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Immunomodulatory effects of sinensetin on macrophage and cyclophosphamide-induced immunosuppression in mice

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Received January 25, 2022, accepted February 25, 2022

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Pharmazie 77: 147-151 (2022) *doi: 10.1691/ph.2022.2321*

In this study, we investigated the immunomodulatory effects of sinensetin (SI) on RAW 264.7 macrophages and cyclophosphamide (CY)-induced immunosuppressed mice. The results showed that SI enhanced macrophage activity and promoted the secretion of NO, IL-1β, and TNF-α *in vitro*. Compared with the CY-induced immunosuppressed mice, in mice treated with SI, the body weights, organ indices, and total lymphocytes increased. Furthermore, SI promoted the secretion and mRNA expression of IFN-γ, IL-2, and IL-6 and reduced the damage caused by CY to the organs of the immune system. Moreover, it increased the activities of GSH-Px, CAT, SOD, and T-AOC and decreased the level of MDA. This study suggests that SI has the potential to be used as an immunity enhancer in the functional food and healthcare industries.

1. Introduction

Immunity is the physiological function of recognizing and destroying foreign bodies such as viruses, bacteria, and contaminants. It can also affect aging, injury, death, and cell degeneration to maintain the health of the body (Sell 2019). Macrophages play an important role in the immune response. When the body is affected by pathogens or injury, macrophages are activated to produce a series of cytokines that resist the invasion of pathogens and protect the host (Gentek et al. 2014). Lymphocytes is an important cellular component of the body's immune response function. The ability of lymphocytes to reflect the immune ability of cells in the body is an index for studying the body's immune ability (Li et al. 2012).

Cyclophosphamide (CY) is a cytotoxic drug and an immunosuppressive alkylating agent. It can destroy the DNA structure and block replication, thus leading to cell death. CY has a strong immunosuppressive effect; therefore, it is commonly used to prepare immunosuppressed models in immunotoxicological studies (Emadi et al. 2009).

Sweet orange is a citrus plant of the Rutaceae family, and sinensetin (SI) is a natural polymethoxy flavone with the molecular formula $C_{20}H_{20}O_7$ (Nakanishi et al. 2019). In recent years, natural flavonoids have attracted increasing attention because of their various biological activities and negligible risk of side effects, and they have been used as lead compound resources with potential medical applications (Jiang et al. 2016). SI inhibits the proliferation of breast cancer cells through the metabolites produced after interacting with cytochrome P450 CYP1 enzyme (Androutsopoulos et al. 2009) and inhibits the inflammatory response induced by influenza A virus, possibly through inhibiting the NF-κB and MAPK signaling pathways activated by influenza A virus infection (Li et al. 2020).

However, the immunomodulatory activity of SI has not been confirmed, and to fill this gap, this study investigated the immunomodulatory effects of SI on macrophages and lymphocytes. Our study provides a theoretical basis for the development of natural immunity-enhancing functional foods.

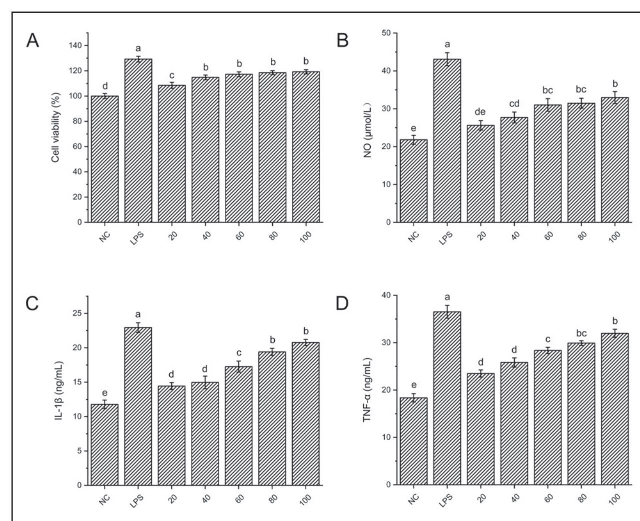

Fig. 1: Effect of SI on RAW 264.7 cells. (A) Cell viability; (B) The secretion of NO; (C) The secretion of IL-1β; (D) The secretion of TNF-α. All experiments were duplicated for three times. All data were expressed as means±SD. Different letters indicate significant differences ($P < 0.05$).

