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Original Research Article

Norcantharidin alleviates cyclophosphamide-induced immunosuppression and leukopenia in mice through NF-κB pathway

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Abstract

Purpose: *To investigate the effect of norcantharidin (NCTD) on cyclophosphamide (CY)-induced immunosuppression and leukopenia in mice.*
Methods: *Cyclophosphamide (80 mg/kg/day) was intraperitoneally administered to mice from day 1 to 3 and day 9, and from day 4 to 10, NCTD (1, 2 and 4 mg/kg) was intraperitoneally administered. The counts of white blood cells (WBC), red blood cells (RBC) and platelets (PLT) in peripheral blood were determined, followed by the evaluation of mouse spleen and thymus indices. Histopathological examination was performed using hematoxylin-eosin (H & E) staining. In addition, the serum levels of immunoglobulins and cytokines were determined using enzyme-linked immunosorbent assay (ELISA), while plenetic T lymphocyte subsets were assessed by flow cytometry. The expression levels of p65, p-p65, IκBα, and p-IκBα in spleen tissues were determined by immunoblotting.*
Results: *Administration of NCTD (1, 2 and 4 mg/kg) increased blood cell counts, and alleviated injuries on splenocytes and thymocytes in CY-treated mice, accompanied by decline in spleen and thymus indices. NCTD ameliorated CY-induced immunosuppression by inhibiting the cytokines but increased the immunoglobulins. CD4+ and CD8+ T lymphocyte expressions were enhanced, especially at the high dose of NCTD (4 mg/kg). Besides, NCTD at high doses (2 and 4 mg/kg) significantly activated NF-κB pathway.*
Conclusion: *Administration of NCTD alleviates CY-induced leukopenia and immunosuppression in mice through activation of NF-κB pathway, thus providing a lead for the potential treatment of chemotherapy-induced leukopenia and immunosuppression.*

Keywords: *Norcantharidin, Cyclophosphamide, Immunosuppression, Leukopenia, Nuclear factor kappa-B*

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INTRODUCTION

Cyclophosphamide (CY), an alkylating agent, is frequently used for cancer therapy, and as an immunosuppressive agent for the treatment of autoimmune and immune-associated disorders in clinics. However, it has been recognized that chemotherapy kills or renders dysfunctional WBC

systemically as the drugs target fast-growing tissues [1]. Administration of CY results in leukopenia, which is considered as a leading cause of opportunistic pathogens-induced infections [2]. In addition, in the process of chemotherapy, CY frequently induces immunosuppression in patients. The therapeutic drugs for leukopenia include leucogen, batyl alcohol, and colony stimulating factors. Although the short-term curative effect of therapeutic drugs for leukopenia is significant, it has the disadvantages of recurrence, uncertain long-term curative effect, and high price. It has been reported that other compounds such as ganoderma atrum polysaccharide and trametes orientalis polysaccharide exert chemoprotective effects preventing CY-induced immunosuppression or oxidative stress [3]. Therefore, it is necessary to develop an alternative drug for leukopenia or immunosuppression. As a conventional animal medicine, cantharides have a long history of more than 2000 years of application in the treatment of skin and other diseases. Cantharidin (CTD) is the major bioactive ingredient of cantharides, and norcantharidin (NCTD) is a methylated compound of CTD. It has been demonstrated that NCTD has extensive antitumor effects via provoking cell apoptosis and death, and autophagy in non-small cell lung cancer [4]. Besides, NCTD induces apoptosis of Tregs and enhances vaccine-induced immunity by increasing CD4+ and CD8+ T cells [5]. However, the role of NCTD in CY-induced leukopenia remains poorly elucidated.

NF-κB signaling pathway has been implicated in the immunosuppression induced by human epithelial ovarian cancer [6]. It has been suggested that NCTD facilitates LPS-mediated immune responses through activation of AKT/NF-κB pathway in macrophages [7]. However, whether NCTD exerts protective effect on CY-induced leukopenia via NF-κB signaling pathway remains unclear. Therefore, in this study, leukopenia was induced by CY injection in mice, followed by NCTD administration. NF-κB pathway-related proteins were evaluated to determine whether NCTD attenuated CY-induced leukopenia by regulating NF-κB pathway.

