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Protective Effect of Zinc on Cyclophosphamide-Induced Hematoxicity and Urotoxicity

Adnan Ayhanci · Ruhi Uyar · Erinc Aral · Selda Kabadere · Sila Appak

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Abstract Cyclophosphamide (CP) is widely used for the treatment of neoplastic diseases; however, its toxicity causes dose-limiting side effects. Zinc (Zn) is an essential trace element and has important biological functions that control many cell processes including DNA synthesis, normal growth, reproduction, fetal development, bone formation, and wound healing. Therefore, the toxicity of CP and the possible protective effect of Zn on blood cells, bone marrow, and bladder of rat were investigated in this study. Intraperitoneal administration of 50, 100, or 150 mg/kg CP for 3 days caused, in a dose-dependent manner, reductions in the number of leukocytes, thrombocytes, and bone marrow nucleated cells and a serious urotoxicity. To explore whether CP-induced damages could be prevented by Zn, other groups of rats were pretreated with 4 or 8 mg/kg $ZnCl_2$ intraperitoneally for 3 days then challenged with respective doses of CP plus $ZnCl_2$ on day 4 for three more days. The results indicated that treatment of rats with Zn could dose-dependently alleviate CP-induced toxicities on blood cells, bone marrow cells, and urinary bladder. We suggest that Zn could be a potentially effective drug in the prevention of CP-related hematoxicity and urotoxicity.

Keywords Zinc · Cyclophosphamide · Hematopoietic system · Bladder · Rat

A. Ayhanci
Department of Biology, Faculty of Medicine, Faculty of Art and Sciences,
Eskisehir Osmangazi University, Eskisehir, Turkey
e-mail: aayhanci@ogu.edu.tr

R. Uyar · S. Kabadere
Department of Physiology, Faculty of Art and Sciences, Eskisehir Osmangazi University,
Eskisehir, Turkey

E. Aral
Department of Histology, Faculty of Art and Sciences, Eskisehir Osmangazi University,
Eskisehir, Turkey

S. Appak (✉)
Department of Molecular Biology and Genetics, Faculty of Science, Izmir Institute of Technology,
35430 Urla, Izmir, Turkey
e-mail: silaappak@iyte.edu.tr

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Introduction

Cyclophosphamide (CP) is one of the most widely administered anticancer agents, and it is also used for its immunosuppressive actions. It is a multifunctional alkylating agent and a prodrug undergoing a complicated process of metabolic activation and inactivation; however, CP activation is often accompanied by a number of potential toxicities [1]. It is toxic to rapidly proliferating tissues such as epithelial cells of digestive system, hair follicles, and gonads [2–4]. In addition to carcinogenic and teratogenic potentials, CP has also well-known toxic effects on heart, bladder, and hematopoietic system, primarily leucopenia and reduction in platelet number [5–7]. Besides contracture, fibrosis, and necrosis of urinary bladder, hemorrhagic cystisis is a major dose-limiting side effect of CP [8]. Probably caused by highly reactive acrolein, CP-induced hemorrhagic cystisis has been observed in patients to range from 2% to 78% [3, 4, 9]. Despite the fact that pharmacokinetics of CP have been studied extensively, there is still an incomplete understanding on the role of CP efficacy and toxicity.

Zinc (Zn) is a trace metal required for the activity of over 300 metalloenzymes, including those involved in nucleic acid and protein synthesis, cellular replication, immune function, and antioxidant systems [10]. Studies performed by Anttinen et al. [11] and Camps et al. [12] suggested a protective effect of Zn on liver changes. Tate et al. [13] have also indicated a protective action of Zn on cultured human retinal pigment epithelial cells. Furthermore, treatment with Zn reduced the decrease in bone formation mediated by ethanol or protein deficiency [14]. It has been recently found that Zn promotes the formation of granular layers in mouse tail [15]. Therefore, Zn would theoretically exert a protective effect in CP-induced toxicity. Additionally, there are about 1,400 zinc-finger proteins that participate in the genetic expression of many proteins [16]. It is known that zinc is less toxic than other metals, e.g. lead, arsenic, mercury, and cadmium, etc [17], and does not easily accumulate in the body [18]. Based on these facts, we decided to investigate the possible protective effect of Zn on the toxic action of CP in blood, bone marrow, and bladder of rat.

