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Research article

Cichoric acid from extracted *Echinacea purpurea* induces the proliferation and apoptosis of peripheral blood mononuclear cells from yaks

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ABSTRACT

Background: Cichoric acid (CA) is extracted from *Echinacea purpurea*. It is well known and widely used for its immunological function. However, the effect of CA on peripheral blood mononuclear cells (PBMCs) from yaks is still unclear. This study investigated the potential influences of CA on the proliferation, cytokine induction, and apoptosis of PBMCs from Datong yak in vivo, and aimed to provide a basis for exploring the pharmacological activities of CA on yaks.
Results: In this study, CA promoted PBMCs proliferation by combining concanavalin A (Con A) and exhibited a dose-dependent effect as demonstrated by a Cell Counting Kit-8. The concentration of 60 μg/ml CA was the best and promoted the transformation from the G0/G1 phase to the S and G2/M phases with Con A. Furthermore, 60 μg/ml CA significantly increased IL-2, IL-6, and IFN-γ levels and PCNA, CDK4 and Bcl-2 expression levels, but it significantly inhibited the TP53, Bax, and Caspase-3 expression levels. Transcriptome analysis revealed a total of 6807 differentially expressed genes (DEGs) between the CA treatment and control groups. Of these genes, 3788 were significantly upregulated and 3019 were downregulated. Gene Ontology and pathway analysis revealed that DEGs were enriched in cell proliferation and immune function signaling pathways. The expression level of some transcription factors (BTB, Ras, RRM_1, and zf-C2H2) and genes (CCNF, CCND1, and CDK4) related to PBMCs proliferation in yaks were significantly promoted after CA treatment. By contrast, anti-proliferation-associated genes (TP53 and CDKN1A) were inhibited.
Conclusions: In summary, CA could regulate the immune function of yaks by promoting proliferation and inhibiting inflammation and apoptosis of PBMCs.
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1. Introduction

Cell death is an indispensable activity in the whole life process of multicellular organisms [1]. Generally, cell proliferation and apoptosis are in balance [2]. Through these processes, superfluous and damaged cells can be removed at a time and play an important role in the establishment of the immune system and the development of various tissues and organs. Well-known cancer genes such as the tumor suppressor p53 (TP53), PCNA, and cell cycle gene CDK4 all can regulate the proliferation of cells. The primary role of TP53 is to control cell cycle progression, which leads to inhibition of cell proliferation and promotion of cell apoptosis. Apoptosis is regulated by conservative genes between species and genera and it is an autonomous cell death that maintains homeostasis in the internal environment. In many immune cells, especially in lymphocytes, some members of the TNF receptor family induce cell death via a special conjunction with their ligands. TNF-α is an extracellular signal protein and serves as the major inducement of apoptosis [3].

Herbal dietary supplement transaction increases by 5–10% annually. Among the top five grossing taxa is *Echinacea* [4]. It is native to North America and widely used for its immune regulatory functions and beneficial effects [5]. Its extracts have been investigated for their

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immune enhancement, anti-inflammatory, antioxidative [6], and anti-cancer effects [7]. The main active ingredients of *E. purpurea* are polyacetylenes, polysaccharides, alkamides, and phenolics (e.g., caftaric acid, chlorogenic acid, caffeic acid, cynarin, echinacoside, and cichoric acid) [8]. Caffeic acid derivatives could elicit immunomodulatory effects [9] and mitigate oxidative stress and inflammation [10]. Cichoric acid (CA), a derivative of caffeic acid, is one of the biologically active constituents in *E. purpurea* [8]. Its content varies in different parts of the plant [11]. CA has been studied for its anti-inflammatory, immune regulatory, and cell apoptosis induction activities [12,13]. It could inhibit hyaluronidase and protect collagen, which is the main component of animal connective tissue and the most abundant and widely distributed functional protein in mammals, from degradation by free radicals [14]. As an effective immunopotentiator, CA has been widely investigated because of its role in inflammation [15], oxidative stress [16], diabetes mellitus [17], nonalcoholic fatty liver [18], and immunity. It can prevent acute alcohol-induced hepatic steatosis in mice by its anti-inflammatory activity and oxidative stress inhibition [19]. However, the expression changes of immune-related genes induced by CA in peripheral blood mononuclear cells (PBMCs) are yet to be studied.

PBMCs, which are an important class of immune cells and play a vital role in immune function, comprise several varieties of cells, including B cells (~15%), T cells (~70%), monocytes (~5%), natural killer cells (~10%), and other immune cells [20]. Ground and root extracts of *E. purpurea* could potently increase the number of lymphocytes, especially T cells, in rabbit peripheral blood [21]. *E. purpurea* can induce the secretion of IL-1, IL-6, IL-10, and TNF-α by human peripheral blood macrophages in vitro [22,23,24]. Phagocytosis in alveolar macrophages of mice fed with *E. purpurea* can be enhanced [25]. In addition, *E. purpurea* extracts could improve the immunity of yaks [26] and promote PBMCs proliferation in humans [27]. It has been proved that CA can induce immunostimulation by promoting the proliferation and activity of immune cells. CA has been used as an immunopotentiator in many European and American countries through its addition to daily feed to enhance the disease resistance of livestock and poultry, reduce the use of antibiotics, and improve the quality and competitiveness of products. The immune effects of CA have been investigated in dairy heifers [26], mice [22], and humans [28], but not in yaks (*Bosgrunniens*). Yaks are less sensitive compared with other livestock and can adapt to the harsh living environment with changing climates in plateau areas because of their immune mechanism. The effect of CA on the immune systems of yaks has been rarely investigated.

In this study, the effects of CA on the proliferation, cytokine secretion (interleukin IL-2, IL-6, and interferon-gamma IFN-γ), and apoptosis of PBMCs from yaks were investigated. Transcriptome differences between the CA treatment group and the control group were explored using RNA-Seq. This study provided valuable information and a theoretical basis for exploring the potential mechanism of CA participating in the immune regulation of yaks and its application as a kind of daily feed addition.

2. Materials and methods

2.1. Experimental animals

Yaks from the Datong Yak Breeding Farm of Qinghai Province were sampled. This study was approved by the Animal Ethics Committee of Qinghai University. Six healthy yaks (average age of 8 months, (2014.3–2014.5)) were included in this study. All animals were kept under the same natural conditions (37°11′–37°22′ N, 100°52′–101°26′ E). The yaks were allowed to freely graze in the pasture during summer and were provided with free access to water. During winter, the yaks received standard fodder to supplement grazing, and *Echinacea* was not part of their diet. All the blood samples were collected after the yaks were kept inside for 1 night, just before they were released outside. They were housed and cared for in accordance with the Chinese guidelines for the care of experimental animals. These guidelines are similar to those of the US National Institutes of Health and the Canadian Council on Animal Care.

2.2. Compounds and reagents

The following compounds/reagents/kits were used: CA and Con A (Sigma, St. Louis, MO, USA) dissolved in RPMI 1640 medium; RPMI 1640 medium and fetal calf serum (FCS; Gibco, Invitrogen Inc., Carlsbad, CA, USA); CCK-8 kit (Beyotime Institute of Biotechnology, Haimen, China); Ficoll bovine lymphocyte separating medium (TBD, Shanghai, China); annexin V-FITC and propidium iodide (PI) staining kit (Lianke Biotechnology, Hangzhou, China); EdU kit (Guangdong Ruibo Biotechnology, Guangdong, China); IFN-γ, IL-2, and IL-6 ELISA kits (MyBioSource, San Diego, CA, USA); TNF-α (Solarbio, Beijing, China); and reverse transcription and fluorescence quantification kits (Tiangen Biotechnology, Beijing, China).

2.3. Preparation of CA and PBMCs isolation

CA (0.18 g, purity >95%) was extracted from *Echinacea* and dissolved in 1 ml of DMSO to avoid the influence of LPS. The compound was then dissolved in 4.88 ml of mother liquor and stored at −20°C.