2. Investigations and results

2.1. *Effects of SI on RAW 264.7 cell viability*

As shown in Fig. 1A, treatment of RAW 264.7 cells with SI significantly increased their viability in a concentration-dependent manner ($P < 0.05$). At SI concentration of 100 μg/mL, the cell viability reached 119.24±1.65%, which indicated that RAW 264.7 cells were activated after SI treatment.

2.2. *Effects of SI on the secretion of NO, IL-1β, and TNF-α in RAW 264.7 cells*

As shown in Fig. 1B-D, the secretion of NO, IL-1β, and TNF-α significantly increased after treatment of RAW 264.7 cells with SI ($P < 0.05$). At the maximum SI concentration of 100 μg/mL, the levels of NO, IL-1β, and TNF-α were 32.96±1.57 μmol/L, 31.98±0.85 ng/mL, and 20.77±0.44 ng/mL, respectively. These results show that SI could regulate the immune activity of RAW 264.7 cells *via* promoting the secretion of various cytokines.

2.3. *Effects of SI on body weight and organ index*

As shown in Table 1, the body weight of mice significantly decreased after CY treatment ($P < 0.05$). However, after 14 days, SI-treated mice recovered from weight loss to varying degrees, indicating that SI could reduce the immune damage caused by CY. The organ indices of the CY group were significantly lower than those of the NC group ($P < 0.05$). After SI treatment, the organ indices of the CY+SIL, CY+SIM, and CY+SIH groups were significantly higher than those of the CY group ($P < 0.05$). The results showed that SI reversed the effects of CY on organ indices.

Table 1: Effects of SI on body weight and organ indices of CY mice

Group	NC	CY	CY+SIL	CY+SIM	CY+SIH
Initial weight (g)	21.09±0.50^{a}	21.06±0.45^{a}	21.41±0.34^{a}	21.20±0.24^{a}	21.38±0.36^{a}
Final weight (g)	24.86±0.47^{a}	22.19±0.59^{c}	23.20±0.31^{b}	23.57±0.32^{b}	24.68±0.53^{a}
Spleen index (mg/g)	4.91±0.23^{a}	2.27±0.20^{e}	3.43±0.24^{d}	3.81±0.16^{c}	4.11±0.29^{b}
Thymus index (mg/g)	2.28±0.14^{a}	1.02±0.12^{e}	1.31±0.13^{d}	1.48±0.09^{c}	1.90±0.08^{b}
GSH-Px (U/mg pro)	243.16±8.77^{a}	145.07±6.54^{e}	186.79±3.71^{d}	198.85±5.47^{c}	214.60±3.46^{b}
SOD (U/mg pro)	116.54±7.87^{a}	70.37±4.30^{d}	81.58±5.52^{c}	96.63±4.94^{b}	98.29±5.50^{b}
CAT (U/mg pro)	11.09±0.48^{a}	3.46±0.52^{e}	6.71±0.31^{d}	7.79±0.49^{c}	9.23±0.52^{b}
MDA (nmol/mg pro)	1.31±0.06^{e}	2.09±0.07^{a}	1.75±0.04^{b}	1.64±0.04^{c}	1.38±0.06^{d}
T-AOC (U/mg pro)	0.90±0.04^{a}	0.28±0.05^{e}	0.47±0.03^{d}	0.61±0.03^{c}	0.75±0.05^{b}

Note: All data were expressed as means ± SD of 10 samples from 10 different mice. Different letters indicate significant differences ($P < 0.05$).

