Protective Effect of Betulinic Acid on Cyclophosphamide-Induced Oxidative Damage to Immune Organs in Mice: Postprint

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Abstract

To investigate the effect of betulinic acid (BA) on cyclophosphamide (Cy)-induced oxidative damage in the immune organs of mice. Fifty healthy male Kunming mice were randomly divided into five groups: control group, Cy group, and 0.05, 0.50, and 5.00 mg/kg BW BA groups. The control and Cy groups were intragastrically administered 1% soluble starch solution, while the remaining groups received BA at different doses for 14 consecutive days. Subsequently, except for the control group which was injected with normal saline, the other four groups were intraperitoneally injected with Cy (50 mg/kg) to induce an oxidative stress model. Serum, spleen, and thymus were collected to measure serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) activities and albumin (ALB), total protein (TP) contents, as well as spleen and thymus superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), catalase (CAT) activities and reduced glutathione (GSH), malondialdehyde (MDA) contents. The results showed: 1) Regarding body weight, there was no significant difference between the control and Cy groups ($P>0.05$), and no significant difference between each BA dose group and the Cy group ($P>0.05$). 2) Compared with the control group, Cy highly significantly increased serum AST and ALT activities ($P<0.05$), while 5.00 mg/kg BW BA highly significantly alleviated this phenomenon ($P<0.05$). 3) Compared with the control group, Cy caused a significant decrease in spleen index and thymus index ($P<0.05$), while 0.05 mg/kg BW BA significantly alleviated the decrease in thymus index ($P<0.05$). 4) Compared with the control group, the Cy group showed significant or highly significant decreases in thymus SOD activity, thymus CAT activity, and spleen GSH content ($P<0.05$ or $P<0.01$), and a highly significant increase in spleen MDA content ($P<0.01$); compared with the Cy group, the 0.50 mg/kg BW BA group significantly or highly significantly increased spleen and thymus GSH-Px and CAT activities and spleen GSH content ($P<0.05$ or $P<0.01$), highly

significantly decreased spleen MDA content (P<0.01), but highly significantly decreased thymus SOD activity (P<0.01); compared with the Cy group, the 5.00 mg/kg BW BA group significantly or highly significantly increased spleen and thymus GSH-Px and CAT activities (P<0.05 or P<0.01), and highly significantly decreased spleen MDA content (P<0.01). These results indicate that BA can ameliorate Cy-induced oxidative stress in mouse immune organs and exerts a preventive protective effect against Cy-induced oxidative damage.

Full Text

Protective Effects of Betulinic Acid on Cyclophosphamide-Induced Oxidative Damage in Mouse Immune Organs

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Abstract: This study investigated the protective effects of betulinic acid (BA) against cyclophosphamide (Cy)-induced oxidative damage in mouse immune organs. Fifty healthy male Kunming mice were randomly divided into five groups: control, Cy, and three BA-treated groups (0.05, 0.50, and 5.00 mg/kg BW). The control and Cy groups received 1% soluble starch solution, while BA groups received different doses of BA via gavage for 14 consecutive days. On day 15, all groups except the control received intraperitoneal injections of Cy (50 mg/kg BW) to induce oxidative stress, while the control group received saline. Serum, spleen, and thymus samples were collected to measure serum alanine aminotransferase (ALT) and aspartate transaminase (AST) activities, albumin (ALB) and total protein (TP) levels, as well as superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and catalase (CAT) activities and reduced glutathione (GSH) and malondialdehyde (MDA) contents in immune organs. The results showed: (1) No significant differences in body weight were observed between control and Cy groups or between Cy and any BA group (P>0.05). (2) Cy significantly elevated serum AST and ALT activities compared to control (P<0.05), while 5.00 mg/kg BW BA significantly attenuated these elevations (P<0.05). (3) Cy markedly reduced spleen and thymus indices (P<0.05), whereas 0.05 mg/kg BW BA significantly mitigated the reduction in thymus index (P<0.05). (4) Cy significantly or extremely significantly decreased thymus SOD and CAT activities and spleen GSH content (P<0.05 or P<0.01), while extremely significantly increasing spleen MDA content (P<0.01). Compared with the Cy group, 0.50 mg/kg BW BA significantly or extremely significantly enhanced GSH-Px and

CAT activities in both spleen and thymus and spleen GSH content ($P<0.05$ or $P<0.01$), extremely significantly reduced spleen MDA content ($P<0.01$), but extremely significantly decreased thymus SOD activity ($P<0.01$). The 5.00 mg/kg BW BA group also significantly or extremely significantly increased spleen and thymus GSH-Px and CAT activities ($P<0.05$ or $P<0.01$) and extremely significantly reduced spleen MDA content ($P<0.01$). These findings demonstrate that BA can ameliorate Cy-induced oxidative stress in mouse immune organs and provide preventive protection against oxidative damage.