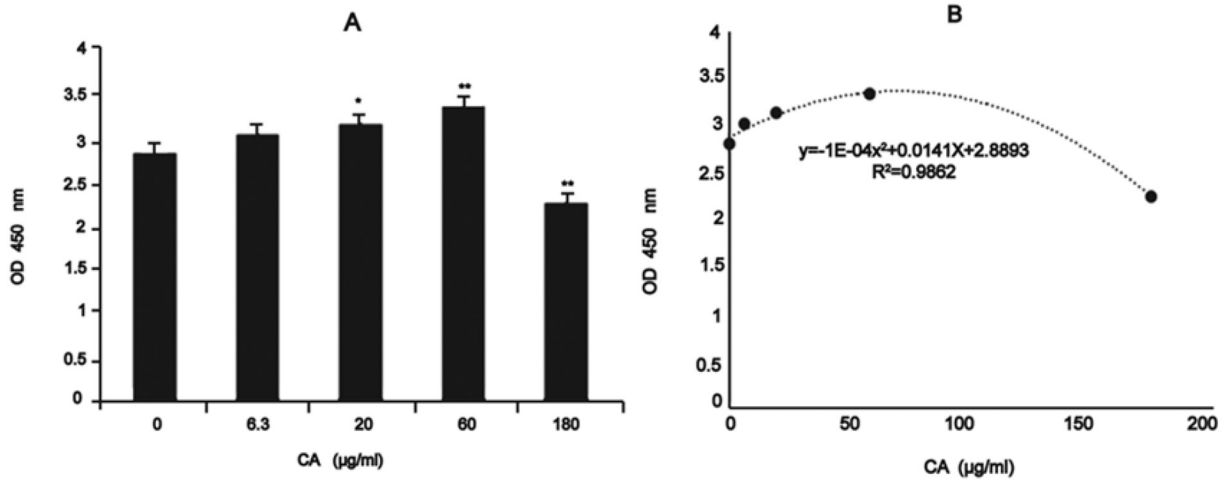

Fig. 1. The effect of cichoric acid on the proliferation of PBMCs in yaks. 0, 6.3, 20, 60, and 180 μg/ml CA induced PBMCs for 48 h. PBMC proliferation was reflected by OD value (A). This gave us the curve of OD value (B). * indicates significant difference compared with the control group ($P < 0.05$), ** indicates that the difference is extremely significant compared with the control group ($P < 0.01$).

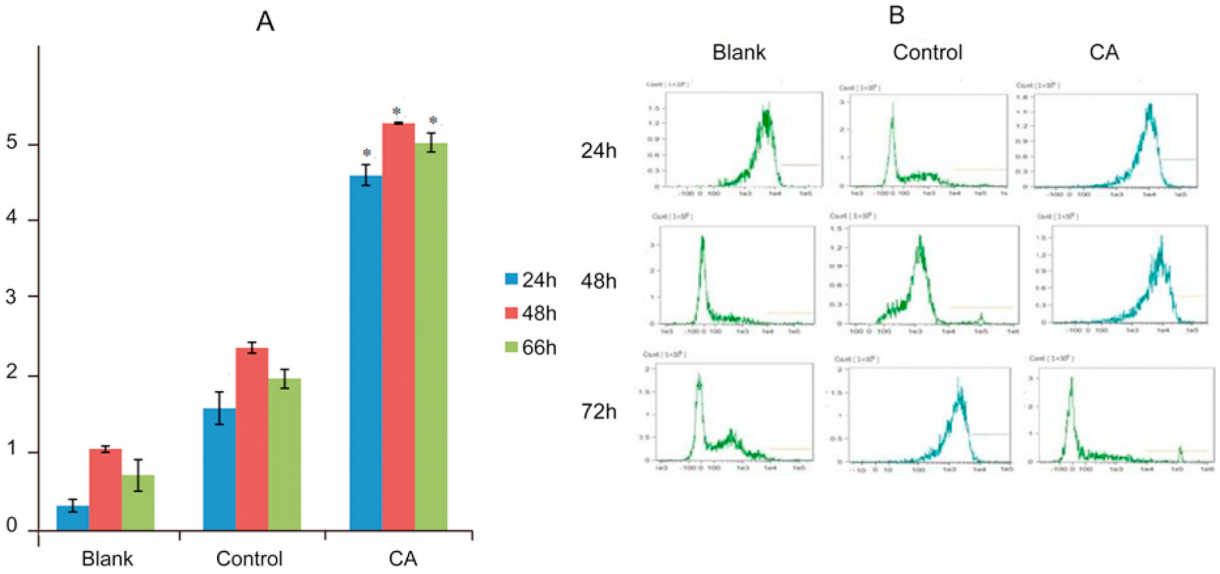

Fig. 2. The effect of cichoric acid on the positive rate of PBMCs in yaks. 60 μg/ml CA induced PBMCs for 24, 48, and 66 h. The data histogram of positive rate was reflected by B (A). The positive rate of blank (PRMI 1640), control (PRMI 1640 + PBMCs), and CA (PRMI 1640 + PBMCs + CA) group were reflected by flow cytometry (B).* indicates significant difference compared with the control group (A; $P < 0.05$).

Subsequently, 20 μl of the mother solution was absorbed and added to the culture medium until 10 ml was reached. The CA concentration was 180 μg/ml, which was initially uniform and then serially diluted to 60, 20, and 6.3 μg/ml.

PBMCs were obtained from the jugular vein of the yaks under sterile conditions, placed in heparinized (20 IU/ml) Vacutainer tubes, and centrifuged at 670 × *g* at 4°C for 20 min. PBMCs were isolated through Ficoll density gradient centrifugation. Within 2 h, an anticoagulant was

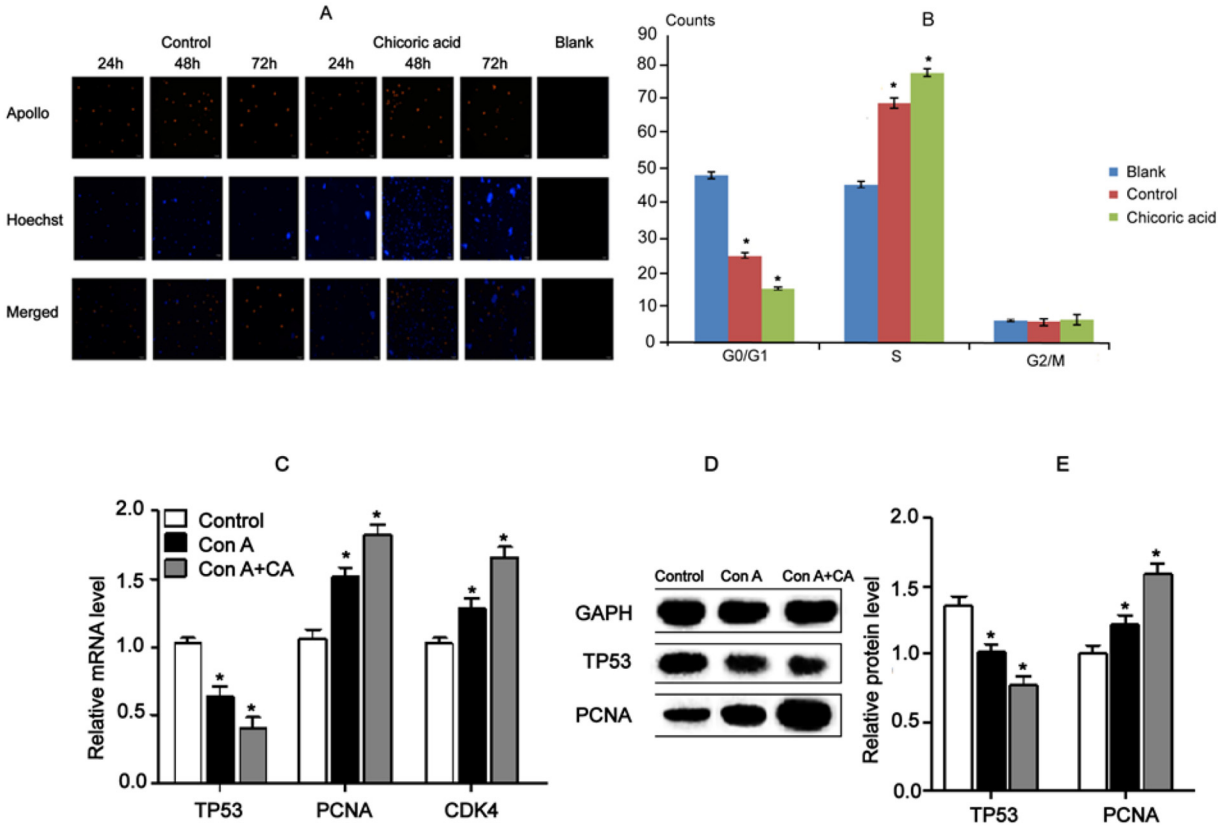

Fig. 3. Further study of cichoric acid on the proliferation of PBMCs in yaks. 60 μg/ml CA induced PBMCs for 24, 48, and 72 h. The observation of blank (PRMI 1640), control (PRMI 1640 + PBMCs), and CA (PRMI 1640 + PBMCs + 60 μg/ml CA) group was made by fluorescence staining (A). PBMCs were induced by 60 μg/ml CA for 48 h. The cell cycle distribution of yak PBMCs was detected by flow cytometry (B). Then the mRNA levels of TP53, PCNA, and CDK4 (C, D) and the protein levels of TP53 and PCNA (E) were measured. CA could significantly promote PCNA and CDK4 expression and arrest TP53 expression at RNA ($P < 0.05$; C) and protein levels ($P < 0.05$; D) to accelerate PBMCs proliferation. *indicates significant difference compared with the control group ($P < 0.05$).

carefully applied to the top of the bovine lymphocyte-separating medium and centrifuged horizontally at 450 × *g* for 45 min. A mononuclear cell band was extracted and washed with the serum-free RPMI 1640 culture medium until the suspension was clear. PBMCs were counted using a cell counting slide and resuspended at a final concentration of 1×10^6 cells/ml in the RPMI 1640 culture medium supplemented with 10% FBS.