Materials and Methods

Spraque–Dawley rats of either sex, weighing 190–220 g, were used for the intraperitoneal injection of CP (endoxan), zinc chloride, and saline (Merc, Germany). The animals were given food and water ad libitum. Besides the control groups, the rats were randomly divided into the following experimental groups, each including six to ten animals:

Groups 1, 2, and 3 treated with 50, 100, and 150 mg/kg CP, respectively
Groups 4 and 5 treated with 4 and 8 mg/kg $ZnCl_2$, respectively
Groups 6, 7, and 8 treated with respective CP plus 4 mg/kg $ZnCl_2$
Groups 9, 10, and 11 also treated with respective CP plus 8 mg/kg $ZnCl_2$

The animals in the first three groups received only CP doses in saline every day for 3 days [19], and the fourth and fifth groups received 4 or 8 mg/kg $ZnCl_2$ for 6 days [19]. The remaining groups [6–11] received respective $ZnCl_2$ for 6 days and then respective doses of CP administered on the fourth day for 3 days. The control groups were injected with the same amounts of saline.

Under the ether anesthesia, the blood samples were collected by cardiac puncture, and then the animals were killed at either day 4 or 7. Both femurs were dissected, and bone marrow was flushed with saline into a test tube. Homogenized bone marrow nucleated cells

and blood cells were counted with a cell counter (Coulter). The bladders were taken out, cleaned, weighed, fixed, and embedded in paraffin. The thin sections were stained with hematoxylin–eosin and examined by a histologist in a single blind fashion.

The results were expressed as means±SEM. Statistical analysis was performed using one-way analysis of variance followed by Tukey multiple-range test and $p<0.05$ accepted as considering statistical significance. Each experiment was repeated at least three times.

Results

Both 4 and 8 mg/kg $ZnCl_2$ did not significantly alter the numbers of leukocytes, platelets, or bone marrow nucleated cells (data not given).

When used alone, doses of 50, 100, and 150 mg/kg CP caused 75%, 88%, and 90% reductions in the number of leukocyte, respectively ($p<0.001$). Administered together with respective doses of CP, comparing to the control, 4 mg/kg $ZnCl_2$ reduced the number of leukocytes by 40%, 74%, and 88%, respectively ($p<0.001$). About a 35% significant recovery was obtained only in the 50+4 mg/kg group. When 8 mg/kg $ZnCl_2$ was combined either with 50 or 100 mg/kg CP, there was a complete and an about 50% improvement; however, with 150 mg/kg CP dose, 8 mg/kg $ZnCl_2$ caused no significant change in the number of leukocytes (Fig. 1).

The number of platelets in rats treated only with 50, 100, and 150 mg/kg CP decreased by 30%, 53%, and 51%, respectively ($p<0.001$, Fig. 2). Compared to the control and to each other, 4 or 8 mg/kg $ZnCl_2$ given together with 50 mg/kg CP did not cause a significant change in the number of thrombocytes; however, with 100 mg/kg CP, both zinc doses caused a 20% reduction. That is, both 4 and 8 mg/kg $ZnCl_2$ doses induced about a 25% recovery when used either with 50 or 100 mg/kg CP. When compared to 150 mg/kg CP group, either respective dose of $ZnCl_2$ did not affect the number of platelets.

Compared to the control group, the number of bone marrow nucleated cells decreased by 78%, 84%, or 91% after the application of 50, 100, or 150 mg/kg CP ($p<0.001$, Fig. 3). When compared with respective three doses of CP, 4 mg/kg $ZnCl_2$ caused about 41%, 30%, and 12% recovery in the number of bone marrow nucleated cells, respectively ($p<0.001$).

