Original Article
The protective effect of cantharidinate sodium on cyclophosphamide-induced leukopenia in mice

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Abstract: Chemotherapy is commonly associated with adverse effects of leukopenia. Cantharidinate sodium, a derivative of cantharidin, is mainly used for cancer treatment due to its immunoregulatory functions. The aim of this study was to evaluate the effects of cantharidinate sodium on chemotherapy-induced leukopenia in mice. The mice were intraperitoneally administered with cyclophosphamide (CY) at 100 mg/kg for three days and on day 4, they were given cantharidinate sodium at 0.3 mg/kg by intravenous injection for ten days. Peripheral blood leukocytes were counted microscopically on days 5–14. 24 hours later after the last drug treatment, and the mice were euthanized. Percentages of CD34⁺ cells in the bone marrow and apoptosis of bone marrow cells (BMCs) were measured using flow cytometer. Secreted levels of GM-CSF and IL-6 in the bone marrow were detected by enzyme-linked immunosorbent assay. The results demonstrate that cantharidinate sodium significantly increased the number of peripheral blood leukocytes compared with CY-treated group. The CD34⁺ cells and released GM-CSF levels were significantly increased on cantharidinate sodium treatment. However, the drug exerted no significant effect on IL-6 levels. In addition, cantharidinate sodium significantly blocked the CY-induced apoptosis of BMCs. Taken together, cantharidinate sodium demonstrated the benefit for mitigating chemotherapy-induced hematological toxicity.

Keywords: Cantharidinate sodium, leukopenia, cyclophosphamide, apoptosis, mice

Introduction
Chemotherapy treatment in cancer patients often induces bone marrow suppression, leading to the hematological side effects such as leukopenia [1, 2]. The suppressed immune function may become life-threatening in case of severe infection and sepsis. Therefore, prevention and recovery of leukopenia is crucial for attenuating the adverse effects of chemotherapy and improving the life quality of cancer patients [3]. Several drugs, including vitamin B4, leucogen, batyl alcohol, as well as cytokines, such as granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) are applied for elevating leukocyte count. However, they failed in some cases and have many side effects [4]. Therefore, there is unmet need to develop new drugs for leukopenia treatment.

Cantharidin, the active ingredient isolated from the blister beetle, has been used as an anti-tumor agent for centuries. It inhibits the action of protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A), resulting in induction of DNA damage and cell apoptosis [5, 6]. However, the intolerable toxicity of cantharidin to mucus membrane and urinary systems promote the discovery and development of many derivatives, such as cantharidinate sodium with decreasing toxicity while retaining its activity [7]. Cantharidinate sodium is clinically used for the treatment of solid tumors, including hepatoma and lung cancer [8, 9]. It also stimulates the proliferation of macrophages and lymphocytes, and production of interleukins (ILs) [10]. However, whether cantharidinate sodium can prevent leukopenia during chemotherapy treatment has not been examined.

In the current study, using a cyclophosphamide (CY)-induced leukopenia model in mice, the effect of cantharidinate sodium on the total number of peripheral blood leukocytes was evaluated. In addition to the CD34⁺ cell count,
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secreted GM-CSF, and IL-6 levels in the bone marrow and apoptosis of bone marrow cells (BMCs) was analyzed.

Materials and methods

Animals

Male KM mice (20 ± 2 g) were purchased from Beijing Vital River Experimental Animal Co., Ltd and maintained in the animal facility under controlled environmental conditions. All protocols were approved by the Animal Care and Welfare Committee of Capital Medical University.

Reagents

The CY was purchased from J&K Scientific Ltd., (Beijing, China). Cantharidinate sodium was supplied by Baiqiang Pharmaceuticals (Guizhou, China). Both agents were dissolved in saline for administration. Fluorescein isothiocyanate (FITC)-anti-mouse CD45 and phycoerythrin (PE)-anti-mouse CD34 were obtained from eBioscience (CA, USA). Enzyme-linked immunosorbent assay (ELISA) kits for IL-6 and GM-CSF were purchased from Biolegend (CA, USA). Annexin V-FITC/propidium iodide (PI) apoptosis detection kit was obtained from TransGen Biotech Co., (Beijing, China).