2.4. Proliferation assay

PBMCs proliferation assays were performed in triplicates by adding 100 μl of cell suspension into 96-well U-bottom plates. The PBMCs were activated with 100 μl of Con A at a final concentration of 2.5 μg/ml. The PBMCs stimulated with Con A were treated with increasing CA concentrations (final concentrations of 6.3, 20, 60, and 180 μg/ml). Afterward, 300 μl of RPMI 1640 medium was used as a blank control. Each experiment was repeated three times. The plates were incubated at 37°C under 5% CO_2 in a humidified incubator for 48 h. Cell proliferation was evaluated using a CCK-8 kit, and cell viability was determined with EdU in accordance with the manufacturer's instructions. The plates were read using a SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale, CA, USA), and the cells were counted after they were scanned under a fluorescence microscope.

2.5. Determination of cytokines in culture supernatants through ELISA

PBMCs were isolated and cultured as described above. Subsequently, 900 μl of the cell suspension was added to a 24-well cell culture plate. The PBMCs were activated with 100 μl of ConA at a final concentration of 2.27 μg/ml and treated with increasing CA concentrations (final concentrations of 6.3, 20, 60, and 180 μg/ml). Afterward, 1.1 ml of RPMI 1640 medium was used as a blank control. Each experiment was repeated four times. The cells were cultured in an incubator at 37°C under 5% CO_2 for 72 h. Cell culture supernatants were harvested and analyzed to determine the presence of cytokines by using commercially available IFN-γ, IL-2, and IL-6 ELISA kits in accordance with the manufacturer's instructions.

2.6. Detection of apoptosis

Apoptosis was evaluated using an annexin V-FITC and PI kit in accordance with the manufacturer's instructions. After being treated for 48 h, 5×10^6 cells were seeded into 96-well plates and cultured with annexin V-FITC and PI at room temperature in the dark for 15 min. Subsequently, the cell suspensions were immediately examined through flow cytometry.

2.7. 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 BSA (the binding reaction of caffeic acid with bovine serum albumin). Equal amounts of protein samples were boiled for 5 min, resolved through 12% SDS-polyacrylamide gel electrophoresis (PAGE), and transferred onto PVDF membranes. The membranes were blocked with 5% nonfat dry milk and incubated with primary antibodies at 4°C for 2 h. The membranes were then incubated with secondary antibodies for 1 h. Protein bands were visualized with the Bio-Rad Chemi Doc XRS + program.

2.8. Real-time PCR

Total RNA was extracted from the PBMCs by using a Trizol reagent, and the RNA concentration was determined using Nanodrop 3000. An M-MLV reverse transcriptase kit was used to reverse transcribe cDNA in accordance with the manufacturer's instructions. cDNA templates were identified using an AceQ qPCR SYBR Green Master Mix. The PCR primers used were as follows: Caspase3, CAGACAGTGGTGCTGAGGATG AC (forward) and TCGAGCCTGTGAGCGTACTTATTC (reverse); Bax, GGCTGGACATTGGACTTCCTTCG (forward) and ATGGTGAGCGAGGCGG TGAG (reverse); and Bcl2, TGGATGACCGAGTACCTGAACCG (forward) and TGCCTTCAGAGACAGCCAGGAG (reverse). Representative gene expression data were quantitated with β-actin expression by using the following formulas: $2^{-\Delta\Delta Ct}$.

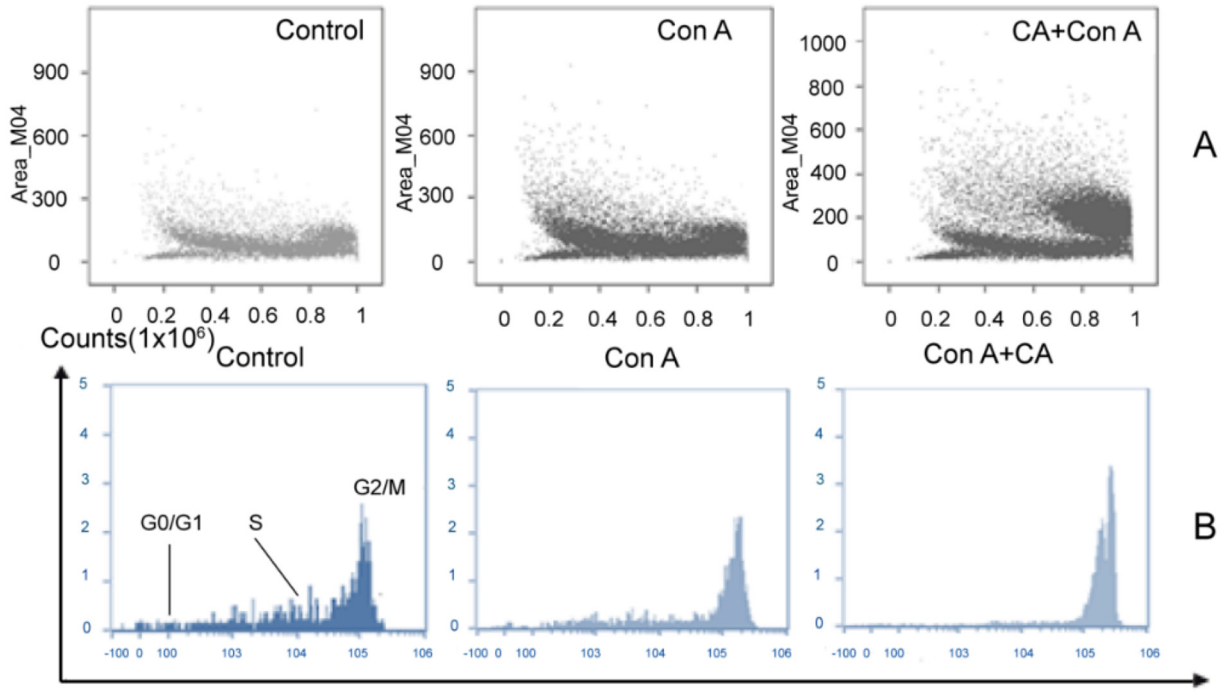

Fig. 4. The flow analysis of cell cycle distribution in yak PBMCs. Cells were induced by 60 μg/ml CA for 48 h. Subsequently, the number of individual nuclear cells (A) and the fluorescence intensity and distribution of DNA content (B) were reflected by flow cytometry.

2.9. RNA-Seq analysis

After the cells were treated with CA for 48 h, they were harvested and their total RNA was extracted.

Raw reads were filtered before data analysis. Clean reads were mapped to the reference sequences with no more than two mistakes. Uniquely mapped data were retained for further analysis. In our study, we defined the high expression gene group as the genes whose reads per kb per million reads (RPKM) values ranged from 10 to 100, the middle expression gene group as the genes whose RPKM values ranged from 1 to 10, the low expression gene group as the genes whose RPKM values ranged from 0 to 1, and the silent group as the genes whose RPKM values were 0, but the zero expression gene in both the experimental (TE) and control (CK) groups were not in the silent gene group. We used FDR ≤ 0.001, P-value ≤ 0.05, and an absolute value of log2 ratio ≥ 1 as the threshold to judge the significance of the difference in gene expression. The biological processes and functions associated with the identified differentially expressed genes (DEGs) were investigated using DAVID (Database or Annotation, Visualization and Integrated Discovery).

2.10. Statistical analysis

The results of each experiment were repeated at least in triplicate independent experiments and expressed as mean ± standard error. Statistical significance was analyzed through one-way ANOVA and LSD post-hoc test. All statistical analyses were performed with SPSS 19.0 (IBM, Armonk, NY, USA), and T-test was used to calculate significant differences, which were represented with asterisks (*$P < 0.05$).

3. Results

3.1. CA concentration screening

Different CA concentrations could promote PBMCs proliferation, and the extent of this effect was related to CA concentration (Fig. 1). The PBMCs proliferation, performed by CCK-8, in the groups treated with 20 μg/ml ($P < 0.05$) and 60 μg/ml ($P < 0.01$) CA significantly increased compared with that in the control group. The group treated with 6.3 μg/ml CA showed no significant difference in PBMCs proliferation ($P > 0.05$). However, 180 μg/ml CA significantly inhibited cell proliferation ($P < 0.01$).