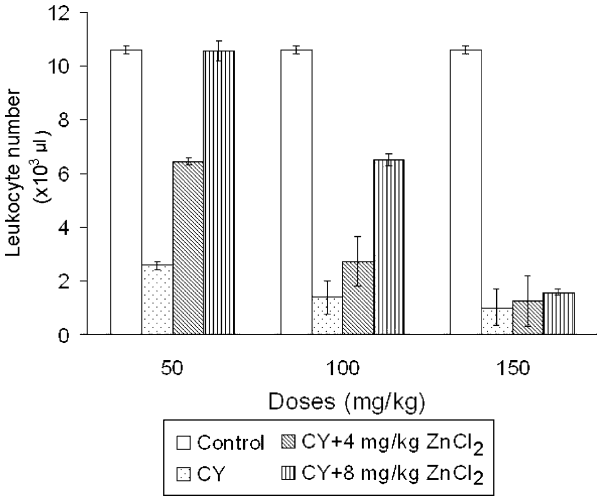

Fig. 1 The number of peripheral leukocytes with the presence of saline, respective doses of CP, or CP plus $ZnCl_2$

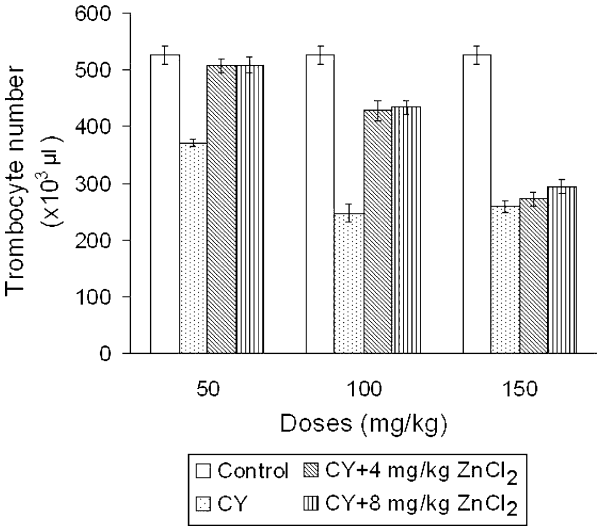

Fig. 2 Blood thrombocyte number of the rats after treatment with saline, respective doses of CP, or CP + $ZnCl_2$

The improvement in the number of bone marrow cells was about 59%, 44%, and 40% when 8 mg/kg $ZnCl_2$ was given together with these doses of CP, respectively ($p<0.001$).

Histological examinations of the bladders of the 4 and 8 mg/kg $ZnCl_2$ groups revealed no change when compared to the control bladders (Fig. 4a). There were epithelial and mucosal degenerations as well as hyperemia, edema, and infiltration of the bladder of 50 mg/kg CP-treated group (Fig. 4b). When 4 or 8 mg/kg $ZnCl_2$ was given together with 50 mg/kg CP, tunica media and epithelial tissue appeared slightly normal with little edema in lamina propria and infiltrative cells near tunica muscularis as well as between the muscle layers (Fig. 4c).

In the 100 mg/kg CP group, the changes in the bladder structure were more severe than 50 mg/kg CP group. There were edema, infiltration, and necrotic epithelium with degenerations. Edema and thickenings were detected in lamina propria (Fig. 4d). Histological

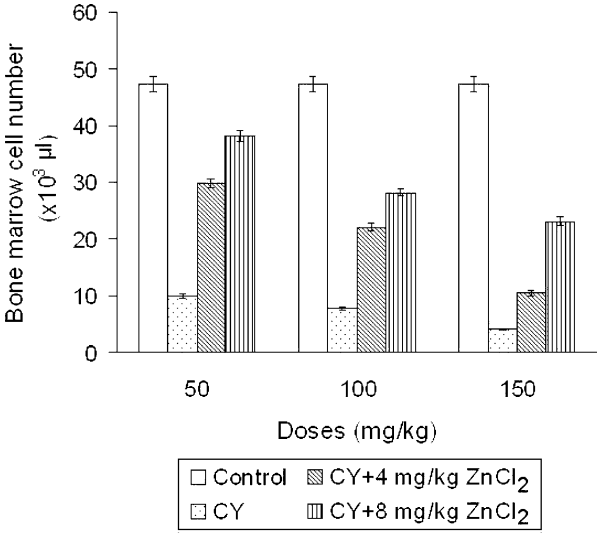

Fig. 3 The number of bone marrow nucleated cells in the control, CP, or CP plus $ZnCl_2$-treated groups

