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In vitro immunomodulatory effects of an oleanolic acid-enriched extract of *Ligustrum lucidum* fruit (*Ligustrum lucidum* supercritical CO_2 extract) on piglet immunocytes

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ABSTRACT

This study was conducted to evaluate the in vitro immunomodulatory effects of supercritical CO_2 *Ligustrum lucidum* extract (LLE) on the immune cells of piglets. The results showed that the LLE enhanced the proliferative activity of piglet blood lymphocytes and up-regulated the $CD4^+$ $CD8^+$ and $CD4^+$ $CD8^-$ cell populations. The LLE also regulated the expression of Th1- and Th2-related cytokines; elevated the levels of IL-2, IFN-γ and TNF-α, which were produced by Th1 lymphocytes; and decreased the levels of IL-4 and IL-10, which were produced by Th2 lymphocytes. Furthermore, the LLE stimulated the NO secretion of lymphocytes. These results indicated that LLE might have potential immunomodulatory effects on the immune system of piglets and provided scientific and experimental foundations for the development of a new kind of LLE immune adjuvant in the pig production.

1. Introduction

Antibiotics have been used extensively in animal feed to overcome infections and to maintain animal health. However, this therapy often results in side effects, including reduced therapeutic effectiveness of antibiotics in treating a variety of bacterial infections in humans [1,2]. Thus, alternatives to antibiotics are an area of active investigation. Chinese herbal medicines are one class of alternatives that has aroused great interest because of their natural origin and their enrichment with biologically active constituents, such as polysaccharides and essential oils. Furthermore, most of them do not leave any drug residue and have minimal side effects in humans [3]. In traditional Chinese medicines, *Ligustrum lucidum* (LL) is used to nourish the liver and kidneys and to brighten eyes. Studies from modern medicine have shown that LL can activate antibody formation, promote complement production and increase T lymphocyte proliferation in broilers [4–7]. However, there have been only a few reports related to the immunomodulatory effects of different forms of LL extracts which concentrated more active ingredients. Previous studies have shown that LL is rich in oleanolic acid (OA) (7–15 mg/g LL), D-mannanoligosaccharides (D-MOS) (6–9 mg/g LL), and specnuezhenide (0.6-1.2 mg/g LL) [8]. OA is the major effective constituent of LL, and it is responsible for different modifications on the immune system [9]. Recently, a LL supercritical CO_2 extract (LLE) has been produced using a certain extraction condition in our lab; the OA content of the new extract is approximately 10 times greater than that of the original LL powder. If it were revealed that the extract has an immunological effect, it would open a new field for various applications, including as an immunoenhancer for animal feed or as a health-protective remedy for humans. Based on the above, in this study, LLE was selected as an immunoregulator, and its immunomodulatory effects in piglets were investigated through a series of in vitro experiments. The purpose of this study is to offer a theoretical basis for developing LLE as new type of immune adjuvant and immunopotentiator.

2. Materials and methods

2.1. Preparation of LLE

The LL used in this study was purchased from the Harbin Chinese medicine market, Heilongjiang, China. After drying the LL at 60 °C and crumbling it to powder with an average granule diameter of 0.38 mm, we employed supercritical CO_2 extraction technology. The optimum conditions used were as follows: the LL was soaked in 80% ethanol for 1 h (the ratio of LL weight and ethanol volume was 1:1) and then extracted for 3 h under a pressure of 18 MPa at 60 °C and a CO_2 flow rate of 4.8 L/h. The extraction yield was approximately 7% (w/w). The content of OA in the LLE, as determined by high-pressure liquid chromatography (HPLC) was 6.87%; OA is considered to be a main active ingredient in LL. The amino acid concentrations in the LLE were analyzed by a Hitachi L-8800 Auto-Analyzer, Tokyo, Japan [10] and were as

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follows: Glu 2.64%, Asp 1.20%, Leu 1.08%, Arg 0.83%, Ser 0.78%, Ala 0.78%, Phe 0.75%, Gly 0.68%, Lys 0.54%, Val 0.54%, Thr 0.47%, Ile 0.35%, Cys 0.33%, His 0.31%, Met 0.26%, and Tyr 0.21%.

The LLE was diluted with RPMI-1640 medium (GIBCO, USA) into 500-mg/L samples by adding DMSO (final volume<1%) as the solution adjuvant. The diluted preparations were filtered through a 0.22-μm filter, and endotoxin was detected using a chromogenic tachypleus amebocyte lysate Endpoint Kit (Chinese Horseshoe Crab Reagent Manufactory Co., Ltd., Xiamen, China). The concentration of bacterial endotoxins (LPS) in the sample solution (500 mg/L) was 0.228 EU/mL [11]; the solution was then stored at −20 °C until it was used to determine the in vitro immunomodulatory effect of LLE on weaned piglets.

