CARPATHIAN JOURNAL OF FOOD SCIENCE AND TECHNOLOGY

journal homepage: http://chimie-biologie.ubm.ro/carpathian_journal/index.html

SIMPLE ANALYSIS OF POTENTIAL IMMUNE REGULATION EFFECT OF CUCURBITACIN E ON PROFESSIONAL ATHLETES ENGAGED IN HIGH INTENSITY TRAINING

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Article history:
Received:
11 February 2016
Accepted in revised form:
19 March 2016

Keywords:
Cucurbitacin E;
Professional athletes;
Immunological regulation;
Peripheral blood mononuclear cells;
Ionomycin;

ABSTRACT

This study was designed to analyze the immune regulation effect and functional mechanism of cucurbitacin E (CucE) taking professional athletes engaged in high intensity training as the main analysis objects, and then to further discuss the potential possibility of CucE to be a kind of immune regulation drug for professional athletes. Phorbol esters and ionomycin were used in this study to activate human peripheral blood mononuclear cells (PBMC) and Jurkat T cells; immunofluorescent staining combined flow cytometry method was adopted for analysis of CD3+ T cell activation antigen expression in human PBMC; quantitative polymerase chain reaction (PCR) method was used to detect the effect of CucE on expression of interleukin-2 (IL-2), tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) mRNA; moreover, immunofluorescence assay was used to observe the nuclear translocation of NF-κB/p65, thus to further discuss whether CucE had regulating effect on the adaptive immunologic function. Results showed that high intensity training could lead to immunosuppression of professional athletes as well as the increase of various kinds of inflammatory cytokines, and CucE could significantly inhibit the expression of cytokines from the mRNA level and protein level through lowering NF-κB signal path, and CucE also had significant regulating effect on adaptive immunity.

1. Introduction

Professional athletes belong to a special group who are of strong physique, and athletes engaged in different sport events have different body shapes. Physiologically, energy consumption of athletes in unit time is large, thus athletes are required to be physically healthy and strong as well as have strong cardio-pulmonary function and anaerobic metabolism ability (Miyagi et al., 2012; Tylee and Walters, 2010). Immunologic function reflects the ability of body to resist diseases, which is an important part of evaluation of human body functions. As a kind of stress stimulation (Jung et al., 2009), long-term high intensity training can have short-term or long-term effect on the overall immunologic function of athletes, such as leading to immunosuppression, decrease of immunologic function of athletes and especially the increase of infection rate of the upper respiratory tract (Dever et al., 2016;

Petrelli, et al., 2016). Therefore, how to relieve sports inflammatory state of athletes is an urgent problem to be solved. In recent years, the anti-inflammatory effect of cucurbitacin E (CucE) has been verified by a large number of experiments. Gursoy et al. (2012) carried out an in vitro cell culture experiment in 2012, which verified that CucE could inhibit the production of nitric oxide (NO) from LPS/INF-γ activated mice macrophage RAW 264.7 and WRL-68 cell; meanwhile, CucE could play an anti-inflammation role through inhibiting the activity of cyclo-oxygenase and active nitrogen. In 2013, Mielgo-Ayuso et al. (2013) carried out an in vivo test and verified that intraperitoneal injection of CucE could effectively relieve the edema of feet of mice induced by carrageenin. In 2014, Ramos et al. (2014) discovered that CucE could inhibit T cells activation and expression of cytokines and had certain regulating effect on adaptive immunologic function.

While strengthening the competitive ability training of athletes and improving their sport performance, it is of great significance to monitor changes of immunologic function of athletes timely to avoid decrease of immunologic function as well as injury and diseases (Bennell et al., 2011; Silva et al., 2013). Through the discussion on regulating effect of CucE on adaptive immunologic function and its possible mechanism, this study mainly aims to explore the potential possibility of CucE to be an anti-inflammatory drug which can inhibit the increase of pro-inflammatory cytokines of athletes (Chew and Ong, 2016), so as to relieve the increase of inflammatory cytokines of athlete to some extent through dietary instruction, which has certain practical significance.

2. Materials and methods

2.1. Reagents and equipment

Reagents used in this study include paraformaldehyde, absolute ethyl alcohol, phorbol esters (PDB), ionomycin (Ion), 2-mercaptoethanol, L-glutamine, et al.; equipment used in this study include a clean bench, a refrigerated centrifuge, a microplate reader, an inverted microscope, an electronic scale, a pipette, et al.