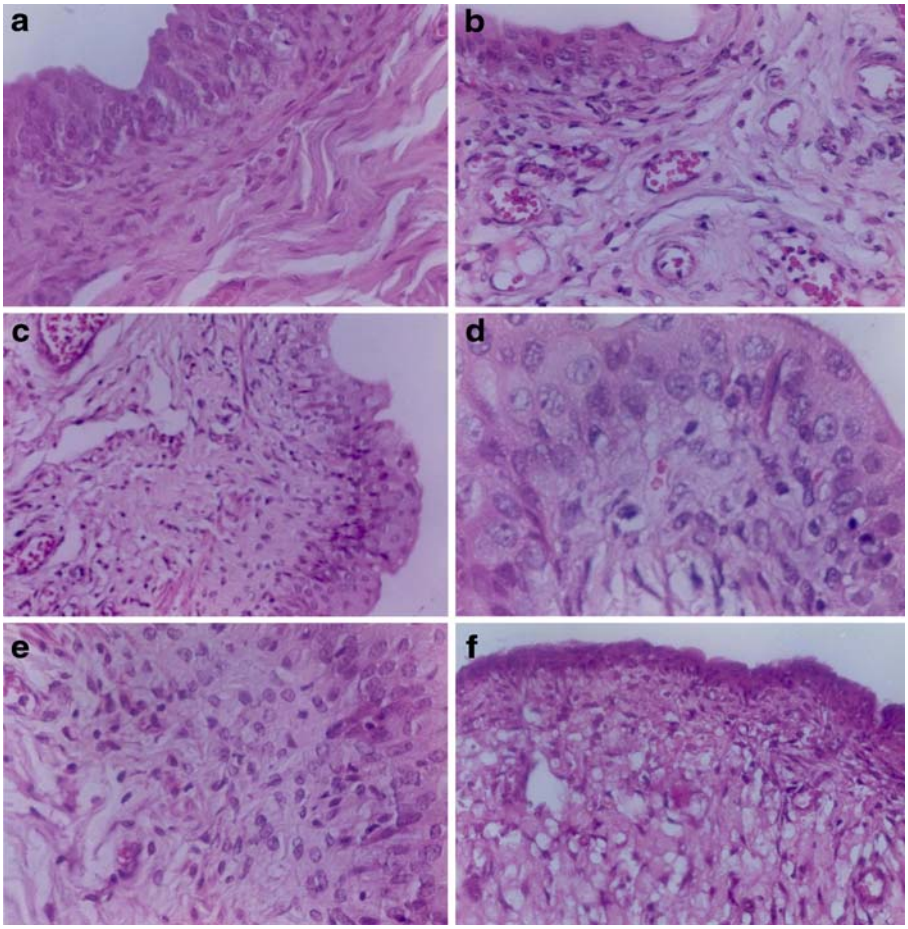

Fig. 4 Photomicrographs of hematoxylin–eosin-stained bladders. **a** Control (×132), **b** 50 mg/kg CP (×100), **c** 50+8 mg/kg CP + $ZnCl_2$ (×66), **d** 100 mg/kg CP (×200), **e** 100+8 mg/kg CP + $ZnCl_2$ (×132), **f** 150 mg/kg CP (×66)

appearance of the bladders in the 100+4 mg/kg CP + $ZnCl_2$ group resembled the 50 mg/kg CP group. In the 100+8 mg/kg CP + $ZnCl_2$ group, there were little changes in the epithelial layer with some vacuolizations, edema, and cell proliferation in lamina propria (Fig. 4e).

The typical tissue structure was altered in the 150 mg/kg CP group as there were desquamation and reduction in the number of layers of the epithelial tissue and atypical cell proliferation, edema, and hyperemia in the lamina propria (Fig. 4f). The histopathological changes in the groups of 150+4 and 150+8 CP + $ZnCl_2$ resembled almost the changes in the 100+8 mg/kg CP + $ZnCl_2$ group (Fig. 4e).

Discussion

Even though there have been sporadic reports on the toxicity of Zn [18], we did not observe any changes in the numbers of leukocytes, thrombocytes, bone marrow nucleated cells, or structure of bladder when only 4 or 8 mg/kg $ZnCl_2$ was injected to the rats.

