Echinacea purpurea extract (cichoric acid) exerts an anti-inflammatory effect on yak PBMCs and regulates the TLR4 signalling pathway

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Abstract

Introduction: Inflammation is one of the main causes of impaired health in livestock and some of its processes weaken animal productivity and impact human health. The present study was conducted to evaluate the effect of echinacea extract (cichoric acid – CA) on yak peripheral blood mononuclear cells (PBMCs), inflammatory-related factors, and the toll-like receptor (TLR)4 signalling pathway induced by lipopolysaccharide (LPS) in these PBMCs. Material and Methods: Yak PBMCs were co-cultured with LPS and CA in vitro. The proliferative activity of cells was detected using the cell-counting kit-8 method, the optimal stimulation concentration of LPS was selected, the effect of CA on the content of inflammation-related factors was evaluated using an ELISA kit, and the mRNA expression of these factors was detected by RT-PCR. Results: CA inhibited the inflammatory response of yak PBMCs induced by LPS. CA inhibited gene and protein expression of key nodes of the TLR4 signalling pathway in yak PBMCs. Conclusion: It is suggested that CA has anti-inflammatory and immunomodulatory effects on yak PBMCs via the TLR4 pathway.

Keywords: CA, yak PBMCs, inflammation, TLR4, LPS.

Introduction

Inflammation is a localised protective response elicited by injury or destruction of tissues that serves to eliminate, dilute, or sequester the injurious agent and injured tissues (10). Inflammation damages the health of livestock frequently and consequentially, thus causing huge economic losses to commercial animal husbandry. Some inflammatory processes not only lead to reduced livestock productivity, but also have a significant impact on human health. Echinacea (Echinacea purpurea) is a perennial herb of the Chrysanthemum genus which is native to North America and Canada. Studies have shown that it has antiviral, anti-inflammatory, and immunoregulatory properties (13, 17, 30). The lipopolysaccharide (LPS) is a strongly pro-inflammatory molecule outside the cell wall of Gram-negative bacteria that is comprised of conserved lipid A and polysaccharides. This molecule activates the toll-like receptor (TLR)4 signalling pathway, which leads to the release of inflammatory cytokines, while the release of LPS causes an inflammatory response (3, 14, 26, 28). The aim of this study was to evaluate the effects of echinacea extract (cichoric acid – CA) on the yak peripheral blood mononuclear cell (PBMC) inflammatory response and these cells’ TLR4 pathway to provide a theoretical basis for the rational development of utilisations of echinacea.

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Material and Methods

Animals. Six grazing yaks were provided by the Datong Breeding Farm (Qinghai, China). Cichoric acid was purchased from Sigma Xi’an Rui Bo Biological Technology Company (Shanxi, China). Roswell Park Memorial Institute (RPMI)-1640 medium (Gibco, Shanghai, China), foetal bovine serum (Hangzhou Sijiqing Co., Ltd., Hangzhou, China), LPS (Solarbio, Beijing, China), a cell-counting kit (CCK)-8 (Jiangsu Haimen Biyntech Biotechnology Research Institute, Jiangsu, China), myeloid differentiation primary response (MyD)88 antibody (Cell Signaling Technologies, Danvers, MA, USA), tumour necrosis factor (TNF) receptor associated factor (TRAF)6 antibody (Bioworld Technology, Bloomington, MN, USA), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) antibody (Abcam, Cambridge, UK), and interferon regulatory factor (IRF)5 antibody (Proteintech Group, Manchester, UK) were used in the experiments described below. In order to avoid non-specific responses of immunocompetent cells, a bacterial LPS-free CA solution was prepared by dissolving 0.18 g of cichoric acid soluble powder in 1 mL of dimethyl sulphoxide (DMSO) solution, and then diluting this with 4.88 mL of medium to make a stock solution which was stored in a refrigerator at −20°C and diluted to the required concentration. In the early stage of this preparation, the research group verified that the concentration of DMSO used (<0.1%) was not toxic to the cells (25).