2.2. Detection of activation surface molecules of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were inoculated in a 24-well plate, 1×10^6 cells for each well and the volume was 0.5 ml. Four groups which were blank group, CucE group, PDB+Ion model group and CucE+PDB+Ion drug group were set and cultured for one day in an incubator (37 °C, 5% CO_2). After that, supernatant was collected for cytometric bead array (CBA) (Malpass, et al., 2013).

2.3. Extraction and inverse transcription of RNA

(1) Jurkat cells were collected and added with 1 ml of Trizol; cells were separated and dissolved and then transferred to an eppendorf (EP) tube for 5-6 min of incubation (at about 25 °C), thus to completely separate the ribonucleoprotein complex.

(2) Then 0.2 ml of chloroform/Trizol mixed liquor was added and the obtained solution was vibrated quickly for 10-20 s and had about 3 min of reaction.

(3) The tube was put in a 5 °C environment and centrifuged in 8000 g for 15 min; then the supernatant was transferred to a new EP tube; then 0.5 ml of isopropanol was added and the reaction lasted for 10 min at room temperature.

(4) After the reaction, the tube was

centrifuged in 12000 g for 10 min at 2-8 °C; the supernatant was removed and 1 ml of 75% diethyl pyrocarbonate-ethyl alcohol was added; sediments were washed once and the solution was shaken up.

(5) After that, the tube was centrifuged in 13000 g for 2-3 min at 2-8 °C and the supernatant was removed; sediments were dried for about 8 min; 20 μL of D.D•H_2O was added and vibrated to fully dissolve RNA (Burns et al., 2015).

2.4. Real-time quantitative polymerase chain reaction (Table 1)

Table 1. Real-time quantitative polymerase chain reaction

	Sense	Antisense
IL-2	5'-TGAAGGACGAGGAGTACGAGC-3'	5'-TGCAGGAACGAGTCTCCGT-3'
TNF-α	5'-CGTGGAACTGGCAGAAGAG-3'	5'-TGAGAAGAGGCTGAGACATAGG-3'
IFN-γ	5'-AGATCTGGCACCACACCTTCT-3'	5'-CTTTGATGTCACGCACGATTT-3'
β-Actin	5'-TGGTACCATGTACCCAG-3'	5'-AAGGGTGTAAAACGCAGCTC-3'

2.5. Extraction of nuclear protein

(1) Blank control group, PDB+Ion model group and drug pretreatment group were set in the experiment. Jurkat T cells in logarithmic phase were selected and cell concentration was adjusted. Cells were inoculated in 75 ml cell culture flasks, 5×10^7 cells for each flask.

(2) In the drug pretreatment group, flasks were added with CucE in 0.1 mol/L, 0.3 mol/L and 1 mol/L respectively for 0.5 h of pretreatment; then PDB+Ion was added in PDB model group and CucE+PBD+Ion drug group; flasks in the two groups were cultured in 5% CO_2 at 37 °C for 0 min, 0.5 h, 1 h and 2 h respectively, then the reaction was terminated.

(3) Lysis buffer was added into cells to remove cytoplasm protein and cells were washed by lysis buffer once.

(4) 2×SDS-PAGE loading buffer was used to extract nuclear protein and nuclear protein was split using boiling water bath; after the centrifugation in 16000 g for 20 min, the supernatant was removed and nuclear protein was obtained (Lentz and Shideler, 2016).

2.6. Immunofluorescence analysis

(1) Blank control group, PDB+Ion model group and CucE+PBD+Ion drug pretreatment group were set in the experiment. Jurkat T cells in logarithmic phase were selected and cell concentration was adjusted. Cells were inoculated in culture dishes in 5% CO_2 at 37 °C overnight, 1.5×10^4 cells for each.

(2) CucE+PBD+Ion drug pretreatment group was pretreated using CucE for 60 min and then added with PDB and Ion and cultured in 5% CO_2 at 37 °C for 120 min, then the reaction was terminated; PDB+Ion model group was stimulated using dulbecco's modified eagle medium (DMEM) complete culture containing PDB and Ion for 120 min (Yamamoto, et al., 2013).

(3) Then culture medium was removed and 4% paraformaldehyde was added to cover cells; cells were fixed for 15 min at room temperature, then stationary liquid was removed and cells were washed twice by cold phosphate buffer saline (PBS); then iced methyl alcohol was added for 10 min of transparence at -20 °C.