3.2. Effect of CA on PBMCs proliferation

Based on Section 3.1, further study was performed to detect the effect of CA on PBMCs proliferation and cell cycles. The positive rate and period distribution of PBMCs were evaluated by flow cytometry. It was found that the positive rate of PBMCs increased significantly (P <

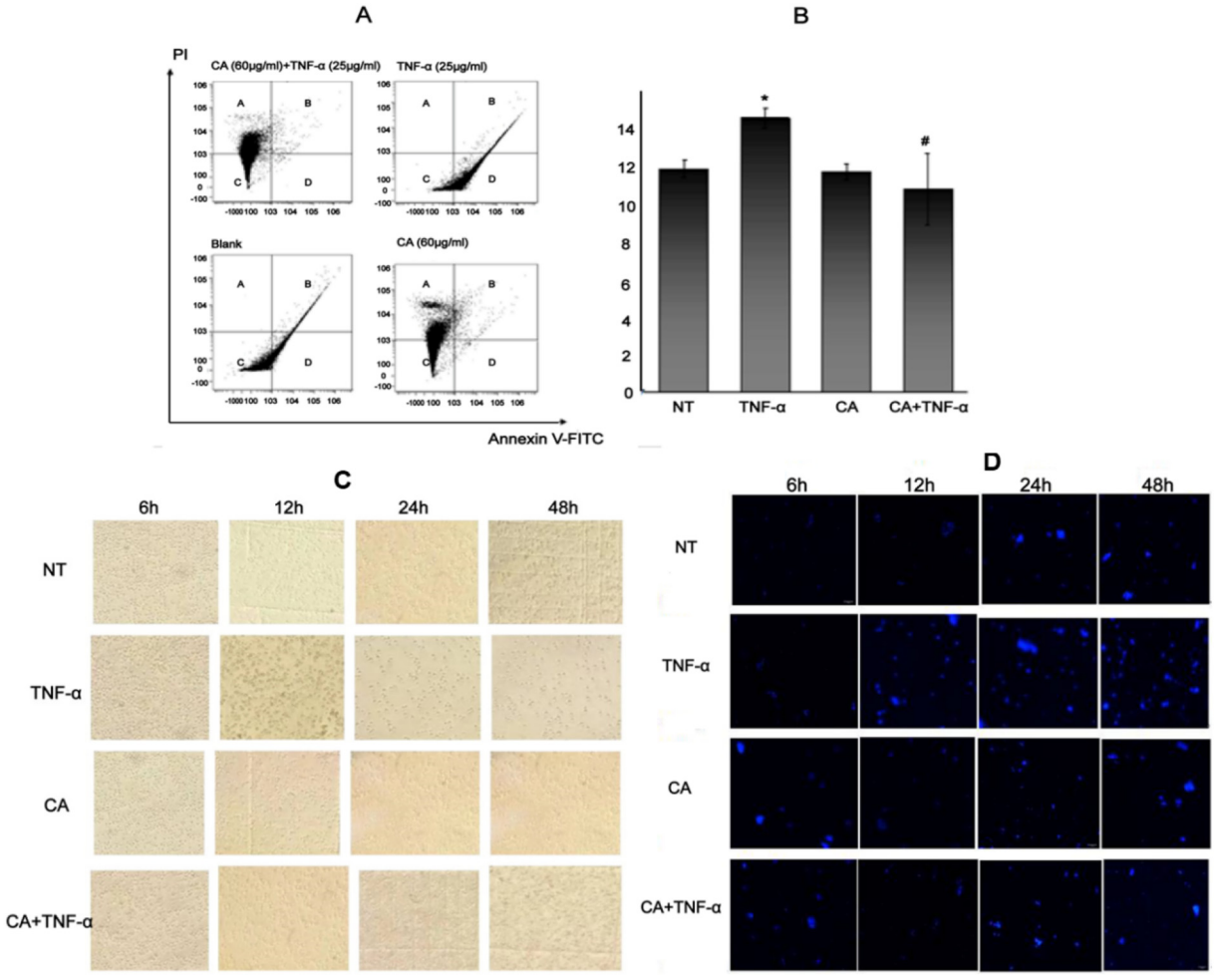

Fig. 5. The effect of CA on apoptosis rate of TNF-α induced PBMCs in yaks. Cells induced by TNF-α, 60 μg/ml CA, and 60 μg/ml CA + TNF-α for 48 h. The rate was performed by flow cytometry (A, B). A represents naked nuclei, B represents late apoptotic or necrotic cells, C represents living cells, and D represents early stage cells of apoptosis. Cells induced by TNF-α, 60 μg/ml CA, and 60 μg/ml CA + TNF-α for 6, 12, 24, and 48 h. Morphological changes of CA to yak PBMCs was detected using an inverted fluorescence microscope (C). Further, the fluorescence microscope observation of CA to yak PBMCs was detected by inverted fluorescence microscope (D). * indicates significant difference compared with the control group ($P < 0.05$), ** indicates that the difference is extremely significant compared with the control group ($P < 0.01$), # indicates significant difference compared with the TNF-α group ($P < 0.05$), ## indicates that the difference is extremely significant compared with the TNF-α group ($P < 0.01$),.

0.05) after they were treated with 60 μg/ml CA for 24 and 72 h and showed an extremely significant difference ($P < 0.01$) for 48 h (Fig. 2; Fig. 3A). This result indicated that CA could increase the positive rate of PBMCs in grazing yaks.

TP53 (MIM #191170) is a tumor suppressor gene and is the most frequently mutated gene in human cancer [29,30]. Low-expression of TP53 preserves the homoeostasis of the cell cycle and cell death [30]. Meanwhile, proliferating cell nuclear antigen (PCNA) lies at the center of the faithful duplication of eukaryotic genomes [31] and acts as a central coordinator of DNA transactions for cell cycle regulation [32]. Additionally, it has been proved that cyclin-dependent kinase 4 (CDK4) plays an important role in the G1/S transition of the cell cycle [33]. According to the experiment, we found that cells in the blank group were mainly in the G0/G1 phase (Fig. 3B). The control and experimental groups showed a significantly reduced G0/G1 phase ($P < 0.05$) and a significantly increased S phase ($P < 0.05$) compared with those in the blank group. Thus, no significant difference was observed in the G2/M phase ($P > 0.05$). CA could significantly promote PCNA and CDK4 expression and arrest TP53 expression at RNA ($P < 0.05$; Fig. 3C) and protein levels ($P < 0.05$; Fig. 3D) to accelerate PBMCs proliferation.

Con A is a plant lectin with strong mitogenic effects on PBMCs in mice and humans. It was the first lectin to be commercially available and has been used to study the regulation of various immune cells [34]. To check the effect of CA on PBMCs mitosis, cell cycle was performed by flow cytometry. It was suggested that CA could effectively furtnerance PBMCs karyomitosis (Fig. 4).

3.3. Effect of CA on apoptosis induced by TNF-α in PBMCs

TNF-α is a pleiotropic cytokine involved in cell proliferation, death, and inflammation through binding to specific receptors on cell membranes [35] and can induce apoptosis [36]. In this study, the apoptotic rate and spherical appearance of PBMCs induced by TNF-α were examined. It was showed that cells treated with TNF-α were evidently enhanced by 1.23 times at 48 h compared with that of the cells in the control group ($P < 0.05$). By contrast, the apoptotic rate of the cells treated with CA + TNF-α remarkably decreased by 1.35 times compared with that of the TNF-α group ($P < 0.05$; Fig. 5A, Fig. 5B). In comparison with the other groups, the number of the cells treated with CA significantly decreased. Their spherical appearance markedly changed to an irregular one. The size of the cells also decreased, and they clustered as observed under an inverted microscope with 10× magnification (Fig. 5C). DNA conjugated with Hoechst33258 and DAPI could permeate a complete cell membrane, bind to a duplex DNA, and present a well-distributed blue fluorescence. The blue fluorescence of apoptotic cells was enhanced. Apoptotic bodies resembled a large crescent or wheel-like mass in the nucleus with prolonged incubation. The cells in the control group were stained normally. The cells and the nuclear chromatin in the TNF-α group contracted, and the nucleus locally showed an intense blue light (Fig. 5D).

Many genes are involved in apoptosis modulation, including caspase-3 [37], bcl-2, and bax [38]. Caspase-3, a convergence of the