Instruments and equipment. A super-clean worktable (Jiangsu Tongji Purification Equipment Co., Jiangsu, China), MU-5810E CO2 incubator (Nuaire, Plymouth, MN, USA), V18R horizontal centrifuge (Dynamica Scientific, Livingston, UK), GelDoc XR Biorad System (Bio-Rad, Hercules, CA, USA), power supply (Bio-Rad), T100 thermal cycler (Bio-Rad), Amnis flow cytometer (Luminex, Seattle, WA, USA), and Power Wave XS2 absorbance microplate reader (Bio-Tek Instruments, Winooski, VT, USA) were used in the experiments described below.

Cell culture. Peripheral blood mononuclear cells were isolated by Ficoll (TBD, Shanghai, China) density gradient centrifugation and cultured in RPMI-1640 medium containing 10% foetal bovine serum and were then placed in a humidified incubator at 37°C, 5% CO2 and 95% humidity. The medium was changed once every 24 h.

CCK-8 assay of cell viability. Yak PBMCs were seeded into a 96-well plate at a density of 1×10⁶ cells/well. Then, 2.5 µg/mL of ConA (100 µL) was added, and the final LPS concentration was adjusted to 0.1 µg/mL, 1 µg/mL, 5 µg/mL, and 10 µg/mL. The concentration of CA (60 µg/mL) was determined as it also had been by this research group in a previous experiment and used at the same concentration in the CA and CA+LPS groups. Each group was provided with three wells and cultured in a 5% CO2 humidified incubator at 37°C for 48 h. The 96-well plate was removed, the instructions of the CCK-8 kit were followed, and the absorbance at 450 nm was measured with the Power Wave XS2 microplate reader.

Inflammatory cytokine determination. The treated cells were used to detect inflammatory cytokines. The cells were inoculated into 96-well plates (1×10⁶ cells/well). The concentration of CA was 60 µg/mL, LPS was 1 µg/mL, and the optimal concentration was determined by CCK-8. Each experiment was repeated three times. The cells were cultured in an incubator at 37°C under 5% CO2 for 48 h. Interleukin 6, 8, 10 and 1β and TNF-α and INF-γ ELISA kits were used according to the protocols provided by the manufacturer (MyBioSource, San Diego, CA, USA). The absorbance values of related factors were determined by the microplate reader.

RT-PCR. Evaluation of the mRNA expression of INF-γ, TNF-α, IL-10, IL-1β, IL-8, NF-κB, IRF5, TRAF6, and MyD88 was made with an RT-PCR. Total RNA was extracted from yak PBMCs using Trizol reagent according to the manufacturer’s instructions (Takara, Japan). An M-MLV reverse transcriptase kit was used to reverse transcribe RNA in accordance with the manufacturer’s instructions (Tiangen Biotechnology, Beijing, China) and detected according to the instructions for the real-time fluorescent RT-PCR kit (Tiangen Biotechnology, Beijing, China). GAPDH expression was included as an internal housekeeping gene control. Primer sequences as listed in Table 1 were designed and synthesised by Shanghai Shenggong (Shanghai, China). The levels of gene expression were analysed using the 2-ΔΔCT method.

Table 1. Gene primer sequences

| Gene       | Forward primer | Reverse primer          |
|------------|----------------|-------------------------|
| GAPDH      | ATCTGACCTGCGCGGCTGGAG | GCAGGCTGAACCTACCCATCC   |
| INF-γ      | CCGACGGCTGAGGATGACGC | CCAAGAGGACCCGAGGATG     |
| TNF-α      | CTGGCGGCAAGGAGGGGTCC  | GAGGAGGAGGAGGAAGGGTGGAG |
| IL-10      | ACCAGCACCACATGTTCTTATCA | CTCTCCACCGCCTCTCTTGG   |
| IL-6       | CACTGACTGCTTGGAGGAGG | CCAGAATGCTTGCAAGGCTTACG |
| IL-1β      | GAGTTGCACCTCTTCCATCACTTC | AGCTACACACTCCTCATC     |
| IL-8       | CATTTAGGGAGGCGCCCTGAGG | CTGGTACTGGCTCGTGTCGTC  |
| NF-κB      | ACAAGGCTGCTGACAGCCAACATG | TGAATGGTGAAGGCTTCAGGGTGG |
| IRF5       | TGCTGCGGCTTCCGACACCTG | CGACCTGTCGCCAGGTCAC    |
| MyD88      | TATCGGCTGAGTGTCGCGTGC | TCAAGGACCACGCCACCATCC  |