(4) After that, cells were washed three

times by cold PBS and blocking buffer was added for 60 min of treatment; then the blocking buffer was removed and 500 μmol/L of Hoechst 33342 was added for about 10 min of staining; a fluorescence microscope was used for observation and recording (Masuda et al., 2015).

2.7. Statistical analysis method

Graphpad Prism 5.0 software was used for one-way analysis of variance; Tukey test was adopted for comparison between groups; $p < 0.05$ was considered to have statistical significance and $p < 0.01$ represented that the difference was highly significant.

3. Results and discussions

3.1. Inhibition effect of CucE on expression of PBMC cytokines (Figure 1)

Activated T cells can secrete IL-2, tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ) and other cytokines which play an important role in congenital immunity and adaptive immunity. Figures 1a-c show that in PDB+Ion model group, the specific value of $CD3^+CD69^+/CD3^+$ increased significantly compared with that of $CD3^+CD25^+/CD3^+$ ($p < 0.01$); in the meantime, in the CucE+PDB+Ion drug treatment group, the expression of CD69 and CD25 of T cells activation surface molecules was inhibited significantly in a dose-dependent way. Compared with the blank group, expression of TNF-α in CucE treatment group was improved, and expression level of IL-2, TNF-α and IFN-γ in PDB+Ion group was increased significantly ($p < 0.01$); compared with the activation model group, expression level of IL-2, TNF-α and IFN-γ in PDB+Ion+CucE drug treatment group was significantly inhibited in a dose-dependent way (Amezquita-Garcia et al., 2015; Yousef et al., 2015).

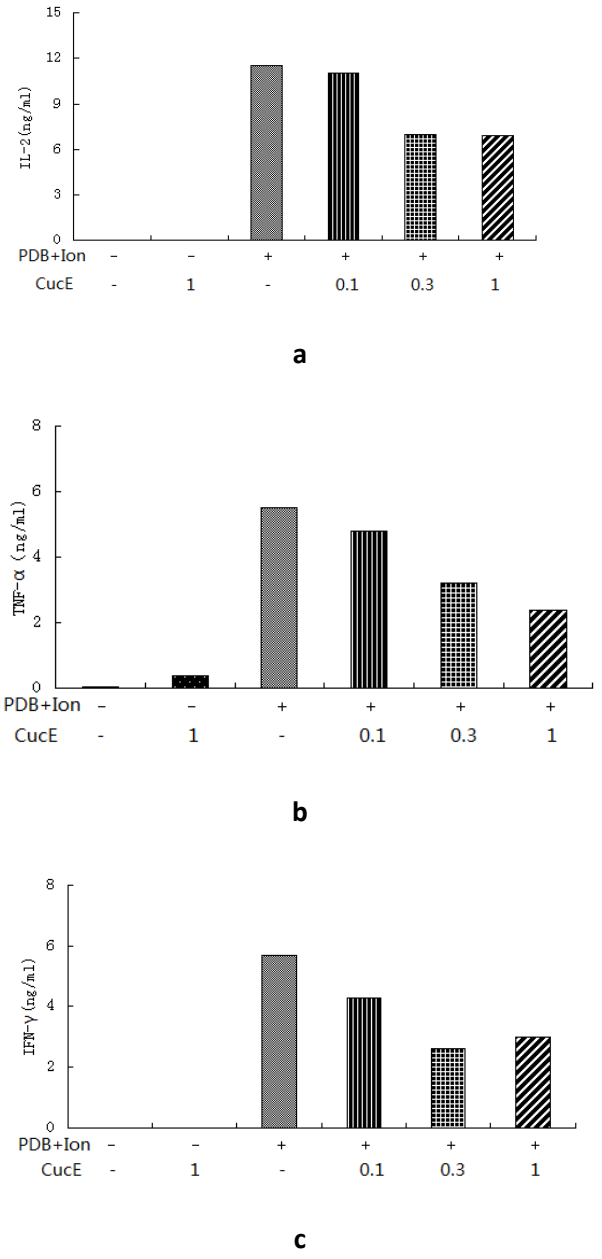


Figure 1. Inhibition effect of CucE on expression of PBMC cytokines (a-c)

3.2. The inhibition effect of CucE on expression of Jurkat T cytokines (Figure 2)

As shown in Figure 2, compared with the blank group, the expression level of IL-2 and IFN-γ after 6 h and 24 h in PDB+Ion model group both increased significantly ($p < 0.01$).