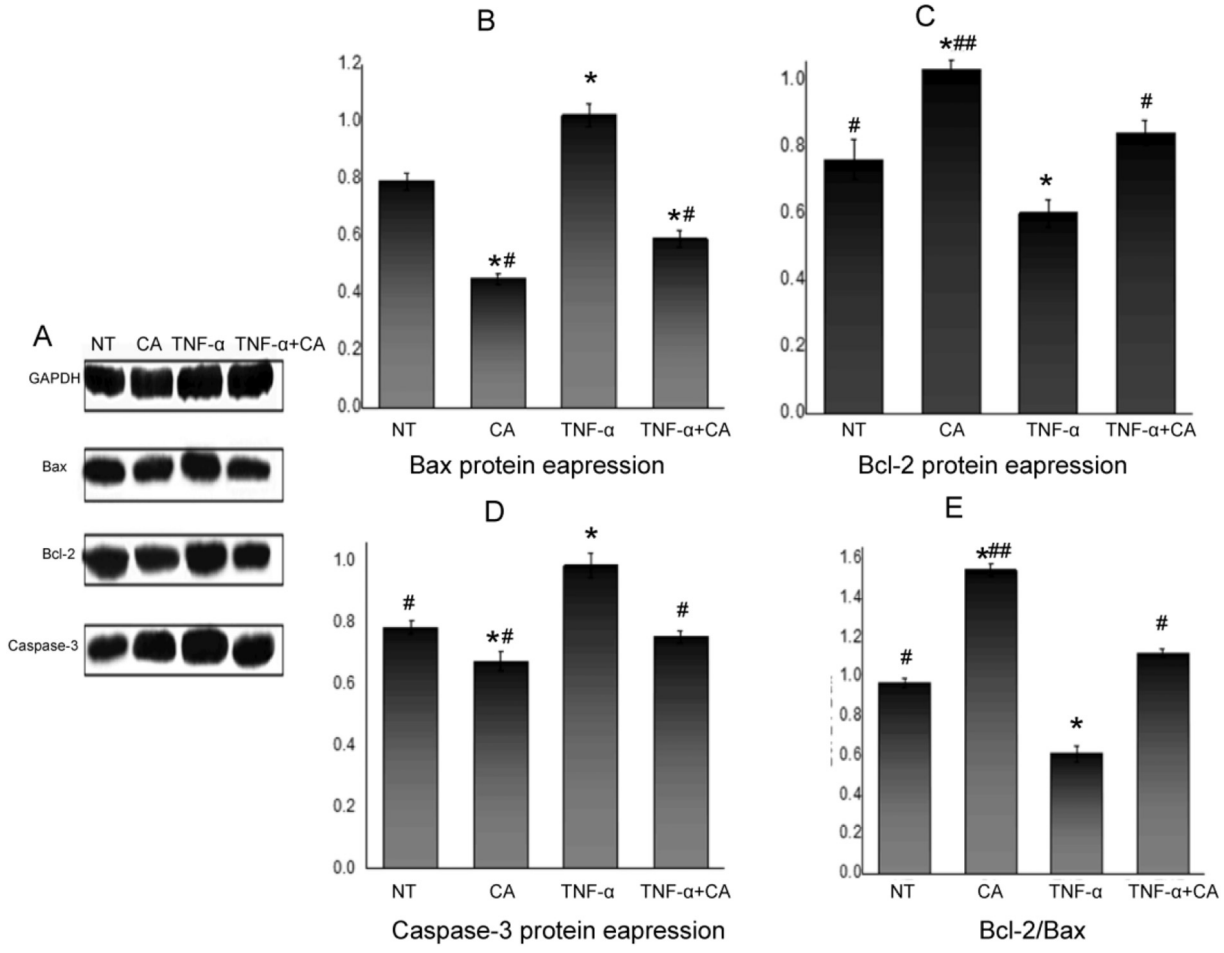

Fig. 6. Expression of TNF-α induced apoptosis-related factor protein in yak PBMCs by CA. Cells induced by TNF-α, 60 μg/ml CA, and 60 μg/ml CA + TNF-α for 48 h. The protein expression of Bax, Bcl-2, and Caspase-3 and the ratio of Bcl-2 and Bax were detected (A). Bax protein expression was significantly attenuated in the CA and CA + TNF-α groups but not in the TNF-α group compared with those in the control group ($P < 0.05$; B). Caspase-3 was extremely arrested in the CA + TNF-α group (D). The Bax expression was inhibited by CA + TNF-α compared with that in the TNF-α group ($P < 0.05$; B). By contrast, the Bcl-2 expression was dramatically increased by the ($P < 0.05$; C). The Bax and caspase-3 expression levels were remarkably downregulated by CA ($P < 0.05$; B, D), whereas the Bcl-2 (C) and Bcl-2/Bax expression levels were extremely upregulated by CA ($P < 0.01$; E).

intrinsic and extrinsic apoptotic pathways, is the main executioner of apoptosis [39]. Futher, a widely accepted model postulates that Bax can promote apoptosis, and that the functional effect of Bcl-2 related proteins is to form competing heterodimers with Bax [40]; however, Bcl-2 and Bax are both able to regulate apoptosis independently [41]. To explore the effect of CA on PBMCs apoptosis, we performed further study. The results showed that the Bax protein expression was significantly attenuated in the CA and CA + TNF-α groups but not in the TNF-α group, in comparison with those in the control group ($P < 0.05$; Fig. 6B). Caspase-3 was extremely arrested in the CA + TNF-α group (Fig. 6D).The Bax expression was inhibited in the CA + TNF-α group compared with that in the TNF-α group ($P < 0.05$; Fig. 6B). By contrast, the Bcl-2 expression was dramatically increased in the former ($P < 0.05$; Fig. 6C). The Bax and caspase-3 expression levels were remarkably downregulated by CA ($P < 0.05$; Fig. 6B, Fig. 6D), whereas the Bcl-2 (Fig. 6C) and Bcl-2/Bax expression levels were extremely upregulated by CA ($P < 0.01$; Fig. 6E).

The RNA expression levels (Fig. 7) of Bax and Caspase-3 in the TNF-α group were significantly promoted ($P < 0.05$) compared with those in the control group. Bcl-2 in the TNF-α group was arrested compared with that in the control group ($P < 0.01$). CA clearly inhibited the Bax expression ($P < 0.05$). Compared with that of the TNF-α group, the expression levels of Bcl-2 ($P < 0.05$) and Bcl-2/Bax ($P < 0.01$) remarkably increased, the expression levels of Bax and Caspase-3 were downregulated by CA + TNF-a ($P < 0.05$), and the RNA expression levels of Bax and Bcl-2 were extremely reduced by CA, whereas the Bcl-2 and Bcl-2/Bax expression levels were extremely enhanced by CA ($P < 0.01$). It was found that RNA expression may led to the final protein content.

3.4. Global analysis of transcriptome for two groups

To explore the further effect of CA on PBMCs, transcriptome analysis for the control group (CK) and CA group (TE) was based on RNA-seq