EXPERIMENTAL

Animals and treatment

A total of 30 Balb/c mice (7-weeks-old) were purchased from Nanjing Experimental Animal Center (Nanjing, China) and kept in a controlled condition (22 ± 2 °C and 50 - 60 % humidity) under a 12 h light/ 12 h dark cycle. All animal experiments were approved by the Ethics Committee of Guizhou Provincial People's Hospital (approval no 2020-521) for the use of animals, and were conducted in accordance with the National Institutes of Health Laboratory Animal Care and Use Guidelines [8] . After 7 days of adaptation, the mice were divided into 5 groups (6 mice in each group). From days 1 to 3 and day 9, mice in CY group were intraperitoneally injected with cyclophosphamide 80 mg/kg/day. From day 4 to 10, the CY-treated mice were treated with NCTD at a dose of 1 mg / kg, 2 mg / kg and 4 mg / kg, respectively. Mice that received the equal volume of saline solution once daily for 10 days served as the control group. Mouse body weight was recorded throughout the experiment. At the end of this experiment, the mice were anesthetized using pentobarbital sodium, and then sacrificed to obtain spleen and thymus tissues.

Measurement of peripheral WBC, RBC and PLT counts

Blood samples were collected from the retro-orbital plexus into heparin tubes.The peripheral red blood cell (RBC), white blood cell (WBC) and platelets (PLT) were counted using an automatic Hematology Analyzer (Coulter, USA).

Histopathology examination

The spleen and thymus samples were fixed in 4 % paraformaldehyde (Qiaoxing, Shanghai) in phosphate buffered saline (PBS) for 2-4 h, and embedded in paraffin after dehydration with gradient concentration of ethanol (50, 70, 80, 95, and 100 %). Subsequently, 5-μm thick sections were prepared, dried in a drying oven overnight, deparaffinated and rehydrated, and then strained with H & E solution. The sections were then dehydrated, and mounted in DPX.

Evaluation of mouse spleen and thymus indices

Twenty-four hours following the last treatment, the body weight of each mouse was recorded. The obtained thymus and spleen organs were weighed. Thymus or spleen index was calculated as the ratio of thymus or spleen weight to body weight (mg/g).

Determination of immunoglobulin and cytokines

Blood samples were obtained after removal of eyeballs of mice, and then centrifuged at 14000 rpm for 15 min to collect serum. The serum levels of immunoglobulins, i.e,. Ig-A, Ig-M and Ig-

G, and cytokine,s i.e., tumor necrosis factor α (TNF-α), interferon-γ (IFN-γ), and interleukin-2 (IL-2) were quantified using ELISA.

Analysis of T lymphocyte subsets in spleen tissues

The thymus and spleen tissues were immediately excised after sacrifice, fixed in 4 % paraformaldehyde (Qiaoxing, Shanghai), and embedded in paraffin after dehydration. A series of 4-μm thick section was prepared, and incubated with anti-mouse CD4 (1:1000, ab183685, Abcam) or CD8 antibodies (1:2000, ab217344, Abcam) overnight at 4 °C, followed by incubation with HRP-conjugated goat anti-rat IgG (1:1000, D110087-0001, Sango Biotech, China) for 1 h at room temperature. Subsequently, 3,3-diaminobenzidine (YB36201ES03, Sigma, USA) was applied to the sections, which were stained with hematoxylin. After dehydration, the sections were mounted with neutral gum, and T lymphocyte subsets were analyzed.

Western blot analysis

Total proteins were extracted from spleen tissues using RIPA lysis buffer (GenStar BioSolutions Co., Ltd, China), and the protein concentration was quantified using a Bradford assay kit (Bio-Rad Laboratories, Inc.). Aliquots of 30 mg protein were resolved on 10 % SDS-PAGE, electroblotted onto polyvinylidene difluoride membranes, which were blocked with 5 % skimmed milk for 2 h. Next, the membranes were incubated with primary antibodies against p65 (1:500, sc-8008, Santa Cruz), p-p65 (1:500, sc-136548, Santa Cruz), p-IκBα (1:1000, AF5851, Betotime, China), and IκBα (1:1000, AI096, Betotime, China) at 4 °C for 6 h and then at room temperature for 4 h, followed by incubation with HRP-conjugated secondary antibodies (1:1000; Santa Cruz, UA) for 1 h at room temperature.β-actin was used as a loading control. Blots were visualized using an ECL chemiluminescence (Pierce Biotechnology, Rockford, IL, USA) recommended by the manufacturers. Image Pro Plus 6.0 (Media Cybernetics, Silver Spring, USA) was employed for densitometry quantification.