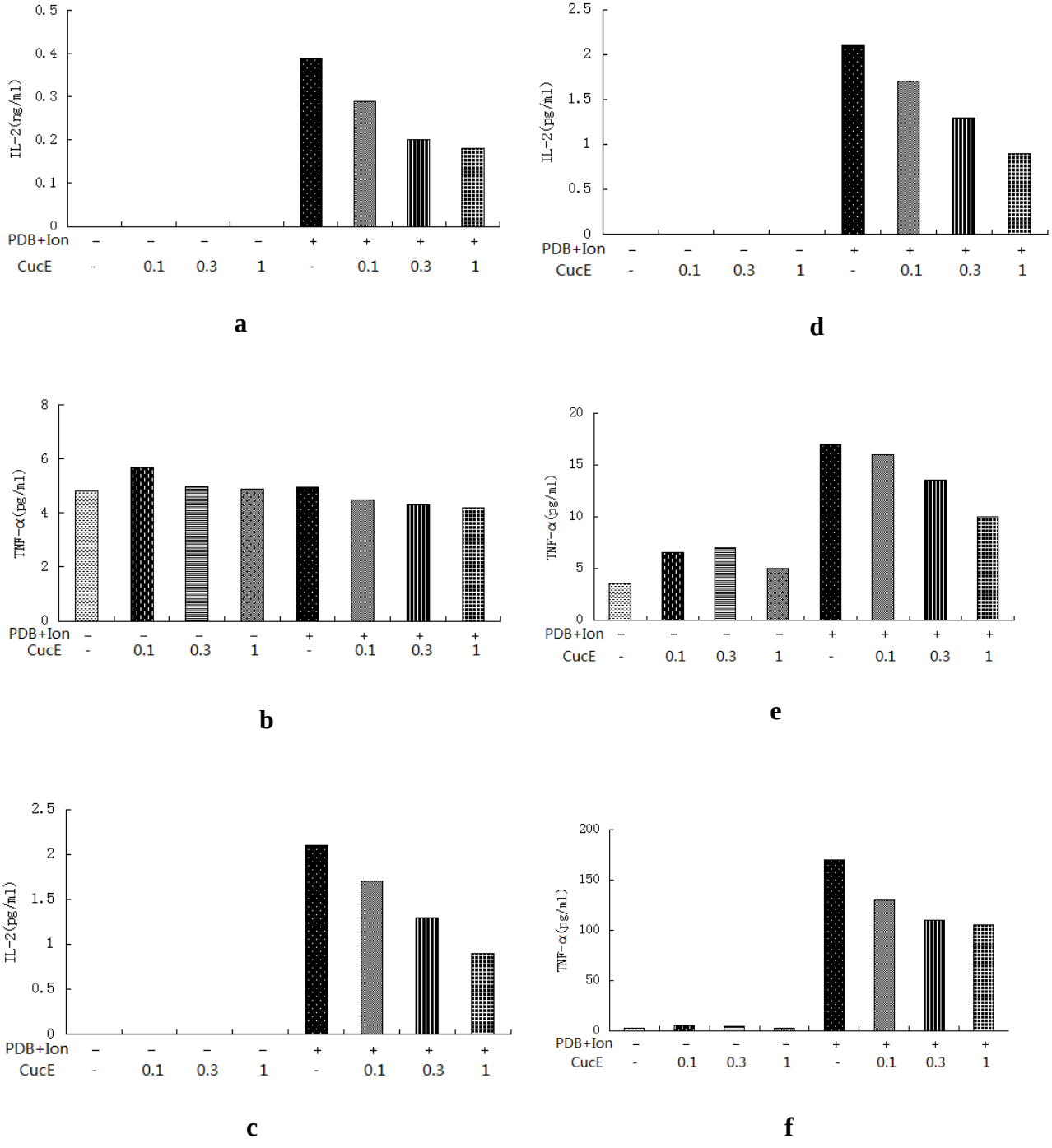
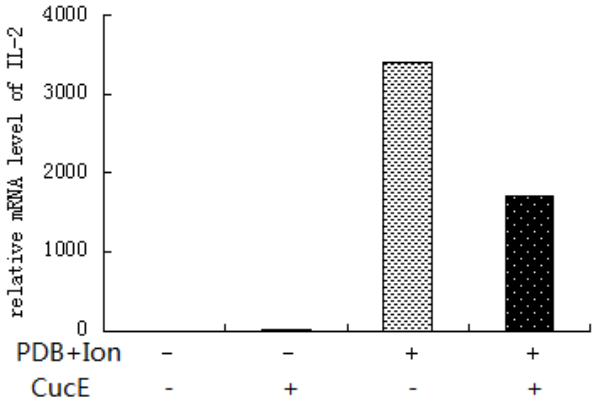