CY-induced leukopenia and drug treatment

The mice were randomly divided into three groups (n = 10/group). The control group received a daily injection of saline. The CY group and cantharidinate sodium-treated group received a daily intraperitoneal injection of CY at 100 mg/kg on days 1–3. However, beginning on day 4, the mice in CY group were administered intravenously with saline and those in cantharidinate sodium-treated group received an intravenous injection of cantharidinate sodium at 0.3 mg/kg once a day for 10 consecutive days. The body weight was recorded every day and the blood samples were taken from the tail vein on days 5–14. The mice were anesthetized and euthanized on day 14.

Leukocyte counting

Total peripheral blood leukocyte counting was performed microscopically using leukocyte diluting fluid containing 3% glacial acetic acid by standard procedure on a hemocytometer.

CD34+ cell counting

A total of 1 × 10^6 BMCs were treated with lysis buffer for 5 minutes at room temperature to remove erythrocytes and then incubated with PE-anti-mouse CD34 (dilution, 1:100) and FITC-anti-mouse CD45 (dilution, 1:100) for 30 minutes on ice. After removing free antibody, the cells were analyzed using a FACSCalibur flow cytometer (BD Biosciences, CA, USA) according to the International Society for Hematotherapy and Graft Engineering recommendations [11]. A threshold was set to exclude debris and include all leukocyte populations. CD34+ cell count was expressed as the percentage of total leukocytes.

Determination of IL-6 and GM-CSF

The BMCs were collected by flushing the femur with 1 ml phosphate-buffered saline (PBS) using a 23-gauge needle. The cell suspensions were centrifuged at 1500 g for 10 minutes and the supernatants were removed for quantification of IL-6 and GM-CSF using ELISA kits according to the manufacturer’s protocols. Absorbance was measured at a wavelength of 450 nm using a SpectraMax M5 Multimode Plate Reader.

Apoptosis assay

BMCs were washed with PBS and then resuspended in 100 μl binding buffer at a concentration of 1 × 10^6 cells/ml. The suspension was incubated with a combination of 5 μl FITC Annexin V and 5 μl PI working solution for 15 min in the dark at room temperature. FITC and PI fluorescence were measured by flow cytometry.

Statistical analysis

The data are expressed as mean ± SD. The differences between two groups were analyzed by one-way ANOVA (Tukey’s post hoc) using SPSS software (version 19.0; SPSS Inc., Chicago, IL, USA). P < 0.05 was considered to be statistically different.

Results

Peripheral blood leukocyte count

As shown in Figure 1A, a significant decrease in the number of peripheral blood leukocytes was induced by CY treatment with a maximal
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value of 84% on day 7. A slight increase of leukocyte count was seen on day 8, and this returned close to the normal levels on day 9 and was not significantly different from control group until day 14. Treatment of cantharidinate sodium significantly increased the leukocyte count on days 7, 9–12 and 14 by 30–130%. In addition, CY caused a significant drop in body weight by 11–25%, which was not obviously affected by the addition of cantharidinate sodium, suggesting its minimal toxicity to the mice (Figure 1B).

CD34+ cell count in the bone marrow

CD34 molecule is a common cell surface marker of hematopoietic stem cells (HSCs). The CD34+ cell percentage in the bone marrow in control group was 3.31% ± 0.9%, which was significantly reduced by 52% by CY treatment. While cantharidinate sodium significantly inhibited this reduction (Figure 2). This indicated a capacity of cantharidinate sodium to stimulate the proliferation of HSCs in the bone marrow.