Statistics

All experimental data were analyzed using SPSS 17.0 (SPSS Inc., Chicago, IL, USA) and presented as mean ± SD from at least three biologically repeated experiments with three replicates. Differences between the groups were compared using one-way analysis of variance. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Norcantharidin alleviates CY-induced leukopenia in mice

The chemical structure of norcantharidin is displayed in Figure 1 A. After CY administration (80 mg / kg / day), WBC, RBC and PLT were counted in peripheral blood. The results revealed that CY induced significant decreases in WBC ($p < 0.001$), RBC ($p < 0.01$) and PLT ($p < 0.001$) when compared with the control group. Administration of NCTD (1, 2 and 4 mg/kg) significantly increased blood cell counts ($p < 0.01$ or $p < 0.001$) (Figure 1 B - D). Thus, NCTD alleviated CY-induced leukopenia in mice.

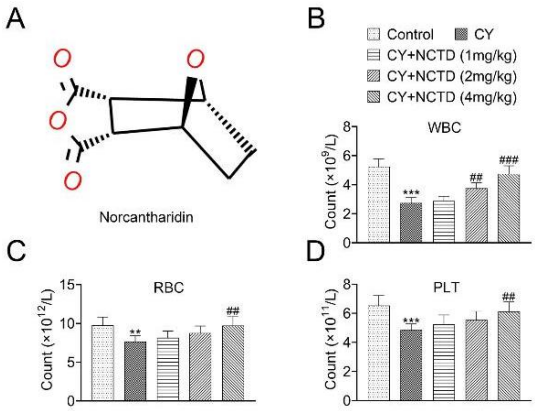

Figure 1: Norcantharidin alleviates CY-induced leukopenia in mice. A, The chemical structure of norcantharidin. B, White blood cell (WBC) counts. C, Red blood cell (RBC) counts. D, Platelet (PLT) counts. $^{**}P < 0.01$ and $^{***}p < 0.001$, compared with the control group; $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$, compared with CY-treated group

Norcantharidin alleviates CY-induced injuries in mouse spleen and thymus

As shown in Figure 2 A, spleen and thymus indices were significantly reduced by CY administration ($p < 0.001$), whereas administration of NCTD (2 and 4 mg/kg) attenuated the decline in spleen and thymus indices ($p < 0.001$). Histopathological examination using H & E staining showed that CY had detrimental effects on spleen and thymus, which manifest as decreased and loosely arranged splenocytes and thymocytes, and necrosis with no cell structures (Figure 2 B). NCTD promoted closely and orderly arrangement of splenocytes and thymocytes, and induced clear nucleus and less intercellular space. Therefore, NCTD alleviated CY-induced spleen and thymus injuries in mice.