Figure 2. The inhibition effect of CucE on expression of Jurkat T cytokines **(**a-f)

After 24 h, compared with the PDB+Ion model group, the expression level of IL-2, TNF-α and IFN-γ in PDB+Ion+CucE drug treatment group was inhibited significantly in a dose-dependent way (Wei et al., 2015).

3.3. The inhibition effect of CucE on mRNA expression level of IL-2, TNF-α and IFN-γ in Jurkat T cells

In the experiment, blank group, CucE treatment group, PDB+Ion model group and CucE+PDB+Ion drug group were all cultured in a 5% CO_2 incubator at 37 °C for 3 h and 6 h; then the supernatant was collected to detect the mRNA expression level of IL-2, TNF-α and IFN-γ (Figure 3). Figure 3 shows that after the addition of PDB+Ion and 3 h of culture, the mRNA expression level of IL-2, TNF-α and IFN-γ increased significantly, while after the addition of CucE, the mRNA expression level of IL-2 and IFN-γ decreased significantly; after the addition of PDB+Ion and 6 h of culture, the mRNA expression level of IL-2, TNF-α and IFN-γ increased significantly, while after the addition of CucE, the mRNA expression level of IL-2, TNF-α and IFN-γ decreased significantly. Therefore, a conclusion could be drawn that CucE could effectively inhibit the mRNA expression level of IL-2, TNF-α and IFN-γ in Jurkat T cells (Suttorp et al., 2013; Chih-Kang et al., 2009).



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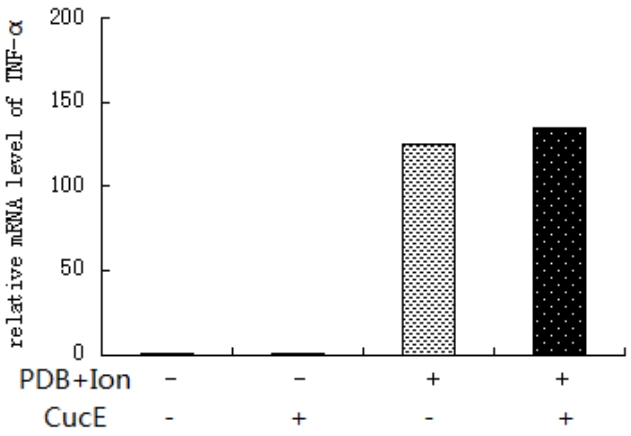

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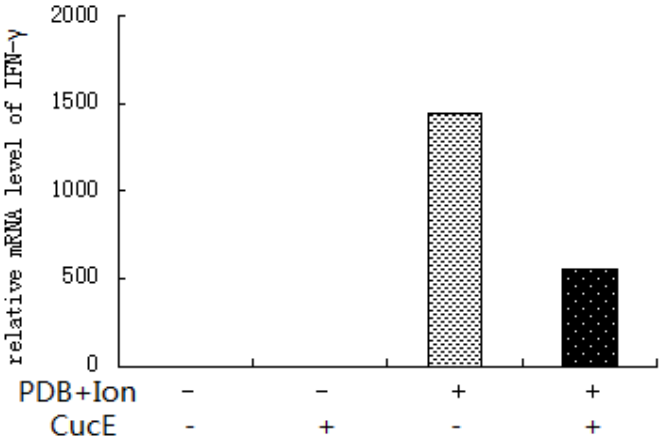