Gene primer sequences.
Western blot analysis. After being treated for 48 h, cells were lysed with a protein extraction solution, harvested, and evaluated in terms of protein concentrations by using BCA (the binding reaction of caffeic acid with bovine serum albumin). Gel was prepared for SDS-PAGE electrophoresis, and samples were added to the wells for electrophoresis. Resolution through 12% SDS-polyacrylamide gel electrophoresis (PAGE) and transfer onto PVDF membranes took place. The membrane was sealed with skim milk at room temperature for 1 h. The primary antibody was diluted and incubated on the PVDF membrane overnight at 4°C, then decolorised and washed three times on the shaker with tris-buffered saline with Tween (TBST) for 5 min at room temperature. The secondary antibody was diluted 3000-fold with TBST, incubated on the PVDF membrane at room temperature for 30 min, then decolorised with TBST and washed three times on the shaker for 5 min each. The electrochemiluminescent A and B reagents were mixed in a medium-volume centrifuge tube with the protein on the PVDF membrane facing the mixture. After 5 min, the residual liquid was removed, wrapped, and put into a cassette for exposure. The Bio-Rad gel imaging system was used for visualisation.

Statistical analysis. Data analysis was performed using SPSS 22.0 (IBM, Armonk, NY, USA). All data were expressed as the mean ± SD. Statistical significance was determined by Student’s t-test or one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison tests, and P<0.05 was considered a statistically significant difference.

Results

Determination of cell survival rate using a CCK-8

Effect of LPS on the yak PBMC survival rate. To determine the effect of different concentrations of LPS on PBMCs, the cell survival rate was measured. As shown in Figure 1, compared with the control group, the cell survival rate decreased significantly in the 0.1, 5, and 10 µg/mL LPS groups (P < 0.01), while the cell survival rate decreased but not significantly in the 1 µg/mL LPS group (P > 0.05).

Effect of the interaction between LPS and CA on the yak PBMC survival rate. As shown in Figure 2, compared with the CA group, the combined action of 0.1 and 5 µg/mL of LPS and CA significantly decreased the survival rate of yak PBMCs (P < 0.05). The combined effect of 10 µg/mL of LPS and CA significantly lowered the cell survival rate (P < 0.01). There was no significant effect on the survival rate of yak PBMCs of 1 µg/mL of LPS and CA (P > 0.05).

Fig. 1. Effects of LPS on the survival rate of yak PBMCs. The survival rate was determined using a CCK-8. The values presented are the means ± SD. * – P < 0.05; ** – P < 0.01. P values are compared with the control group

Fig. 2. Effects of LPS and CA on the survival rate of yak PBMCs. The survival rate was determined using a CCK-8. The values presented are the means ± SD. * – P < 0.05; ** – P < 0.01. P values are compared with the control group
Effects of CA on LPS-induced yak PBMC inflammatory factors. As shown in Figure 3, the IL-6, IL-8, IL-1β, IFN-γ, TNF-α and IL-10 levels in the CA treatment group were higher than those in the control group, but there was an insignificant difference between these levels in the CA treatment and control groups (P>0.05). The IL-6, IL-8, INF-γ and IL-10 levels in the LPS treatment group were significantly higher than those in the control group (P<0.05). In the CA+LPS treatment group, the IL-6, IL-8, IL-1β, INF-γ, and TNF-α levels increased, but the difference was not significant (P>0.05), while the IL-10 level increased significantly (P<0.01). Compared with those of the LPS treatment group, in the CA treatment group the IL-1β and TNF-α levels were lower and statistically significantly so (P<0.01), and IL-6, IL-8, INF-γ, and IL-10 were inhibited (P<0.05). In the CA+LPS treatment group the IL-6, IL-8, IL-1β, and INF-γ levels fell (P<0.05), the TNF-α level decreased (P<0.01), but the IL-10 level increased (P<0.05).