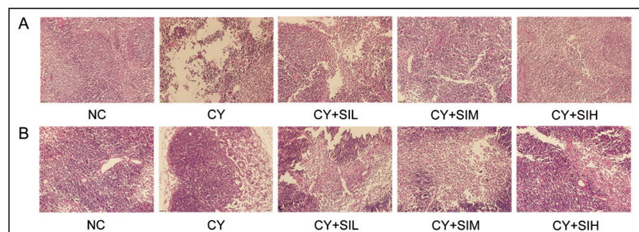

Fig. 2: Histological observation in CY mice by SI. (A) Spleen; (B) Thymus. Original magnification: ×100. Scale bar: 50 μm.

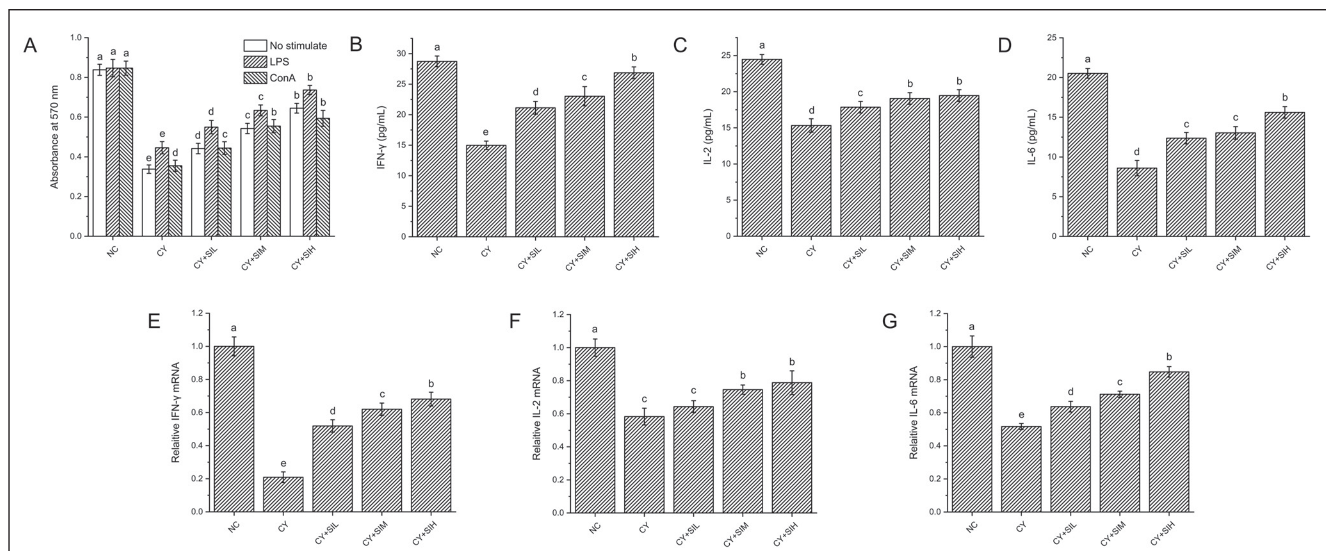

Fig. 3: Effect of SI on lymphocytes. (A) Cell proliferation; (B) The secretion of IFN-γ; (C) The secretion of IL-2; (D) The secretion of IL-6; (E) The mRNA expression of IFN-γ; (F) The mRNA expression of IL-2; (G) The mRNA expression of IL-6. All data were expressed as means±SD of 10 samples from 10 different mice. Different letters indicate significant differences ($P < 0.05$).

2.4. *Effects of SI on histology of thymus and spleen*

As shown in Fig. 2, thymocytes and splenocytes in the NC group were normal with clear nuclei. The numbers of thymocytes and splenocytes in the CY group decreased, and large acellular necrotic areas were observed. However, thymocytes and splenocytes in the CY+SIH group were arranged in an orderly fashion, with clear nuclei and less intercellular spaces, which was the closest to that in NC group. The results showed that SI could promote the recovery of CY-induced damage to the thymus and spleen.