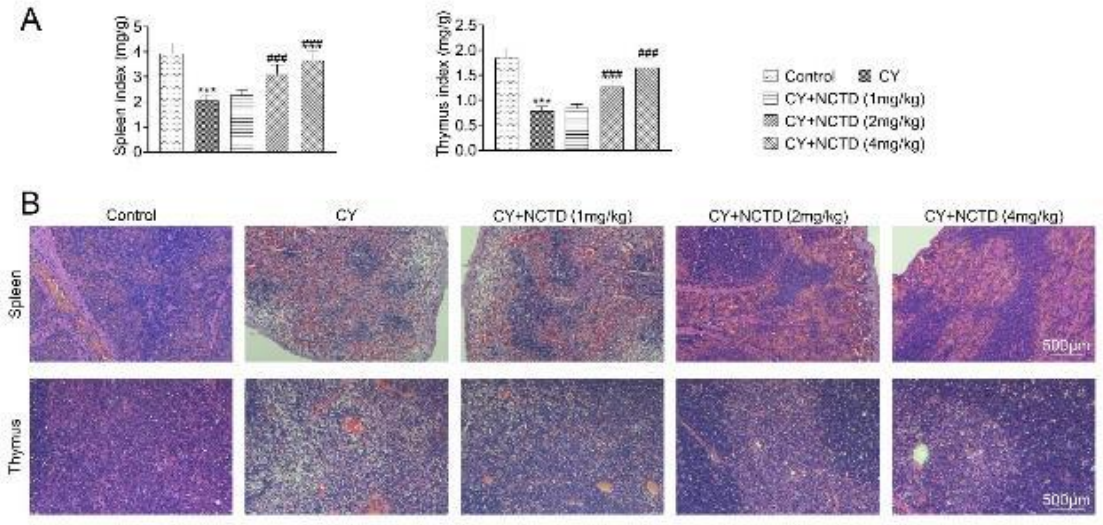

Figure 2: Norcantharidin attenuates CY-induced spleen and thymus injuries in mice. A, The spleen and thymus indices. B, Histopathological changes in the spleen and thymus using H & E staining. $^{***}P < 0.001$, compared with the control group; $^{###}p < 0.001$, compared with CY-treated group

Norcantharidin ameliorates immunosuppression caused by CY in mice

To evaluate the effects of NCTD on immune responses, serum levels of immunoglobulins and cytokines were determined. Figure 3 A shows that serum levels of Ig-A, Ig-M and Ig-G were significantly reduced by CY treatment compared to the control group ($p < 0.001$), whereas administration of high-dose NCTD (4 mg/kg) apparently increased the levels of immunoglobulins compared with untreated CY mice ($p < 0.01$). Similar to immunoglobulins alterations, CY treatment significantly suppressed serum levels of TNF-α, IFN-γ, and IL-2 when compared with control group ($p < 0.001$), while high-dose NCTD (4 mg/kg) significantly increased cytokine expressions compared with untreated CY mice ($p < 0.01$ or $p < 0.001$; Figure 3 B). These findings suggest that NCTD ameliorated immunosuppression caused by CY in mice.

Norcantharidin increased T lymphocyte subsets in spleen tissues

T lymphocyte subsets in spleen tissues were evaluated using immunohistochemistry after CY and NCTD administration. The results portrayed that CD4+ and CD8+ T lymphocyte expression in spleen tissues were significantly inhibited on exposure to CY compared with the control group. On the other hand, NCTD provoked CD4+ and CD8+ T lymphocyte expression, especially at high dose of NCTD (4 mg/kg) when compared with the untreated CY mice (Figure 4). Collectively, NCTD provoked CD4+ and CD8+ T lymphocyte expression in spleen tissues of CY mice.

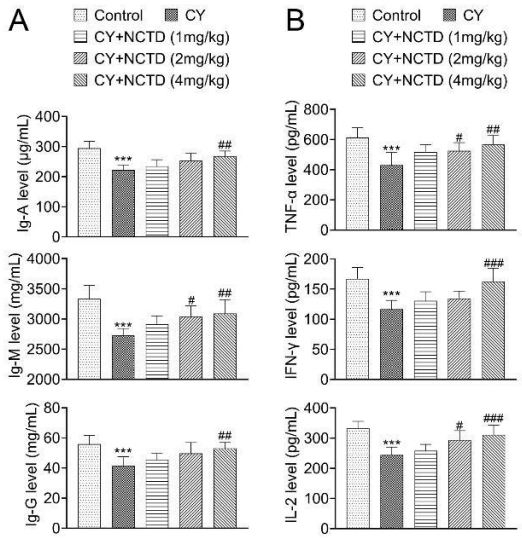

Figure 3: Norcantharidin ameliorated CY-induced immunosuppression in mice. A, Serum levels of immunoglobulins including Ig-A, Ig-M and Ig-G as determined using ELISA. B, Serum levels of cytokines TNF-α, IFN-γ, and IL-2 as measured using ELISA. $^{***}P < 0.001$, compared with the control group; $^{###}p < 0.001$, compared with CY-treated group

Norcantharidin activates NF-κB pathway

To investigate whether NCTD affects the NF-κB pathway, the expression levels of p65, p-p65, IκBα, and p-IκBα in spleen tissues were determined using immunoblotting. As shown in Figure 5, the ratio of p-p65 to p65, the level of p-IκBα were significantly down-regulated after CY

treatment compared with the control group (p < 0.001). On the other hand, the level of IκBα was significantly up-regulated when compared with the control group (p < 0.001). Furthermore, high-dose of NCTD (2 and 4 mg/kg) significantly reversed the effects of CY on NF-κB pathway-related proteins compared with untreated CY mice (p < 0.01 or < 0.001). Therefore, NCTD activated NF-κB pathway in CY mice.