Next, the effect of CA on the TLR4 signalling pathway induced by LPS in yak PBMCs was investigated. As shown in Figure 5A, the levels of NF-κB, TRAF6 and IRF5 expression in the CA treatment group were insignificantly higher than in the control group (P>0.05), and the expression of MyD88 was upregulated (P<0.05). The expression of MyD88, NF-κB and TRAF6 mRNA in the LPS treatment group was higher than it was in the control group (P<0.01), and the expression of IRF5 mRNA was higher than it was in the control group (P<0.01). The expression of MyD88 and TRAF6 mRNA was significantly stronger in the CA+LPS treatment group than in the control group (P<0.05). Compared with the LPS group, the expression of MyD88 and TRAF6 mRNA was nearly completely inhibited (P<0.01) and the expression of NF-κB mRNA was weaker in the CA treatment group (P<0.05). The expression of MyD88, NF-κB and TRAF6 mRNA was significantly diminished in the CA+LPS treatment group (P<0.05).

As shown in Figure 5C, compared with the control group, the protein expression of MyD88, NF-κB and TRAF6 was upregulated in the CA group, and the expression of IRF5 protein was downregulated (P>0.05). The expression of MyD88 protein in the LPS treatment group was higher than this expression in the control group (P<0.01), and the expression of NF-κB and TRAF6 protein was upregulated (P<0.05). The expression of MyD88, NF-κB and TRAF6 protein in the CA+LPS group was more potent than it was in the control group (P<0.05). Compared with the LPS group, the expression of MyD88 protein was nearly completely inhibited (P<0.01) and the expression of NF-κB and TRAF6 protein was less potent (P<0.05) in the CA treatment group. The expression of MyD88 and TRAF6 protein was significantly reduced in the CA+LPS treatment group (P<0.05).

**Fig. 4.** Effects of CA on inflammatory factor mRNA expression of yak PBMCs. Cells were induced by 1μg/mL LPS, 60μg/mL CA, and 60μg/mL CA + 1μg/mL LPS for 48h. The RNA expression of IL-6, IL-8, IL-1β, IFN-γ, TNF-α and IL-10 was measured. *P < 0.05; **P <0.01. P values are compared with the control group, #P <0.05; ##P <0.01. P values are compared with the LPS group.

**Effect of CA on the expression of yak PBMC inflammatory factor mRNA.** As shown in Figure 4, the expression of IL-6, IL-8, IL-1β, IFN-γ, TNF-α, and IL-10 mRNA in the CA treatment group was higher than the control group, but the difference was not significant (P>0.05). The expression of IL-6, IL-8, IL-1β, IFN-γ, and TNF-α mRNA in the LPS treatment group was higher than that in the control group (P<0.01), and the expression of IL-10 mRNA increased in the LPS treatment group (P<0.05). The expression of IL-6, IFN-γ and TNF-α mRNA was significantly greater in the CA+LPS treatment group than in the control group (P<0.05). Compared with the LPS group, the expression of IL-6, IL-8, IL-10, IL-1β, IFN-γ, and TNF-α mRNA was decreased in the CA treatment group (P<0.01 and P<0.05). The expression of IL-6, IFN-γ and TNF-α mRNA was significantly diminished in the CA+LPS treatment group (P<0.05), and the expression of IL-8 and IL-1β mRNA was also significantly suppressed (P<0.01); however, the expression of IL-10 mRNA was augmented (P<0.05).
Discussion