2.5. *Effect of SI on lymphocyte proliferation*

As shown in Fig. 3A, the number of lymphocytes, B cells, and T cells in the CY group was significantly decreased when compared with that in the NC group ($P < 0.05$). The proliferation of lymphocytes, B cells, and T cells in the CY+SIL, CY+SIM, and CY+SIH groups significantly increased in a dose-dependent manner ($P < 0.05$). The number of lymphocytes, B cells, and T cells in the CY+SIH group were 0.645±0.024, 0.736±0.023, and 0.594±0.040, respectively. This shows that SI could reverse immunosuppression in mice by promoting lymphocyte proliferation.

2.6. *Effect of SI on IFN-γ, IL-2, and IL-6 secretion*

As shown in Fig. 3B-D, the secretion of IFN-γ, IL-2, and IL-6 in the CY group significantly decreased when compared with that in the NC group ($P < 0.05$). The secretion of cytokines was significantly increased in the CY+SIL, CY+SIM, and CY+SIH groups ($P < 0.05$) compared with that in the CY group. The best effect was observed in the CY+SIH group where the secretion of IFN-γ, IL-2, and IL-6 reached 26.87±0.96, 19.74±0.82, and 15.61±0.73, respectively. The results showed that SI increased the production of immunity-modulating cytokines to improve immune response.

2.7. Effect of SI on IFN-γ, IL-2, and IL-6 mRNA expression

As shown in Fig. 3E-G, the expression of IFN-γ, IL-2, and IL-6 mRNA in the CY group was significantly reduced when compared with that in the NC group ($P < 0.05$). The mRNA expression of these cytokines significantly increased in the CY+SIL, CY+SIM, and CY+SIH groups ($P < 0.05$) when compared with that in the CY group. The highest effect was observed in the CY+SIH group where the expression of IFN-γ, IL-2, and IL-6 mRNA reached 0.68±0.04, 0.79±0.07, and 0.85±0.03, respectively. The results showed that SI could restore CY-induced immunosuppression in mice by increasing the cytokine mRNA expression in lymphocytes.

2.8. Effect of SI on the antioxidant status of spleen

As shown in Table 1, the activities of GSH-Px, SOD, CAT, and T-AOC in the CY group significantly decreased when compared with those in the NC group ($P < 0.05$), whereas the levels of MDA increased significantly ($P < 0.05$). The activities of GSH-Px, SOD, CAT, and T-AOC in the CY+SIL, CY+SIM, and CY+SIH groups were significantly increased ($P < 0.05$) compared with those in the CY group, whereas the MDA levels were significantly decreased ($P < 0.05$). These results showed that SI enhanced the antioxidant status of the spleen of immunosuppressed mice.

3. Discussion

The immune system consists of immune organs (spleen, thymus, bone marrow), immune cells (lymphocytes, macrophages, neutrophils), and immunocompetent substances (IL, TNF, IFN) (Black et al. 2016). In this study, SI was found to enhance the immunity and maintain the physiological balance by coordinating the components of the immune system.

As important immune cells, macrophages play a major role in phagocytosis and digestion of cell fragments and pathogens as well as the activation of other immune cells (Tardito et al. 2019). LPS can induce the macrophage activation and B-lymphocyte proliferation and promote IL-6, IL-12, and TNF-α secretion (Hoogland et al. 2015). NO is a non-specific molecule that plays an important role in pathophysiological processes of the body. It activates the cellular secretion and inhibits the growth of pathogenic microorganisms (Humberto et al. 2018). IL-1β has a wide range of biological effects such as promotion of immune response, participation in inflammatory reactions, and promotion of wound healing (Lim et al. 2019). TNF-α can kill and inhibit tumor cells, promote neutrophil phagocytosis, and participate in pathological damage caused by autoimmune diseases (Hoogland et al. 2015). *Astragalus* flavonoids can activate macrophages, increase the expression of IL-6 and IL-1β mRNA, and regulate the immune function of RAW264.7 cells by activating the NF-κB signaling pathway (Li et al. 2018). Blueberry flavonoids reduce the secretion of TNF-α and phosphorylation of the NF-κB signaling pathway in RAW 264.7 cells stimulated by LPS (Shi et al. 2017). *In vitro* studies have shown that SI can promote the activation of macrophages; increase the secretion of NO, IL-1β, and TNF-α in a dose-dependent manner; and improve immune function by enhancing the function of macrophages.

