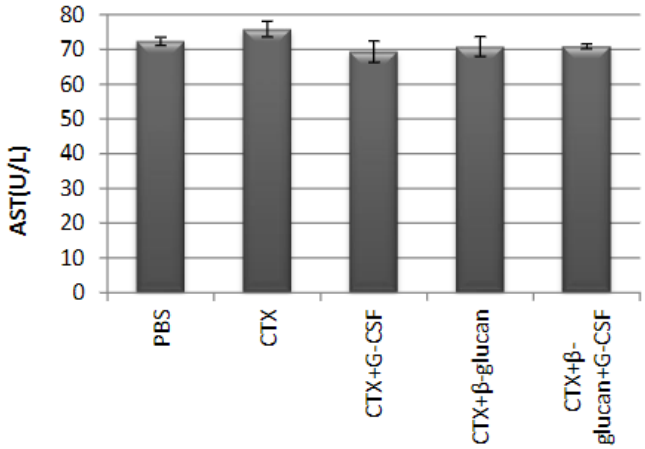

Figure 2: Changes in liver AST activity after different treatments.

Histological observations:

Sections of the control liver of mice stained with H&E dye showed normal histological pattern. Hepatocytes are polyhedral in shape. Their cytoplasm is acidophilic with numerous basophilic granules and rounded centrally located nuclei. Hepatocytes are arranged in well-organized hepatic cords radiating from the central vein, these cords are separated by narrow blood sinusoids; the sinusoidal wall islined by two types of cells a thin endothelial cell and Kupffer cells (Fig. 3). The sections of liver of mice injected with CTX (4 mg i.p) alone showed irregular hepatic architecture, vacuolation of the cytoplasm of hepatocytes, karyolitic and pyknotic nuclei. Also, necrosis of many hepatocytes and inflammatory leucocytes infiltration around the portal area were noticed (Fig.4). The liver sections of mice injected with CTX (4 mg i.p) + β-glucan (100µg i.p) + neupogen (5µg / mouse subcutaneous) revealed approximately normal hepatocytes with normal nuclei and well defined blood sinusoids with normal Kupffer cells. Also, normal appearance of portal area was noticed (Fig. 5).

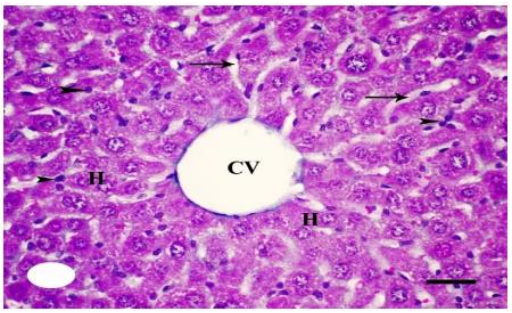

Figure 3:High magnified section of the liver of a control mouse showing normal architecture of hepatocytes (H) radiating from the central vein(CV) and separated by blood sinusoids (arrows)with normal Kupffer cells (arrow heads). (H&E, Bar = 6.25 µm).

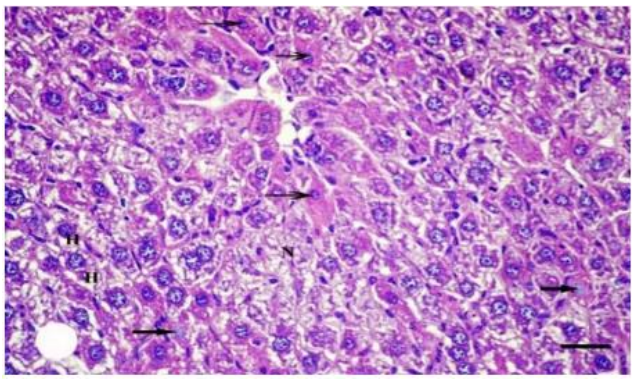

Figure 4:Section of the liver of a mouse injected with CTX showing irregular hepatic architecture with hydropic degeneration of the cytoplasm (H), karyolitic (thick arrows) and necrotic nuclei (N).(H&E, Bar = 6.25 µm)

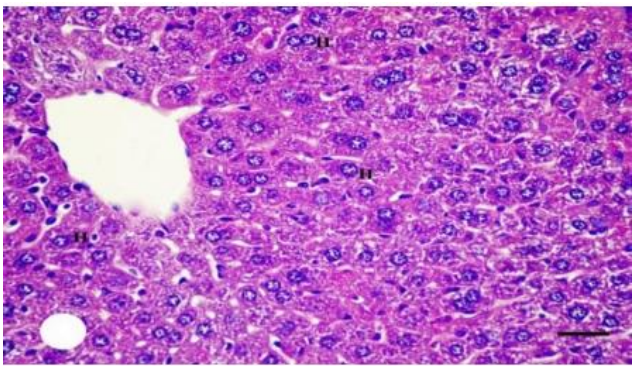

Figure 5: Section of the liver of a mouse injected with CTX + neupogen + β-glucanshowing approximately normal appearance of hepatocytes (H) with normal cytoplasmic inclusions and normal nuclei. Well defined blood sinusoids with normal Kupffer cells are too seen. (H&E, Bar = 6.25 µm)

DISCUSSION

Mice treated with CTX showed a sharp decrease in both the total number of WBCs as well as in the number of lymphocytes. These data are consistent with data reported on the effect of CTX in mice [15].

Also, the results showed that the numbers of WBCs and lymphocytes increased during G-CSF injection alone or in combination with β-glucan post CTX treatment, Consistent with other results [16]. The present results are consistent with the results revealed by [17] that the ability of G-CSF to consistently induce increases in white blood cell counts and its role as a potent hematopoietic growth factor, particularly for cells of the granulocytic lineage. Furthermore, many studies indicate that CTX leads to oxidative stress, as it is recognized that the strong depletion of anti-oxidant enzymes is associated with the high production of pro-oxidant molecules [18] In line with these reports, our data shown in Table 2 demonstrated that CTX increased the level of TBARS and XO activity in hepatocytes, which was associated with a decrease in the GPx activity comparing to that in the control group.In addition, the hepatotoxicity effect of CTX was shown in our experiment due to the status of hepatic oxidative stress which characterized by elevation of prooxidant (TBARS and XO activity), and associated with depletion of GPX activity [19].

It is reported that CTX can alter liver functions by modulating all liver enzymes. The liver is the richest source of both AST and ALTenzymes [20].And thus, the levels of both these enzymes are expected to increase as result of damage to the liver cells [21]. In support of these reports, our data indicate that CTX treatment resulted in a significant increase in the activities of AST and ALT in sera as compared to the control group.

The histological observations confirmed data in the current study. The present results revealed that treatment with G-CSF after CTX in combination with β-glucan, resulted in the improvement of many hepatocytes with the restoration of normal nuclei and well defined blood sinusoids with normal Kupffer cells and also normal appearance of portal area consistent with these results [22].

In conclusion;

β-glucan has the best antioxidant effect, as demonstrated by the return of the number of white blood cells, red blood cell distribution width and lymphocytes count of CTX group to the normal range in comparable to control PBS group, and also it returned the changes in liver enzymes to normal level.Therefore, the present study recommends using the β-glucan as a natural product such for patients put on CTX therapy to reduce its toxicity.

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