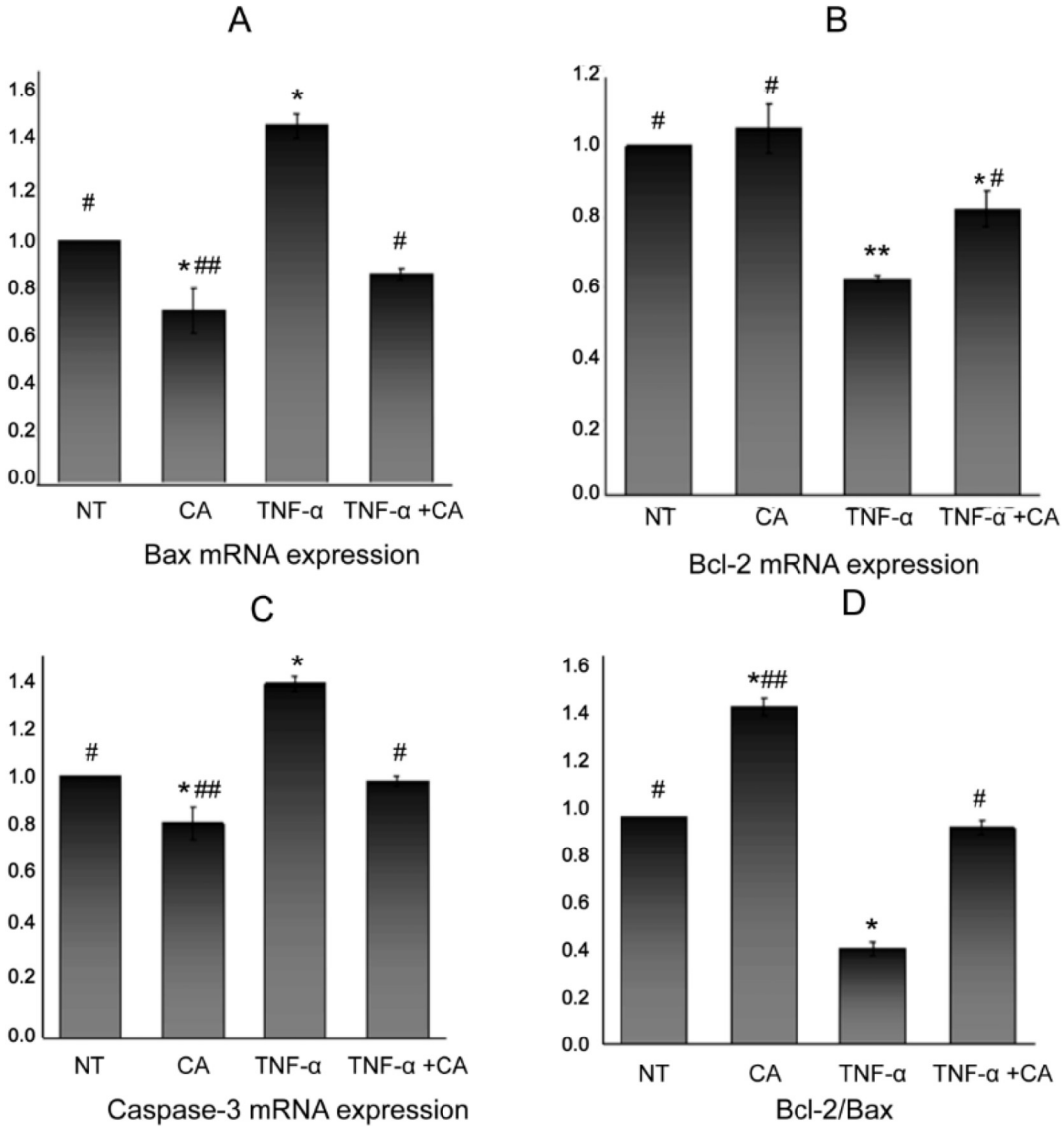

Fig. 7. Effect of CA on mRNA expression of PBMC apoptosis-related factors in yaks induced by TNF-α. Cells induced by TNF-α, 60 μg/ml CA, and 60 μg/ml CA + TNF-α for 48 h. The RNA expression of Caspase-3, Bax, and Bcl-2 and the ratio of Bcl-2 and Bax were measured. The RNA expression levels of Bax and Caspase-3 in the TNF-α group were significantly promoted ($P < 0.05$; A, C) compared with those in the control group. Bcl-2 in the TNF-α group was arrested compared with that in the control group ($P < 0.01$; B). CA clearly inhibited Bax expression ($P < 0.05$; A). Compared with that of the TNF-α group, the expression levels of Bcl-2 ($P < 0.05$; B) and Bcl-2/Bax ($P < 0.01$; D) were remarkably increased and the expression levels of Bax and Caspase-3 were downregulated by CA + TNF-a ($P < 0.05$; A, C); and the RNA expression levels of Bax and Bcl-2 were extremely reduced and the Bcl-2 and Bcl-2/Bax expression levels were extremely enhanced by CA ($P < 0.01$; B, D).

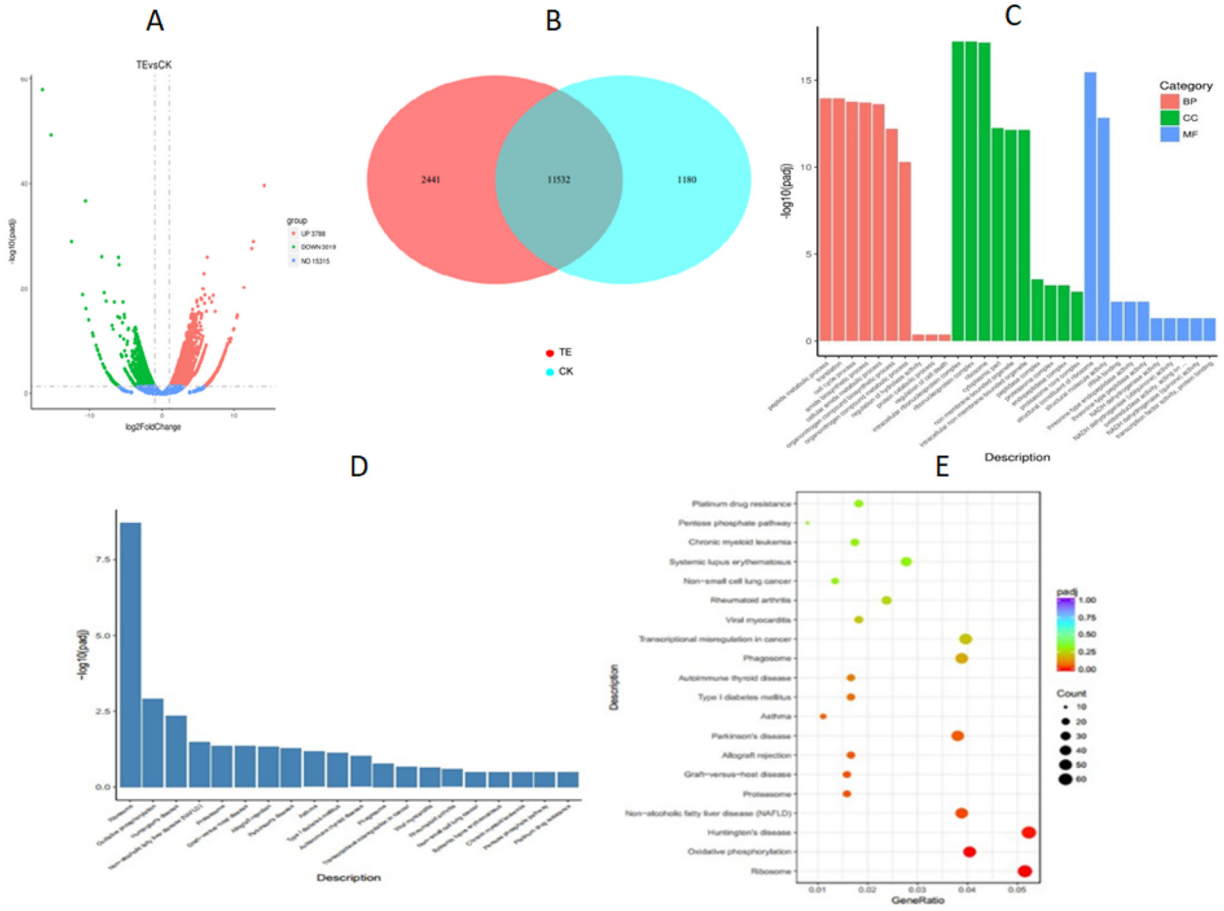

Fig. 8. RNA-seq data of yaks. Differentially expressed gene volcano map included the red dot in the figure indicated that the TE group (CA group, 60 μg/ml CA + Con A) was significantly upregulated compared to the CK group (control group, Con A), the green dot indicated that the TE group had a significantly downregulated gene compared to the CK group, and the blue dot indicated no significant difference (A). In the screening process of differentially expressed genes, $\log_2$ Fold Change≥2 and false discovery rate FDR conditions for differentially expressed genes in burdock-treated yak peripheral blood mononuclear cells. Differentially expressed genes was diagramen into wayne analysis (B). GO function analysis and enrichment analysis was performed based on DEGs (C, D). The first 20 metabolic pathways of yaks were reflected by TE and CK differentially expressed genes' KEGG enrichment analysis (E).

data. We compared the transcriptomes of the two groups, measured the expression rate changes, and identified DEGs in CK and TE. Finally, we obtained 22,122 DEGs in CK and TE, of which 3788 were upregulated, 3019 were downregulated, and 11,532 showed no remarkable difference (Fig. 8A). Further analysis was carried out, and 11,532 DEGs were both upregulated and downregulated in the two groups (Fig. 8B).

3.5. Gene ontology and metabolic pathways analysis

Gene Ontology and metabolic pathways analysis was performed to investigate the biological processes and pathways associated with DEGs. DAVID analysis was performed by running queries for each DEG against the DAVID database. The results of the DAVID analysis showed that translation, cell cycle, ribosome, cytoplasmic part, proteasome complex, and ribosome structural constituent were significantly enriched in both TE and CK with GO entries (Fig. 8C). Ribosome, oxidative phosphorylation, proteasome, and non-alcoholic fatty liver disease were evidently enriched in the TE and CK of pathways by KEGG (Fig. 8D). The results of the first 20 KEGG metabolic pathways of TE and CK showed that ribosome, phagosome, and proteasome were enriched in a large number of genes with a high reliability. These results indicated that the differentially expressed genes were remarkably enriched in proliferation and immunity with CA (Fig. 8E).

To further verify the effect of CA on PBMCs proliferation, proliferation-associated factors analysis was performed by RNA-seq data. BTB, S, RRM_1, and zf-C2H2 were expressed after the cells were treated with CA, indicating that PBMCs proliferation could be regulated by CA through related transcription factors (Fig. 9A). CCNF, CCNT1, CCND1, CCND3, CDK6, CDK1, CDKN1A, CDKN2A, and NPDC1 were important for the PBMCs proliferation pathway (Fig. 9B). To verify the effect of CA on PBMC proliferation, we detected the RNA expression levels of TP53, CCNF, CCND1, CDKN1A, and CDK4D. The expression levels of CCNF, CCND1, and CDK4 increased ($P < 0.05$), whereas the expression levels of TP53 and CDKN1A decreased ($P < 0.05$; Fig. 9C). These results indicated that CA was responsible for PBMCs proliferation.