CY is a cytotoxic drug that can inhibit mitosis through its effects on the cell cycle, destroy DNA structure, and exert a strong immunosuppressive effect (Nosadini et al. 2015). The thymus regulates the immune balance of the body and maintains immune stability (Piray et al. 2021). In this study, SI reversed the effects of CY on body weight and immune system of mice; CY-induced organ damage was significantly improved by SI. Improvement in the spleen was more obvious than that in the thymus.

Lymphocytes are an important component of the cell-mediated immune response function and reflect the immune ability of cells in the body (Crooke et al. 2019). ConA can selectively activate T-cell proliferation and plays an important role in regulating immune responses (Niu et al. 2020). IFN-γ plays an important role in preventing acute infection by inducing the production of antiviral proteins and enhancing their antitumor ability (Russell et al. 2017). IL-2 is a growth factor for all T-cell subsets and promotes the proliferation of activated B-cells; therefore, it is an important factor in the regulation of immune responses (Kariminik 2016). IL-6 plays an anti-infection immune role and can induce the expression of cell surface receptors and enhance mitosis (Russell et al. 2020). Baicalin can stimulate lymphocyte proliferation and upregulate the expression of IFN-γ, IL-2, and IL-12 mRNA (Gong et al. 2011). Genistein can promote lymphocyte proliferation and increase IFN-γ levels (Ghaemi et al. 2012). *In vivo* studies have shown that SI can significantly promote lymphocyte proliferation; increase the mRNA expression and secretion of IFN-γ, IL-2, and IL-6; and enhance immune function.

CY can stimulate the body to produce a large amount of ROS, and excessive ROS reduce the body's antioxidant capacity (Zaffanello et al. 2007). GSH-Px, CAT, and SOD play key roles in scavenging active oxygen free radicals in the body and are important indicators of the body's antioxidant capacity (Ju et al. 2017; Farzaei et al. 2018). Excessive MDA content damages body cells, and T-AOC can reflect the overall antioxidant level of the body (Liu et al. 2018; Wu et al. 2019). Hesperidin can promote the antioxidant activity of the kidney and liver and inhibit apoptosis and inflammation in the kidney (Ahmad et al. 2012). Quercetin has been shown to inhibit the increase in body weight, lipid peroxidation levels, and inflammatory expression (Sikder et al. 2014). *In vivo* studies have shown that SI can restore oxidative damage by increasing antioxidant levels, thereby effectively preventing oxidative stress.

In conclusion, ours is the first report on the immunomodulatory effects of SI *in vitro* and *in vivo*. SI activates macrophages and promotes cytokine secretion *in vitro*; it can enhance the immune response by improving the health of the organs of immune system, stimulating lymphocyte proliferation, and enhancing cytokine secretion and mRNA expression *in vivo*. It can also reduce weight loss, tissue damage, and oxidative stress induced by CY. Although the exact molecular mechanism and signaling pathways are not fully understood, SI has a potential immunomodulatory effect and a certain protective effect on the spleen and thymus. Future research on the mechanism of action of SI may allow its clinical utilization and promote its use as a food supplement for immunity enhancement.