Keywords: betulinic acid; cyclophosphamide; immune organ; oxidative stress

Introduction

Natural bioactive compounds can regulate oxidative stress and immune responses, maintaining cellular homeostasis and normal physiological functions. Betulinic acid (BA) is a plant-derived pentacyclic triterpenoid widely distributed throughout the plant kingdom, with numerous reports documenting its presence across many species. BA exhibits protective effects on normal cells and organisms, likely mediated through antioxidant activity and immunomodulation. Previous studies have demonstrated that BA can activate mouse peritoneal macrophages, enhancing phagocytic capacity and energy metabolism while strengthening antioxidant defenses. Further research has shown that BA significantly enhances cellular, humoral, and non-specific immune functions in mice. Notably, BA demonstrates extremely low toxicity, with no obvious toxic effects observed in animals at doses up to 500 mg/kg. These findings confirm BA' s potential as an antioxidant and immunomodulatory agent for animal health applications.

Cyclophosphamide (Cy) is an alkylating antineoplastic agent that serves as both a broad-spectrum anticancer drug and one of the most potent and widely used immunosuppressive agents. However, Cy causes adverse effects including bone marrow suppression, alopecia, leukopenia, and teratogenicity. Research indicates that Cy reduces lymphocyte counts and significantly decreases the number and percentage of CD25+ and CD4+ T cells in spleen and lymph nodes, demonstrating potent immunosuppressive effects that make it commonly used for establishing immunosuppression models. Additionally, Cy promotes tissue oxidation and releases free radicals, causing oxidative damage, which has led to its use in oxidative stress models. Whether BA can protect against Cy-induced oxidative damage in immune organs and consequent immunosuppression remains unknown. This study employed intraperitoneal Cy injection to induce an oxidative stress model in mouse immune organs and investigated BA' s protective effects, aiming to provide a theoretical basis for BA' s clinical application.

1.1 Reagents

Betulinic acid was synthesized from white birch bark collected in Changchun, Jilin Province in spring 2016. The bark was dried at 60°C and stored in dark, dry conditions. Betulin was extracted from the bark, oxidized with Jones reagent to produce betulonic acid intermediate, then reduced with sodium borohydride to synthesize BA, following previously described procedures. High-performance liquid chromatography (HPLC) analysis determined BA purity at 96.53%. Cyclophosphamide was manufactured by Jiangsu Hengrui Medicine Co., Ltd. Assay kits for alanine aminotransferase (ALT), aspartate transaminase (AST), total protein (TP), and albumin (ALB) were purchased from Shenzhen Mindray Bio-Medical Electronics Co., Ltd., along with the BS-200 automatic biochemical analyzer. Kits for measuring superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), catalase (CAT), reduced glutathione (GSH), and malondialdehyde (MDA) were obtained from Nanjing Jiancheng Bioengineering Institute.

1.2 Animals

Fifty healthy male Kunming mice, 4 weeks old, specific pathogen-free (SPF) grade, weighing 20±2 g, were used in this study. Mice were fed a standard growth diet provided by Hunan Slack Jingda Laboratory Animal Co., Ltd. The diet consisted primarily of wheat, corn, soybean oil, bran, soybean meal, fish meal, maltodextrin, yeast, grass meal, and premix, with nutritional levels (air-dry basis) of 20.50% crude protein, 4.62% crude fat, 1.23% calcium, 0.91% phosphorus, 1.30% lysine, and 0.68% methionine + cysteine.