3.6. Validation of CA-regulated immune gene interaction and expression regulation in PBMCs from grazing yaks

In response to the call of prevention over treatment, it is necessary to detect the immune system of yaks. Interleukin 2 (IL-2) exerts both stimulatory and regulatory functions in the immune system and is central to immune homeostasis [42]. Interleukin 6 (IL-6), which is promptly and transiently produced in response to infections and tissue injuries, contributes to host defense through the stimulation of acute phase responses, hematopoiesis, and immune reactions by transcriptional and posttranscriptional mechanisms and has a pathological effect on chronic inflammation and autoimmunity [43]. Interferon-gamma (IFN-γ), a key player in driving cellular immunity, is a potent immunomodulatory cytokine [44]. It can induce apoptosis and regulate anti-proliferation [45]. We studied the effect of CA on the

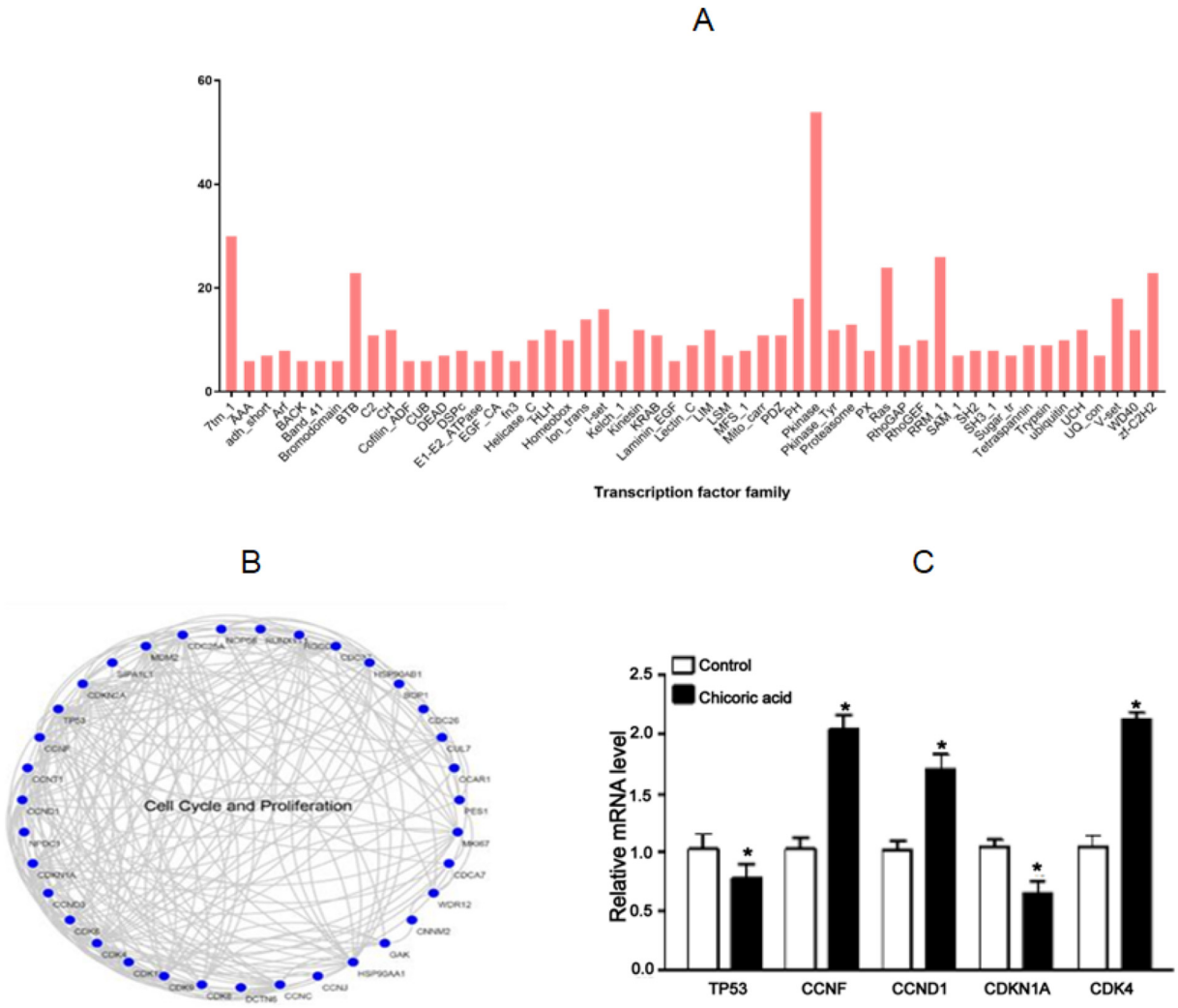

Fig. 9. Cytotoxic acid-regulated proliferation-related transcription factors. FoxO, E2F, CREB, OLR1, and PPARγ expression levels. Note (A): The values represent the mean ± standard error of 3 samples. Verification of expression and regulation of proliferation of PBMCs in yaks after CA treatment was based on RNA-seq (B). PBMCs proliferation genes mRNA levels of TP53, CCNF, CCND1, CDKN1A, and CDK4D in yaks after CA treatment were diagramed. The expression levels of CCNF, CCND1, and CDK4 increased ($P < 0.05$), whereas the expression levels of TP53 and CDKN1A decreased ($P < 0.05$; C). The value represented 3 Mean ± standard error of one sample. *P Note: Each dot in the interaction network represents a gene, and the line indicates the interaction between genes. The more the proliferating genes in the interaction, the more important the role played by the cell proliferation process.

immune system of yaks and we found that different CA concentrations could significantly increase the induction effects of Con A on IL-2, INF-γ, and IL-6 secretion compared with those of the control ($P < 0.01$, Fig. 10). The best effect was observed in the group treated with 60 μg/ml CA ($P < 0.05$). IL-6 was significantly increased by 20 ($P < 0.01$) and 60 ($P < 0.001$) μg/ml CA compared with that in the control group. The best effect was observed in the cells treated with 60 μg/ml CA ($P < 0.001$). IFN-γ was significantly increased by 20 and 60 μg/ml CA compared with that of the control group ($P < 0.05$). It shows that the most effective CA doses obtained in terms of their optimal effect on IFN-γ, IL-2, and IL-6 secretion were 70.5 μg/ml, 87.6 μg/ml, 106.9 μg/ml, and 92.7 μg/ml.

In addition, as detected by transcriptome sequencing, IL-2, IL-6, IL5, IL-17A, CD19, and STAT3 had vital functions in the CA-regulated immune pathway of PBMCs from grazing yaks (Fig. 11A). We detected the mRNA expression levels of IL-2, IL-6, AIF2, and CD97 to test the effect of CA on the immunity of PBMCs. The results showed that CA could specifically upregulate IL-2, IL-6, AIF2, and CD97 compared with that of the control ($P < 0.05$). Thus, CA could promote the immune function of PBMCs from grazing yaks (Fig. 11B).

4. Discussion

CA from *E. purpurea* is a daily nutraceutical that can be used to treat and prevent diseases by activating immune cells to improve the autoimmune system [29]. CA can stimulate lymphocyte proliferation and release inflammatory factors [22,46]. The proliferation and cyclic distribution of T cells play an important role in immune regulation and inflammation [47]. Therefore, studies should be performed to explore drugs that can inhibit T cell activation and proliferation and potently control harmful immunity. Cell cycle changes directly affect hyperplasia. PBMCs' proliferative response is a reflection of overall immunity. The effects on the immune system and inflammatory responses are usually detected in PBMCs, and T cell proliferation can be mainly induced by Con A [48]. Therefore, in this study, PBMCs were used to evaluate the role of immunopotentiators. Different concentrations of CA combined with Con A have various influences based on CA activity. This study revealed that 180 μg/ml *E. purpurea* extract-CA in combination with Con A had an inhibitory effect on PBMCs proliferation. However, at 20 60 μg/ml and 60 μg/ml, CA promoted PBMCs proliferation. In comparison with the control cells, the cells treated with 60 μg/ml CA