4. Experimental

4.1. Materials

SI (purity ≥ 98%, CAS Accession Number: 2306-27-6, batch number: A0584) was purchased from Chengdu Must Biotechnology Co., Ltd. (Chengdu, China). Limulus reagent was purchased from Zhanjiang A&C Biological Co. Ltd. (Zhanjiang, China). CY, levamisole, ConA, LPS, FBS, PBS, RPMI-1640 medium, red blood cell (RBC) lysis buffer, MTT, and hematoxylin eosin (HE) staining kits were purchased from Beijing Solarbio Technology Co., Ltd. (Beijing, China). ELISA kits for mouse NO, IL-1β, TNF-α, IFN-γ, IL-2, and IL-6 were purchased from Shanghai Mlbio Co. Ltd. (Shanghai, China). Reverse transcription and real-time PCR kits and primers for mouse IFN-γ, IL-2, and IL-6 were purchased from Japan Takara Biological Engineering Co., Ltd. (Kyoto, Japan). Assay kits for GSH-Px, CAT, SOD, MDA, and T-AOC were purchased from Beyotime Biotechnology Co., Ltd. (Shanghai, China).

4.2. Endotoxin test

Endotoxins in SI were detected using kinetic turbidimetry. The results showed that SI did not interfere with Limulus amebocyte lysate detection, and the endotoxin content was less than the minimum detection limit (0.031 EU/mL), indicating that SI was safe for the next experiment.

4.3. Cell culture and preparation of RAW 264.7 cells in vitro

RAW 264.7 cells were purchased from the Chinese Academy of Sciences (Shanghai, China) and cultured in RPMI-1640 medium containing 10% FBS at 37 °C with 5% CO_2 (SHELLAB, Japan).

4.4. Viability of RAW 264.7 cells in vitro

RAW 264.7 cells were seeded into 96-well plates (5×10^5 cells/mL) and divided into seven treatment groups. Normal control group (NC) was cultured on 100 μL RPMI-1640 medium containing 10% FBS; positive control group (LPS) was cultured on 100 μL RPMI-1640 medium containing LPS (1 μg/mL); SI groups were cultured on 100 μL of different concentrations of SI (20, 40, 60, 80, and 100 μg/mL) and incubated at 37 °C for 24 h. Then, 20 μL MTT (5 μg/mL) was added to each well,

and the culture was incubated for 4 h. The supernatant was discarded, 150 µL DMSO was added, and the optical density (OD) was determined at 570 nm using a Varioskan Flash microplate reader (Thermo Fisher Scientific, USA).

4.5. Cytokine secretion by RAW 264.7 cells in vitro

RAW 264.7 cells were seeded into 12-well plates (5×10^5 cells/mL), and the grouping of the cells was the same as that described in Section 2.3.2. Secretion of NO, IL-1β, and TNF-α by RAW 264.7 was detected using a kit according to the manufacturer's instructions and quantified using Varioskan Flash microplate reader (Thermo Fisher Scientific, USA).

4.6. Animals model design

All animal experimentation protocols were approved by the Ethics Committee and the Experimental Animal Center of Jinzhou Medical University and performed in accordance with the local ethical guidelines (No. SCXK (Liao) 2014-0004, SPF level). Male BALB/c mice (aged 6–8 weeks and weighing 18–22 g) were provided by the Life Science College of Jinzhou Medical University (Jinzhou, China). The mice were housed in a sterile environment at 22–26°C, 45%–55% humidity, and a 12/12 h light/dark cycle, with access to food and water *ad libitum*.

Fifty mice were divided into 5 groups with 10 mice in each group (n = 10). The normal control (NC) and model control (CY) groups were administered normal saline for 14 days. The low-dose SI (CY+SIL), middle-dose SI (CY+SIM), and high-dose SI groups (CY+SIH) were intragastrically administered 50, 100, and 200 mg/kg bw SI, respectively for 14 days. CY, CY+SIL, CY+SIM, and CY+SIH mice were intraperitoneally injected with CY (100 mg/kg bw) on days 1–3, whereas the NC mice were similarly injected with normal saline. All experimental mice were euthanized 24 h after the final intragastric administration.