1.3 Experimental Methods

After one week of acclimatization at 21-25°C and 50-70% relative humidity, 50 healthy Kunming mice were randomly divided into five groups: control, Cy, and BA groups (0.05, 0.50, and 5.00 mg/kg BW). BA was suspended in 1% soluble starch solution and administered at 0.01 mL/g BW via gavage once daily for 14 consecutive days. Control and Cy groups received equivalent volumes of 1% soluble starch solution. On day 15, all groups except control received intraperitoneal Cy injections for two consecutive days to establish the oxidative stress model, while the control group received saline injections. After 16 hours of fasting (with free access to water), mice were euthanized by orbital blood collection. Blood samples were allowed to clot at room temperature for 2 hours, then centrifuged at 3,000 rpm for 10 minutes to collect serum for biochemical analysis. Spleen and thymus were aseptically removed and weighed to calculate immune organ indices using the formula: immune organ index = organ weight/body weight. Tissue homogenates (10% w/v) were prepared in ice-cold physiological saline and centrifuged at 3,000 rpm for 15 minutes. Supernatants were stored at -80°C for determination of SOD, CAT, GSH-Px activities and GSH, MDA contents.

1.4 Detection Indicators

Serum ALT and AST activities and TP and ALB contents were measured using an automatic biochemical analyzer. Spleen and thymus SOD activity was determined by xanthine oxidase method; GSH-Px activity by dithionitrobenzoic acid (DTNB) method; MDA content by thiobarbituric acid colorimetric method; GSH content by DTNB method; and CAT activity by ammonium molybdate colorimetric method. All assays were performed strictly according to kit instructions.

1.5 Statistical Analysis

Data were analyzed using SPSS 17.0 software. One-way ANOVA was performed followed by q-test for pairwise comparisons. Results are expressed as mean ± standard deviation. $P<0.05$ was considered statistically significant.

Results

2.1 Body Weight

During the 14-day BA administration period, mice showed no obvious abnormalities or mortality, indicating no apparent toxicity. As shown in Table 1 , body weight did not differ significantly between control and Cy groups ($P>0.05$), nor between Cy and any BA dose group ($P>0.05$).

2.2 Serum Biochemical Parameters

Tables 2 and 3 show that Cy treatment extremely significantly increased serum AST and ALT activities compared to control ($P<0.01$), while ALB and TP levels remained unchanged ($P>0.05$). The 5.00 mg/kg BW BA group extremely significantly reduced both ALT and AST activities compared to the Cy group ($P<0.01$), without significantly affecting ALB or TP contents ($P>0.05$).

2.3 Immune Organ Indices

Table 4 demonstrates that Cy significantly decreased both spleen and thymus indices compared to control ($P<0.05$), indicating immune organ atrophy. BA treatment at 0.05 mg/kg BW significantly alleviated the reduction in thymus index ($P<0.05$), while all BA doses showed a trend toward improved immune organ indices, though differences were not statistically significant ($P>0.05$).

2.4 SOD Activity in Spleen and Thymus

As presented in Table 5 , Cy extremely significantly reduced thymus SOD activity compared to control ($P<0.01$), while spleen SOD activity remained unchanged ($P>0.05$). BA treatment decreased thymus SOD activity further com-

pared to Cy group, with the 0.50 mg/kg BW dose showing an extremely significant reduction (P<0.01), whereas spleen SOD activity was not significantly affected by BA.

2.5 GSH Content in Spleen and Thymus

Table 6 reveals that Cy significantly decreased spleen GSH content compared to control (P<0.05). The 0.50 mg/kg BW BA group showed extremely significantly higher spleen GSH content than the Cy group (P<0.01), indicating that Cy induces oxidative damage by reducing GSH levels while appropriate BA supplementation enhances GSH content and antioxidant capacity.

2.6 GSH-Px Activity in Spleen and Thymus

Cy alone did not significantly alter GSH-Px activity in either spleen or thymus compared to control (P>0.05). However, as shown in Table 7 , both 0.50 and 5.00 mg/kg BW BA treatments significantly or extremely significantly elevated GSH-Px activity in both organs compared to Cy group (P<0.05 or P<0.01), demonstrating BA' s protective effect through enhanced GSH-Px activity.

2.7 CAT Activity in Spleen and Thymus

Cy treatment reduced CAT activity in both spleen and thymus, with an extremely significant decrease in thymus (P<0.01) as shown in Table 8 . Both 0.50 and 5.00 mg/kg BW BA treatments significantly or extremely significantly increased CAT activity in both organs compared to Cy group (P<0.01 or P<0.05), indicating BA' s ability to improve antioxidant capacity and protect immune organs.