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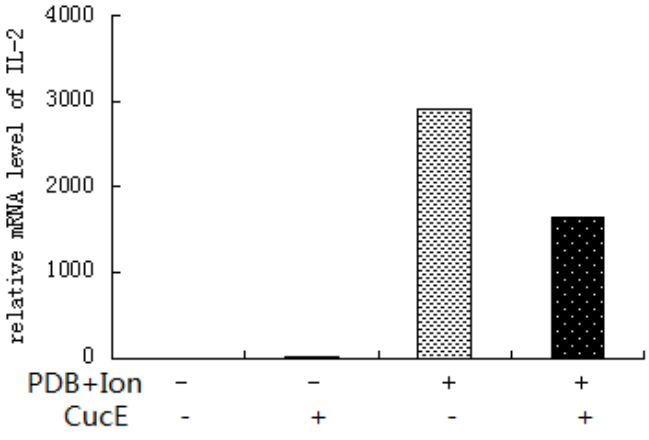

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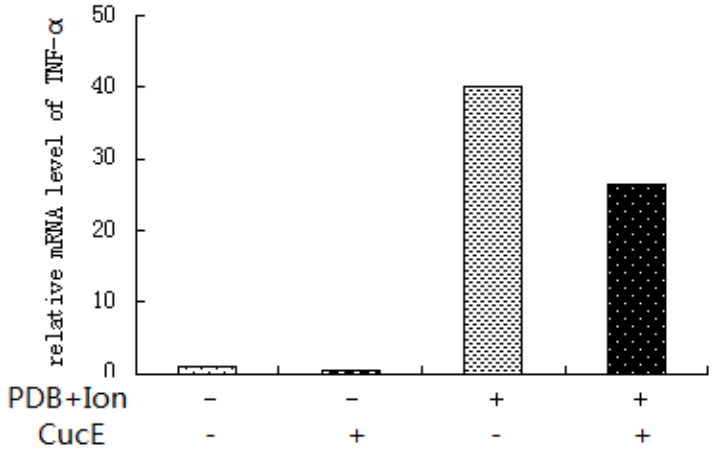

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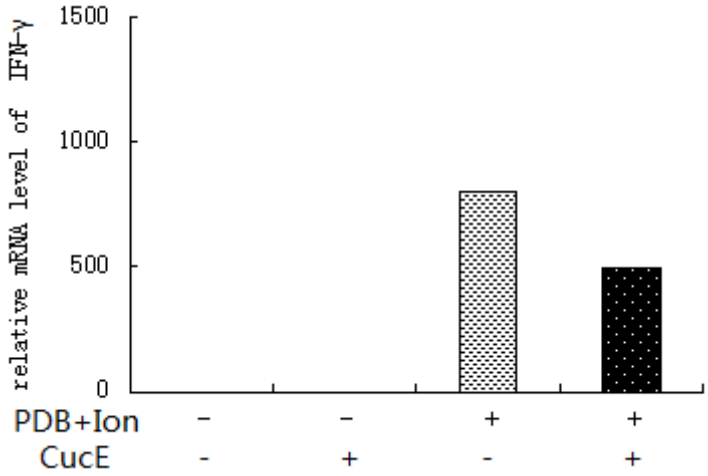

f

Figure 3. The inhibition effect of CucE on decreasing mRNA expression level of Jurkat cytokines

3.4. The influence of CucE on NF-κB signal path and MAPKs signal path

After the 1 h of pretreatment using CucE in 1 μmol/L final concentration, PBD and Ion was added; Protein expression of NF-κB signal path and MAPKs signal path was analyzed using Western Blot method. Results are shown in Figure 4 and Figure 5.

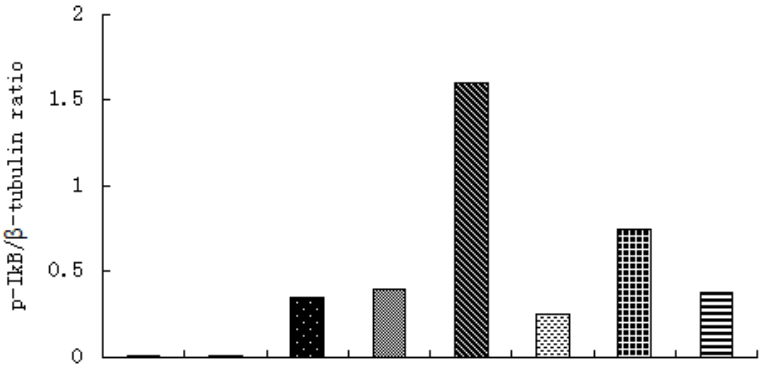

Figure 4. Analysis of protein expression of NF-κB signal path using Western Blot method

Figure 4 indicates that in CucE drug group, phosphorylation level of IκB and NF-κB/p65 decreased significantly at 30 min and 60 min, suggesting that CucE could significantly inhibit the phosphorylation level of IκB and NF-κB/p65.