Secreted levels of IL-6 and GM-CSF in the bone marrow

The hematopoietic cells could produce cytokines to regulate hematopoiesis. In the current study, as shown in Figure 3A, the secreted GM-CSF levels in the bone marrow were lower by 32% in CY-treated group than in the control group (41.7 ng/L ± 8.1 ng/L), which was significantly relieved by cantharidinate sodium. However, no obvious difference in IL-6 levels was observed following treatment with CY or cantharidinate sodium compared with the control group (13.6 ng/L ± 3.3 ng/L) (Figure 3B).

Apoptosis of BMCs

Bone marrow is the primary site for hematopoiesis. The apoptotic rate of BMCs in control group was 3.42% ± 0.29%, which was significantly elevated by CY by ~12%. While cantharidinate sodium brought the change back to normal level (Figure 4).

Discussion

CY, acting by alkylating DNA to form cross-links and thereby inhibiting cell growth, kills both
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Figure 3. Effect of cantharidinate sodium on the secreted (A) GM-CSF and (B) IL-6 levels in the bone marrow. The femur was flushed out with 1 ml PBS to collect the bone marrow cells. The supernatants were isolated by centrifugation and subjected for quantification of GM-CSF and IL-6 by ELISA method. \(##P < 0.01\), compared with control group; \(^*P < 0.05\), Compared with model group.

Figure 4. Effect of cantharidinate sodium on the apoptosis of bone marrow cells. The apoptotic ratio was measured by Annexin V-FITC/PI double staining using flow cytometry. \(^*P < 0.05\), compared with control group; \(^*P < 0.05\), compared with model group.

tumor and rapidly dividing normal cells. It is a commonly-utilized agent for the establishment of chemotherapy-induced leukopenia model [12-14]. Previous studies reported two CY-induced leukopenia models in mice: one is established by daily i.p. injection at 50 mg/kg for 3 days while another by a single i.p. injection at 165 mg/kg weekly for two weeks [15]. However, in the current study, daily i.p. injection of 50 mg/kg CY for 3 days was not sufficient to induce a significant decline in leukocyte count for an extended time in mice, and hence 100 mg/kg CY was used. The number of peripheral blood leukocytes was significantly decreased by CY treatment and did not exceed the normal levels during the recovery phase. Then the CY-treated mice were administered with cantharidinate sodium by daily intravenous injection for 10 days (standard treatment lasts for two weeks) [16], and the cantharidinate sodium significantly elevated peripheral blood leukocyte count without severe body weight loss compared with the model group, suggesting its capacity to stimulate the proliferation of leukocytes. In addition, the effect of cantharidinate sodium at different doses on each category of leukocytes, including neutrophils, lymphocytes and monocytes, should be evaluated in our future studies.

It is known that the blood cells originate from proliferation and differentiation of HSCs or progenitor cells in the bone marrow and immune organs while the stromal cells in the hematopoietic microenvironment release various cytokines to modulate the hematopoiesis [17]. HSCs are often represented by Sca-1, CD117, and CD34 markers [18]. In addition, hematopoietic cytokines, including GM-CSF and IL-6 could stimulate proliferation and maturation of myeloid progenitor cells [19, 20]. The results showed that cantharidinate sodium can elevate the CD34\(^+\) cell count and enhance the secreted levels of GM-CSF in the bone marrow compared the model group. It also blocked the CY-induced apoptosis of BMCs. However, the effect of cantharidinate sodium on IL-6 levels was not significant. Other cytokines, such as IL-3 and G-CSF are involved in the proliferation of hematopoietic stem cells [15, 21]. Further investiga-
tion as to whether cantharidinate sodium impacts these cytokines is warranted.

In conclusion, cantharidinate sodium promotes proliferation of CD34<sup>+</sup> cells and the release of GM-CSF in the bone marrow, along with the suppression of the apoptosis of BMCs, and consequently attenuates CY-induced leukopenia in mice. This suggests that beneficial effects of cantharidinate sodium lead to alleviation of chemotherapy-induced side effects in addition to its antitumor activities.

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Disclosure of conflict of interest

None.

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