4.7. Body weight and organ index

The mice were weighed before and after treatment. After treatment and weighing, the mice were sacrificed, and the thymus and spleen were removed immediately and weighed. Thymus and spleen indices were calculated using the following formula:

$$\text{Organ index (mg/g)} = \frac{\text{organ weigth (mg)}}{\text{body weight (g)}}$$

4.8. Histochemical examination

The spleen and thymus from mice of each experimental group were fixed in formalin, embedded in paraffin, and stained using an HE staining kit. Histological changes were observed using an Olympus GX71 microscope (Olympus, Japan).

4.9. Cell culture and preparation of lymphocytes

The spleens of mice in each group were aseptically separated, minced in PBS, and filtered through a 200-mesh sieve to collect the lymphocytes. RBC lysis buffer was added to lyse the RBC, and the lymphocytes were then cultured on RPMI-1640 medium containing 10% FBS at 37 °C with 5% CO_2 (SHELLAB, Japan).

4.10. Cell proliferation of lymphocytes

Lymphocytes (100 µL) were seeded into 96-well plates (5×10^5 cells/mL) with or without ConA (5 µg/ml) or LPS (5 µg/ml) and cultured at 37 °C under 5% CO_2 for 48 h. Then, 20 µL MTT (5 µg/mL) was added to each well, and the culture was continued for 4 h. The supernatant was discarded, 150 µL DMSO was added, and the optical density (OD) was determined at 570 nm using a Varioskan Flash microplate reader (Thermo Fisher Scientific, USA).

4.11. Cytokine secretion of lymphocytes

Lymphocytes (1 mL) were seeded in 12-well plates (5×10^5 cells/mL). Secretion of IFN-γ, IL-2, and IL-6 by lymphocytes was detected using a kit according to the manufacturer's instructions and determined using Varioskan Flash microplate reader (Thermo Fisher Scientific, USA).

4.12. Cytokine mRNA expression of lymphocytes

Lymphocytes (1 mL) were seeded in 12-well plates (5×10^5 cells/mL). Total RNA was extracted from mouse spleen lymphocytes using the TRIzol method, and reverse-transcription PCR was conducted using Takara Reverse-transcription commercial kits. The synthesis conditions were 37 °C for 15 min and 85 °C for 5 s. Using the Takara commercial kits, real-time PCR was performed using the Eppendorf AG 22331 (Eppendorf, Germany) with the following customized primers: IFN-γ, 5′-CGGCACAGTCATTGAAAGCCTA-3′ and 5′-GTTGCTGATGGCCTGATTGTC-3′; IL-2, 5′-CCCAGGATGCTCACCTTCA-3′ and 5′-CCGCAGAGGTCCAAGTTCA-3′; IL-6, 5′-CCACTTCACAAGTCGGAGGCTTA-3′ and 5′-CCAGTTTGGTAGCATCCATCATTTC-3′; and β-actin, 5′-CATCCGTAAAGACCTCTATGCCAAC-3′ and 5′-ATGGAGCCACCGATCCACA-3′. The reaction conditions for real-time fluorescence PCR were 95 °C for 30 s, followed by 40 cycles at 95 °C for 5 s and 60 °C for 30 s. The $2^{-\Delta\Delta Ct}$ method was used to calculate target gene expression.

4.13. Antioxidant activity detection of spleen

The spleen homogenate was prepared in 0.1 g/mL cold physiological saline, centrifuged at 10000 × *g* at 4 °C for 15 min, and the supernatant was collected. The MDA, SOD, CAT, GSH-Px, and T-AOC activities in the supernatant were determined using the corresponding kits according to the manufacturer's instructions.

4.14. SPSS analysis

Results were expressed as mean±standard deviation (SD). SPSS version 22 (IBM, Armonk, NY, USA) was used to analyze the data. One-way ANOVA with Tukey's test was used for all analyses, and results with $P < 0.05$ were considered significant.

Acknowledgements: We would like to thank Northeast Agricultural University and Jinzhou Medical College for providing us with the necessary research facilities.

Funding: There is no funding for this article.

Conflicts of interest: We have no conflict of interest to declare.

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