CP is widely used, alone or together with other drugs, for the treatment of neoplastic diseases [1]. Its modulation in immune reactivity is well known in mammals, and the drug is regarded as a flexible means to manipulate host responsiveness to malignancies and infections in a variety of ways, but among the therapy-limiting toxicities of CP are immunosuppression and primarily leukopenia [2, 4, 9]. Depending on the dose used in this study, CP showed up to 90% toxicity on the circulating white blood cells of normal rats. Similarly, after a single dose of CP injection, leukocyte counts begin to fall [3, 5, 6]. Furthermore, administration of a single dose of 40 mg/kg CP to baboons resulted in transient reduction in white blood cell count [20]. When 4 mg/kg $ZnCl_2$ was used together with 50 mg/kg CP, a recovery of about 35% was observed; however, 8 mg/kg $ZnCl_2$, with the same dose of CP, caused a complete recovery of leukocytes in number. Either 4 or 8 mg/kg $ZnCl_2$ did not cause a significant protection of leukocytes from the toxic effect of 150 mg/kg CP. It is concluded that Zn is known to be critical for cellular immune function through the action of the thymic hormone zinc-thymulin [21], and Zn replenishment restores normal tymic morphology and cellularity in aged mice [22].

Besides being a cytotoxic drug, CP also affects the immune system by causing acute damage to the blood-forming tissues in bone marrow, thereby causing transient reduction in circulating leukocytes [23]. The toxic effect of our doses of CP on the bone marrow nucleated cell was similar to the effects on leukocytes, but Zn appeared to be more effective on bone marrow. The best recovery of about 60% was obtained with 8 mg/kg $ZnCl_2$ used together with 50 mg/kg CP, but even with the highest (150 mg) CP dose, there was about 40% recovery of the bone marrow cells. In support of our results, the frequency of occurrence of erythrocytes with micronuclei in bone marrow of mice was increased by CP treatment, but micronucleus formation was significantly prevented by pretreatment with $ZnCl_2$ [24]. Similarly, 50 mg/kg CP increased more than five times the number of micronucleated erythrocytes in bone marrow of rat; however, with the treatment of 4 mg/kg zinc acetate, a high percent recovery was achieved [18].

The least toxic effect of CP was observed on the circulating thrombocytes, and 150 mg/kg CP caused only about 50% reduction in blood platelet count. A similar result was reported, indicating that reduction in platelets occurs only at high doses of CP [7]. There was a complete protection when we used 4 or 8 mg/kg $ZnCl_2$ together with 50 mg/kg CP; however, there was no significant change in the number of platelets when 4 or 8 mg/kg $ZnCl_2$ was given together with 150 mg/kg CP. Further studies are needed to substantiate the effect of CP on thrombocytes.

Cyclophosphamide induced bladder damage with the incidence of up to 78% which is a major dose-limiting side effect in patients [3–5, 8, 9]. The urological side effects vary from transient voiding symptoms to life-threatening hemorrhagic cystitis. In accordance with other studies, the damage caused in this study by CP to the structure of the bladders increased as the dose rose. Similar structural damage was obtained when 100 mg/kg CP was injected to rats [25]. Both zinc doses that we used improved bladder morphology in a dose-dependent manner. It is known that CP is a prodrug that produces, among others, acrolein which is thought to be responsible for the dose-limiting urotoxicity of CP [26]. Besides other protective drugs recently, amifostine and glutathione were reported to prevent acrolein-induced hemorrhagic cystitis in mouse bladder; however, the efficacy of these agents in humans has yet to be determined [27]. Zinc is an essential component of biomembranes and is necessary for maintenance of membrane structure and function. Because of its antioxidant and membrane-stabilizing properties, Zn appears to be crucial for the protection against CP toxicity.

In conclusion, Zn alone is not toxic to bone marrow or blood cells; but CP is dose-dependently toxic to bone marrow, leukocytes, platelets and bladder; however, depending on the dose, Zn protects the animals from this toxic effect. The best recovery was obtained when 8 mg/kg $ZnCl_2$ was given in combination with 50 mg/kg CP. Our findings suggest that at convenient concentration Zn could be a potentially effective drug in the treatment of CP-induced damage and could provide us with the hope in prevention and treatment of CP toxicity. We believe that additional experimentation should be performed to at least initially explore the underlying mechanism of Zn protection against CP toxicity.

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