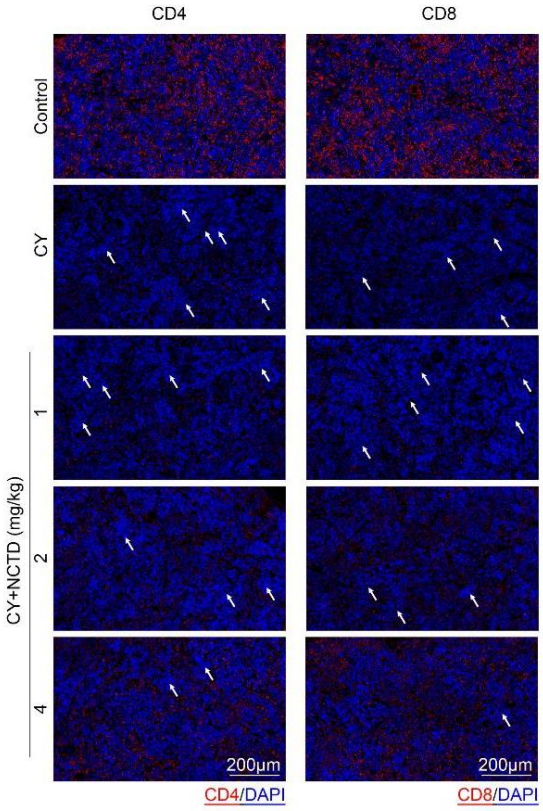

Figure 4: Norcantharidin provoke T lymphocyte subsets in spleen tissues. CD4+ and CD8+ T lymphocyte expressions in spleen tissues were evaluated using immunohistochemistry after CY and NCTD administration

DISCUSSION

The immune system is a complex regulatory system in which the thymus and spleen, as known as immune organs, play essential role in immune-related abnormalities. Spleen and thymus harbour distinct organizational structures, and the homeostasis maintenance of spleen and thymus are regulated through different mechanisms. The thymus is the central immune organ where lymphocytes mature and induce immune tolerance, while the spleen is the largest lymphoid organs where lymphocytes exert immune functions [9].

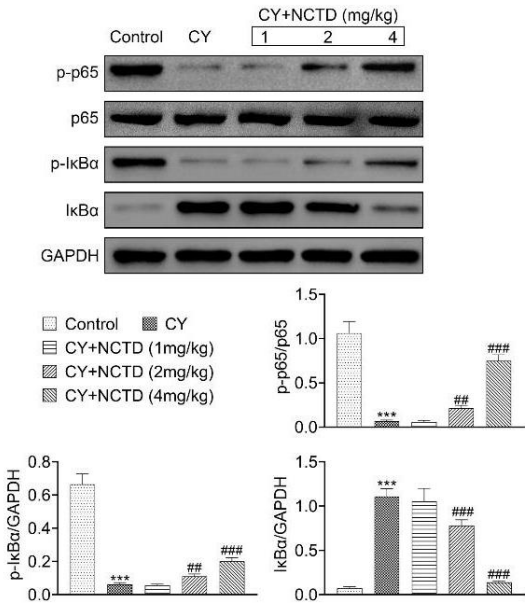

Figure 5: Norcantharidin activated NF-κB pathway. Western blot analysis was employed to determine the levels of NF-κB pathway-related proteins including p65, p-p65, IκBα, and p-IκBα in spleen tissues. $^{***}P$ < 0.001, compared with the control group; $^{\#\#}p$ < 0.01 and $^{\#\#\#}p$ < 0.001, compared with CY-treated group

It has been reported that stable expression ratios of pyroptosis-associated cytokines in spleen or thymus is of great importance for regulating immune homeostasis and adapting to the environment. Therefore, in this study, spleen and thymus injuries were evaluated by H & E staining, and T lymphocyte subsets were detected in spleen tissues after CY and NCTD administration. The results revealed that CY injection had detrimental effects on the spleen and thymus, accompanied with apparent CD4+ and CD8+ T lymphocyte reductions. On the other hand, NCTD attenuated spleen and thymus injuries and provoked T lymphocyte expression in the spleen, suggesting that NCTD may restore the function of lymphocytes.