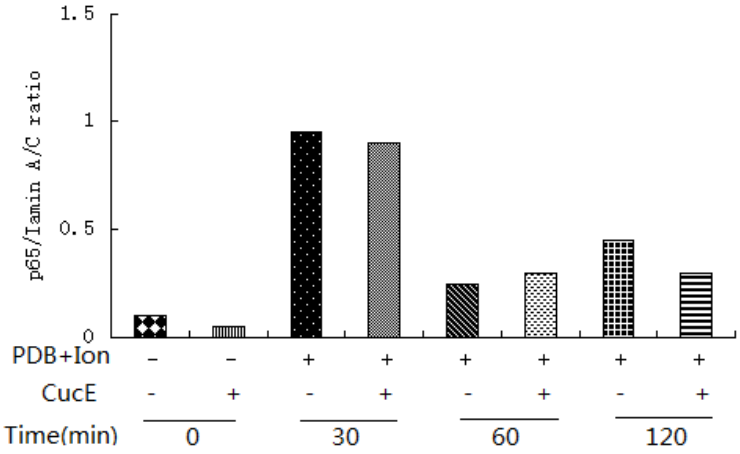

Figure 5. Analysis of intra-nuclear expression level of NF-κB/p65 using Western Blot method

Figure 5 shows that in CucE drug group, the intra-nuclear phosphorylation level of NF-κB/p65 at 2 h decreased significantly, thus CucE could significantly inhibit the intra-nuclear expression level of NF-κB/p65.

In addition, results of using Western Blot method to detect the effect of CucE on protein expression of MAPKs signal path indicated that, after the activation of Jurkat cells through PDB+Ion stimulation, phosphorylation level of JNK, Erk1/2 and p38MAPK increased significantly (Xiao et al., 2011).

4. Conclusions

(1) Researches showed that PBMC in vitro could be activated by PDB+Ion, expression of Jurkat T cells surface molecules CD69 and CD25 increased significantly, and the expression of cytokines IL-2, TNF-α and IFN-γ also increased significantly; however, CucE could significantly inhibit the activation of T cells as well as the expression of IL-2, TNF-α and IFN-γ (Petrelli et al., 2016). Results of Western Blot method showed that such kind of

anti-inflammatory effect might be realized through inhibiting NF-κB signal path (Carvalho et al., 2015), suggesting that CucE might inhibit the expression of IL-2 and its co-stimulatory effect through inhibiting the expression of surface molecules like CD69, thus to inhibit the activation of T cells; CucE has cetatin regulating effect on adaptive immunologic function (Zhao et al., 2016; Molander et al., 2015).

(2) IFN-γ is mainly produced by activated T cells and natural killer (NK) cells, which can regulate immunologic functions, activate NK cells, accelerate differentiation of T cells, induce cells to produce antiviral protein and induce expression of MHC-I and II-type molecules in antigens, etc. (Omokoko et al., 2016).

(3) Cucurbitacin can regulate NF-κB signal path and affect nuclear translocation of NF-kB/p65; CucE can not only inhibit the nuclear translocation of NF-kB/p65, but also can significantly inhibit the phosphorylation level of IkB and p65 (Maroni et al., 2015).

(4) CucE has certain potential medicinal value in regulating adaptive immunologic function and inhibiting inflammatory reaction (Fujita et al., 2015). In conclusion, CucE can significantly inhibit the activation of PBMC in vitro as well as inhibit the protein expression level and mRNA expression level of IL-2, TNF-α and IFN-γ, and it has good immunomodulatory effect on professional athletes engaged in high intensity training in particular (Ye et al., 2014; Miloski et al., 2014). The inhibition effect of CucE on expression of cytokines is realized by decreasing NF-κB path, suggesting that CucE can regulate adaptive immune response, prevent cell fatty degeneration and inhibit fibroplasias, etc.; in addition, it can also eliminate jaundice, lower serum alanine transaminase, zinc sulfate turbidity, eliminate ascitic fluid and improve protein metabolism, which can be used as a new-type drug to relieve sports inflammation of professional athletes.

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