2.8 MDA Content in Spleen and Thymus

Cy extremely significantly elevated spleen MDA content compared to control (P<0.01). All BA dose groups extremely significantly reduced spleen MDA content compared to Cy group (P<0.01). Although thymus MDA showed similar trends, the changes were not statistically significant (P>0.05). These results suggest BA alleviates Cy-induced oxidative damage by reducing lipid peroxidation.

Discussion

Cyclophosphamide is a commonly used alkylating antineoplastic and cytotoxic drug that remains inactive until metabolized in vivo by phosphamidase or phosphatase into active phosphoramide mustard. This metabolite forms cross-links with DNA, inhibiting DNA synthesis and reducing DNA content, thereby affecting immune function. As an immunosuppressant, Cy inhibits both humoral and

cellular immune responses in various animal models. Previous research demonstrated that Cy reduces cell numbers and CD3+ and IgA+ cells in intestinal Peyer's patches while enhancing apoptosis, indicating induction of intestinal mucosal immune dysfunction. Other studies showed that Cy treatment significantly decreased bursal index, B lymphocyte proliferation, and mRNA expression of interleukin-1 in bursa and interferon- in spleen and bursa of broiler chickens. Additional work confirmed that excessive Cy severely damages splenic tissue, likely by disrupting the oxidative-antioxidant balance.

Our findings revealed that high-dose Cy markedly reduced antioxidant enzyme activities (SOD, GSH-Px, CAT) and GSH content while increasing MDA levels in immune organs. This indicates that excessive Cy consumption depletes antioxidant enzymes, disrupts redox balance, induces lipid peroxidation, damages lymphocytes, and ultimately causes oxidative stress. The observed elevation of serum AST and ALT activities following Cy treatment suggests liver injury under oxidative stress conditions. Furthermore, Cy-induced reduction in immune organ indices demonstrates immune organ atrophy and consequent immunosuppression resulting from oxidative damage.

Betulinic acid, a plant-derived pentacyclic triterpenoid, exhibits multiple bioactivities including anti-inflammatory, antioxidant, and immunomodulatory effects. In vitro studies showed BA protects vascular endothelial cells by inhibiting tumor necrosis factor-induced reactive oxygen species production. In vivo research demonstrated that BA prevents alcohol-induced liver injury by improving hepatic redox status, protecting antioxidant systems, and reducing lipid peroxidation. Our results similarly showed that BA significantly reduced serum AST and ALT activities elevated by Cy, indicating hepatoprotective effects. BA treatment significantly alleviated Cy-induced reductions in spleen GSH-Px and CAT activities and GSH content, while markedly decreasing spleen MDA content and enhancing thymus GSH-Px and CAT activities. These findings demonstrate BA' s protective effects against Cy-induced oxidative damage in lymphoid organs, likely mediated through its intrinsic antioxidant properties that enhance antioxidant enzyme activities, reduce lipid peroxide formation, and relieve oxidative stress.

Previous reports indicated that BA inhibits myeloperoxidase activity in carrageenan-induced pleurisy mouse paw tissue by 50.5% while increasing GSH-Px and glutathione reductase activities, suggesting anti-inflammatory and immunomodulatory effects through inhibition of inflammatory cell infiltration and enhanced antioxidant capacity. Our results align with these findings. Notably, 0.50 mg/kg BW BA extremely significantly reduced thymus SOD activity compared to Cy group, suggesting BA does not protect against Cy-induced oxidative damage through SOD modulation. This contrasts with our previous finding that BA increased lymphocyte SOD activity in dexamethasone-induced oxidative damage, possibly due to different oxidative stress inducers. Additionally, BA' s protective effects may relate to enhanced immunity. This study showed BA improved immune responses in immunocompromised mice,

alleviated Cy-induced thymic atrophy, and significantly restored immune organ indices. Our previous work demonstrated that BA enhances lymphocyte activity and modulates T and B lymphocyte populations, thereby improving cellular immunity. These results suggest BA' s immunomodulatory effects depend on its antioxidant capacity, protecting against free radical damage by increasing antioxidant enzyme activity and reducing lipid peroxidation. The specific antioxidant mechanisms of BA require further investigation.

Conclusion

Betulinic acid provides preventive protection against cyclophosphamide-induced oxidative damage by enhancing catalase and glutathione peroxidase activities, increasing glutathione content, and reducing malondialdehyde levels in mouse immune organs.

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