Chemotherapy-induced hematotoxicity such as leukopenia, is considered as one of the major concerns for cancer patients. The severity of chemotherapy-caused leukopenia shows different categories, in which grades 1 - 2 leukopenia may improve whereas grades 3 - 4 leukopenia may result in dose reduction, delay or interruption of therapy, which may reduce the therapeutic effect of cancer. It has been proved that T cell function is closely related to the therapeutic responses [10, 11]. In patients with acute myeloid leukemia, treatment with cytarabine and idarubicin apparently reduced the proportion of Tregs, but increased the rate of CD4+/CD8+ T cells, indicating that the function

of T cells had been restored [12]. In addition, CY damaged the function of peritoneal macrophage, inhibited T and B lymphocytes activities, decreased the levels of CD4+ and CD8+, disturbed the balance of Th1/Th2; whilst administration of bergenin attenuated CY-induced toxicity through improving functions of macrophage and T and B lymphocytes [13]. In agreement with these results, this study found that CD4+ and CD8+ T lymphocyte expression in spleen tissues were significantly inhibited when exposed to CY, while administration of NCTD provoked CD4+ and CD8+ T lymphocyte expression, especially at the high dose of NCTD (4 mg/kg), suggesting that administration of NCTD enhances the function of T lymphocytes.

Cytokines are fundamental in cell-cell communication and modulation of innate immunity, and various cytokines exert synergistical effects. A recent study demonstrated that administration of CY significantly induced immunosuppression via inhibition of the expression of the inflammatory cytokines IL-2, IL-4, TNF-α, and IFN-γ, accompanied by the decreases in serum hemolysin, IgG and IgM in mice [14]. Besides, in CY-treated splenocytes, the levels of TNF-α, IFN-γ, IL-2, and IL-12, as well as IgG and IgA were suppressed, whilst *Platycodon grandiflorum* application reversed the immunosuppressive effects of CY [15].

As is widely known, IL-2, TNF-α and IFN-γ are secreted by Th1 cells, which enhance cell-mediated immune response. In the present study, the serum inflammatory cytokines TNF-α, IFN-γ, and IL-2 were inhibited by CY, whilst high-dose NCTD (4 mg/kg) significantly promoted cytokines production. NCTD also apparently increased the immunoglobulins including Ig-A, Ig-M and Ig-G. The results indicated that NCTD might alleviate CY-induced immunosuppression through regulation of Th1 cells.

The NF-κB pathway is a target for immunosuppressive response, and it has been reported that polysaccharides extracted from *Cordyceps gunnii* mycelia prevented CY-induced immunosuppressive response by regulating TLR4/TRAF6/NF-κB pathway [16]. The electroacupuncture approach may allievate immunosuppression through increasing the levels of p-IκBα and NF-κB while inhibiting DREAM and IκBα, indicating the activation of NF-κB pathway [17]. Moreover, sulfated *Cyclocarya paliurus* polysaccharide (SCP3) enhanced the phosphorylation of Akt, p65, IκB-α, and decreased the expression of IkB-α, suggesting that SCP3 promoted immune activity through the activation of NF-κB pathway [18]. In this study, high-dose NCTD (2 and 4 mg/kg) significantly enhanced the phosphorylation of p65 and IκB-α, but increased the expression of IkB-α. The results imply that NCTD inhibited CY-induced immunosuppression and leukopenia by activating NF-κB pathway.

CONCLUSION

The results of this study reveal that NCTD alleviates CY-induced leukopenia and immunosuppression in mice through activation of NF-κB pathway, thus providing a promising strategy for chemotherapy-induced treatment of leukopenia and immunosuppression.

DECLARATIONS

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Funding

None provided.

Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. All authors contributed to the study conception and design. Material preparation and the experiments were performed by Guochuan Wang and Xiaolu Zhou. Data collection and analysis were performed by Mei Yan and Xiaoyu Ma. The first draft of the manuscript was written by Xin Liu and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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