LPS is an important component of the outer membrane of Escherichia coli and is considered to be the main pathogenic factor in Gram-negative bacteria (9). The inflammatory response is considered to be a potential biological protective mechanism for epithelial cell damage, which may be caused by chemical factors (such as toxic organic compounds) and foreign bodies (including various metals, wood chips, and sawdust and dust entering the body) (16). Echinacea extract enhances immunity, exhibits anti-inflammation, anti-oxidation, anti-virus, and anti-tumour activities, and regulates cell apoptosis. Echinacea is one of the few medicinal plants currently known to have immunity-enhancing and anti-inflammatory effects. It has a wide range of uses in treatment, and stable and discriminating curative effects (2, 4). Studies have shown that macrophages, peripheral blood monocytes and ATCII synthesise and secrete many cytokines and inflammatory mediators, including TNF-α, IL-6, and IL-8 (6, 24). Tumour necrosis factor-α leads to the loss of epithelial barrier function and enhances the permeability of monocytes, and also acts as a pro-inflammatory cytokine to promote the secretion of IL-1β, IL-6, and IL-8 (27). Interleukin-1β activates macrophages and neutrophils (7). Interleukin-6 activates the proliferation of neutrophils and B lymphocytes (21), and IL-8 is a chemokine factor that activates neutrophils and changes their morphology, thus enabling neutrophils to migrate to the reaction site (19). In the current study, yak PBMCs treated with LPS had raised levels of IL-6, IL-8, IL-1β, INF-γ and TNF-α, and the expression of each gene in the CA+LPS treatment group was significantly lower than in the LPS treatment groups. The inhibitory effect of CA on LPS-induced inflammatory factors was verified.

Toll-like receptor 4 is the most important member of the TLR family. It resists the invasion of pathogenic microorganisms via the innate immune response by mediating the expression of NF-κB (12), which is a group of important transcription factors with a wide range of biological functions. The cellular biological effects include stress-induced, immune, and inflammatory responses, and the development of haematopoietic cells, lymphatic organs, and keratinocytes, as well as cell proliferation and apoptosis, tumorigenesis, and neuronal synaptic formation (23). Interferon regulatory factor 5 plays an important role in regulating the transcription of interferon, immune response to pathogens, signal transduction of cytokines, and immune regulation (1, 15, 18). Myeloid differentiation primary response 88, as the first identified toll-like/IL-1 (TIR) domain containing adaptor protein, is recruited by all TLRs except TLR3 in mammals (11). The principal functional domains of MyD88 consist of the C-terminal TIR domain and the N-terminal death domain (8). The TIR domain interacts with the TIR domain of activated TLRs, and the death domain is involved in recruiting the downstream molecule IL-1 receptor-associated protein kinase (IRAK)
(22). Subsequently, the MyD88-IRAK complex induces the auto-ubiquitination of TRAF6, which is the only member of the TRAF family that participates in the MyD88-dependent pathway (5, 20). Then, the MyD88-IRAK complex activates NF-κB and activator protein 1, and inflammatory cytokines are produced and eventually eliminate pathogens (29). In addition, the TRAF6 gene is a key node gene in the TLR pathway and can directly affect the cell cycle, apoptosis, and growth. These structures indicate that CA may directly or indirectly affect the immune regulatory mechanism through the TLR pathway. The results showed that in yak PBMCs treated with LPS, the levels of MyD88, NF-κB and TRAF6 were augmented, and their levels in the CA+LPS treatment group were significantly diminished from those in the groups treated only with LPS. The results also showed that CA can regulate the TLR4 signalling pathway in yak PBMCs and that it enhances immune regulation and anti-inflammatory mechanisms. Immune regulation was mainly manifested in inhibition of the immune response, thereby enhancing the immune function of grazing yak.

In summary, CA inhibited the LPS-induced inflammatory response of yak PBMCs and gene and protein expression of key nodes of the TLR4 signalling pathway in these cells. This study suggests that CA has anti-inflammatory and immunomodulatory effects on yak PBMCs through the TLR4 pathway.

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**Animal Rights Statement:** All animal procedures were performed according to the guidelines laid down by the China Council on Animal Care, and the protocols were approved by the Experimental Animal Management Committee (EAMC) of Qinghai University. All animal experiments were conducted according to the guidelines established by regulations. Ethical approval for this study and its procedures was obtained from the Ethical Committee of Chinese Animal Care at Qinghai University.

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