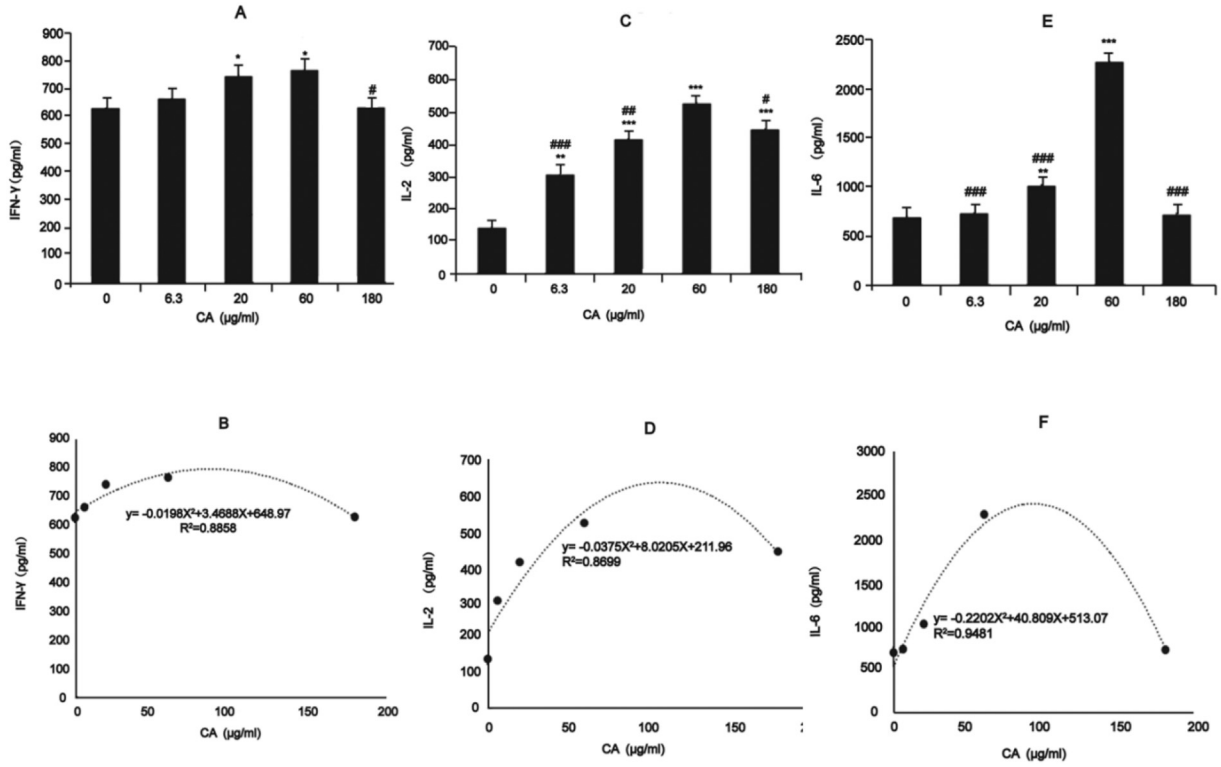

Fig. 10. Effect of cichoric acid on cytokines secretion. 0, 6.3, 20, 60, and 180 μg/ml CA induced PBMCs for 48 h. IFN-γ, IL-2, and IL-6 secretion of PBMCs in yak were detected by ELISA. Cytokine secretion of PBMCs was reflected by OD value (IFN-γ (A); IL-2 (C); IL-6 (E)). This gave us the curve of OD value (IFN-γ (B); IL-2 (D); IL-6 (F)). * indicates significant difference compared with the control group ($P < 0.05$). * *indicates significant difference compared with the control group ($P < 0.01$), *** indicates that the difference is extremely significant compared with the control group ($P < 0.001$), # indicates significant difference compared with the 60 μg/ml group ($P < 0.05$), ## indicates that the difference is extremely significant compared with the 60 μg/ml group ($P < 0.01$), ### indicates that the difference is extremely significant compared with the 60 μg/ml group ($P < 0.01$).

showed a significantly different promoting effect. PCNA, CDK4, and PCNA protein expression levels increased, whereas TP53 and its protein expression were inhibited, suggesting that CA could accelerate PBMC proliferation in grazing yaks. These results indicated that CA could promote PBMC proliferation, which could be beneficial to the increase in PBMCs activity and the improvement of cellular immunity and anti-infection activity. Consequently, the immune function of yaks could be improved. In addition, the distribution of cell cycle phases was measured with PI staining, and the results showed that the number of cells in the G0/G1 phase decreased, but significantly increased in the S phase ($P < 0.05$) after the cells were treated with CA + Con A for 48 h. This finding confirmed that CA + Con A could advance the cell cycle from G0/G1 to S and G2/M. CA might enhance the DNA synthesis of PBMCs.

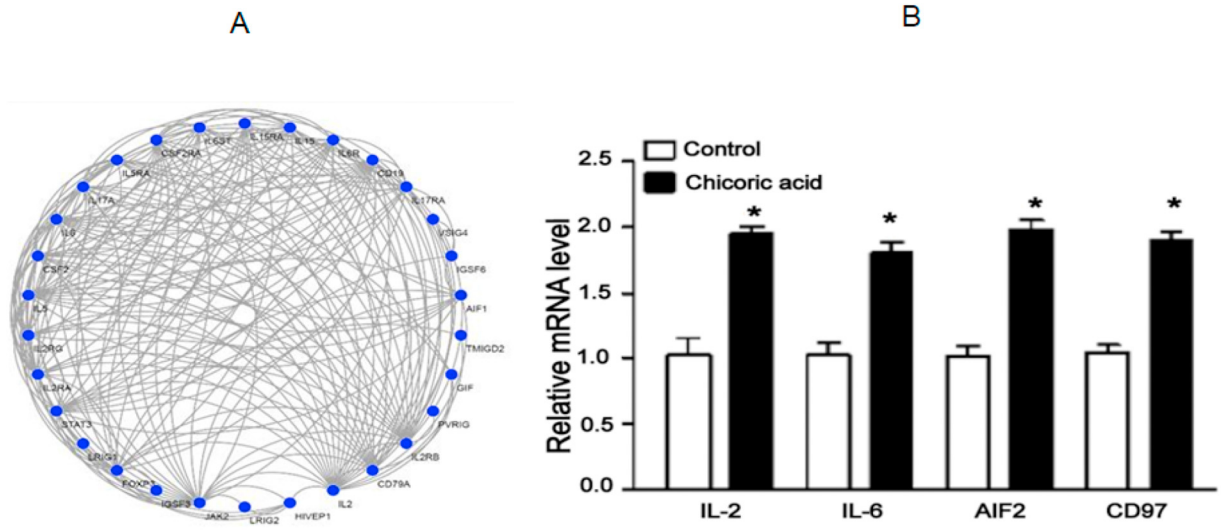

Fig. 11. Immune gene interaction map of PBMCs in yaks after CA treatment. Immune gene interaction map was based on RNA-seq (A). mRNA levels of IL-2, IL-6, AIF2, and CD97 in simian PBMCs after CA treatment represented the mean ± standard error of 3 samples (B). CA specifically upregulated IL-2, IL-6, AIF2, and CD97 compared with that of the control ($P < 0.05$; B). *$P < 0.05$ compared to the control group.

Cytokines, the main signaling molecules of the immune system, can be excreted from immune cells and are implicated in immune regulation [49,50,51]. They are secreted by inflammatory T cells, and the immune system produces various functionally diverse cytokines to maintain homeostasis [52]. This study examined the secretion of IL-2, IFN-γ, and IL-6 by yak PBMCs. The results revealed that Con A-induced IL-2 secretion was significantly promoted by different CA concentrations. In particular, 60 μg/ml CA significantly induced the secretion of IL-2, IL-6, and IFN-γ and showed the most significant effect, indicating that *Echinacea* extract could increase not only the contents of IL-2 and IFN-γ produced by lymphocytes in yak but also the content of IL-6 produced by monocytes/macrophages. Thus, CA addition could increase IL-2, IFN-γ, and IL-6 contents in PBMCs from yaks, enhance the humoral immune function, and promote PBMCs proliferation. Thus, CA effectively improves the immune function of yaks.

TNF-α from monocytes and macrophages is an extracellular signal protein important for proliferation and apoptosis [53,54]. In this study, PBMCs were strained, and the results demonstrated that the cells treated with TNF-α showed contraction, nuclear chromatin pyknosis, and strong blue fluorescence in the nucleus. These results indicated that TNF-α was a potent inducer of PBMCs apoptosis. Furthermore, the apoptotic characteristics of cells treated with CA were not evident, indicating that CA could inhibit apoptosis. Flow cytometry revealed that the apoptotic rate of the TNF-α group was higher than that of the CA + TNF-α group ($P < 0.05$). In addition, Western blot analysis showed that the mRNA and protein expression levels of Bax and Caspase increased, whereas those of Bcl-2 decreased ($P < 0.05$). The expression levels of Bax and Caspase-3 decreased, while the expression level of Bcl-2 increased ($P < 0.05$) with CA. Therefore, CA could promote the proliferation and apoptosis inhibition of PBMCs in grazing yaks by raising Bcl-2 and decreasing Bax and Casepase-3. Its mechanism of immunity improvement was closely related to the proportion of Bcl-2, Bax, and Bcl-2/Bax.

The CA and non-CA groups of grazing yaks were subjected to second-generation sequencing. The results showed different gene expression profiles and pathways. Of the 6807 differentially expressed genes, 3788 were upregulated and 3019 were downregulated in the treatment group. The effect of CA suggested that genes related to the immune system and the cell cycle may be present. Differential gene function analysis suggested that translation, ribosome, cell cycle, and cytoplasmic parts, which were closely related to the cell cycle, proteasome complex, and oxidative phosphorylation, were significantly enriched in the control and treatment groups. Family proteins, such as BTB, Ras, RRM_1, and zf-C_2H_2 were remarkably increased. CCNF, CCNT1, CCND1, CCND3, CDK6, CDK1, CDKN1A, CDKN2A, and NPDC1 played a major role in PBMCs proliferation. Therefore, CA could regulate the proliferation of PBMC in grazing yaks by regulating proliferation-related transcription factors and genes.

In conclusion, CA is an active factor that can promote cell proliferation and the cell cycle and inhibit PBMC inflammation and apoptosis in grazing yaks.

5. Conclusion

We have studied the proliferation, cytokine induction, and apoptosis induced by CA in PBMCs. It has been identified that CA could increase the cell activity and positive rate and reduce some cytokines secretion of PBMCs. Further, CA could activate or inactivate the key proliferating protein and apoptotic protein to promote proliferation and inhibit apoptosis of PBMCs. Furthermore, Gene Ontology and pathway analysis revealed that DEGs were enriched in cell proliferation and immune function signaling pathways, which showed that the expression level of some transcription factors and genes related to PBMCs proliferation in yaks were significantly activated or inactivated after CA treatment. Therefore, it can be concluded that CA could improve proliferation and inhibit inflammation and apoptosis of PBMCs, and consequently promote the immune function of grazing yaks.

Conflict of interest

No conflicts of interest, financial or otherwise, are declared by the authors.

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