2.2. Isolation of peripheral blood lymphocytes

The experiment was carried out in accordance with the Chinese guidelines for animal welfare and experimental protocols [12]. Blood peripheral lymphocytes were prepared using a percoll gradient as described by Huang et al. (2007) [13]. Blood samples were collected by venipuncture of the jugular vein from crossbred (Duroc× Landrace×Yorkshire) healthy piglets that had initial weights of 8.2± 0.8 kg and were transferred immediately into aseptic capped tubes with sodium heparin. The anticoagulated blood samples (2 ml) were diluted with an equal volume of Hanks' solution and carefully layered on the surface of 3 ml of the lymphocyte separation medium. After centrifugation for 20 min at 2000 ×*g*, a white cloud-like band was observed on the lymphocyte separation solution interface. The lymphocyte band was collected and washed twice with an RPMI 1640 medium without fetal bovine serum, then resuspended in the complete RPMI-1640 medium (RPMI 1640 media supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, penicillin 100 IU/mL, streptomycin 100 IU/mL). The cell number was adjusted to 1×10^6 cells/mL. Total lymphocyte counts were performed following standard techniques using a microscope and hemocytometer. The cell viability was tested using the trypan-blue dye exclusion method.

2.3. Proliferation measurement of peripheral lymphocytes

Lymphocyte proliferation was determined using a 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [14–16]. A 100-μl cell suspension was transferred to each well of 96-well cell culture plates. The cells were stimulated by final concentrations of 0, 5, 25, 50 or 100 mg/L of LLE alone or combined with either concanavalin A (Con A, Sigma, USA), at a final concentration of 5 mg/L, or lipopolysaccharide (LPS, Sigma, USA), at 20 mg/L, and each treatment was dosed into four wells. The total volume of solution in each well was 200 μl. After 44-h incubation at 37 °C, 5% CO2 in the CO2 incubator, 20 μl MTT (5 mg/ml) in PBS was added into each well, and incubation continued for another 4 h. Then, the supernatants were discarded by centrifugation at 1800 ×*g* at 37 °C for 10 min. One hundred fifty microliters of DMSO was added into each well, followed by shaking for 10 min to completely dissolve the purple formazan crystals. The light absorbance was measured at 570 nm using a microplate reader (TECAN, Austria). The viability and number of peripheral lymphocytes were determined at A570 nm.

2.4. T-lymphocyte subpopulations assay by flow cytometry

The peripheral blood lymphocytes were prepared as described earlier and treated with LLE at different concentrations (0, 25, 50, 100 mg/L) alone or combined with Con A (final concentration: 5 mg/L) for 48 h. The T cell surface markers were determined by staining the cells with PE-conjugated anti-CD4/L3 T4 and FITC-conjugated anti-CD8/Lyt-2 (BD Pharmingen, USA) [17]. Briefly, 1×10^6 cells were washed twice with wash buffer (PBS containing 0.1% NaN_3). The samples were then incubated with the conjugated antibodies for 30 min at 4 °C. Each sample was resuspended in 0.5 mL of the fix solution (PBS that contained 2% formaldehyde and 0.05% NaN_3). The T cell surface markers were analyzed using a BD FACS Calibur flow cytometer (Becton-Dickinson, San Jose, USA) with a 488 nm argon laser and detected fluorescence at 530 and 575 nm. Cell Quest software (Becton-Dickinson) was used to identify and quantify the distinct cell populations by mean fluorescent intensity (MFI). A minimum of 10,000 cells were analyzed for each sample [18,19].

2.5. Detection of cytokines by ELISA

Aliquots of the peripheral blood lymphocyte suspension (1000 μl) were transferred to each well of a 24-well, flat-bottomed microplate, and then, complete RPMI-1640 medium and different final concentrations of LLE (0, 5, 25, 50, 100 mg/L) were added to wells. The total volume of solution in each well was 2000 μl. The cells were incubated at 37 °C with 5% CO_2 for 48 h, and the culture supernatants were collected and stored at −20 °C for measurement of the cytokine levels. The amount of each cytokine in the supernatants was determined using ELISA kits (BD Pharmingen) following the manufacturer's instructions [20].

2.6. Phagocytic activity measurement of peripheral monocytes

The cells that were prepared as described above were seeded into 96-well plates at 100 μl/well and incubated in 5% CO_2 at 37 °C for 4 hours. Then, the nonadherent cells were removed by two washes with PBS, and the adherent cells in each well were purified peripheral monocytes. The phagocytic activity of the monocytes was determined using the neutral red phagocytosis test [20]. The purified monocytes were suspended in 200 μl/well of complete RPMI-1640 medium that contained various concentrations of LLE (0, 5, 25, 50 or 100 mg/l). Each treatment was dosed into four wells. The monocytes were cultured at 37 °C and subjected to 5% CO_2 for 24 h. Then, the culture media were removed, 100 μL/well of a 0.1% neutral red saline solution was added, and incubation was continued for another 30 min. The media were discarded, and the monocytes were washed twice with PBS. The washed monocytes were resuspended in 100 μL/well of the cell lysing solution (acetic acid:ethanol = 1:1 V:V) and incubated at room temperature overnight. The absorbance (A540 nm) was measured using an ELISA reader (TECAN, Austria).

2.7. Measurement of NO production

The previously prepared peripheral cells suspensions were placed in a 96-well plate (100 μl/well) and incubated in complete RPMI 1640 medium containing various concentrations of LLE at 37 °C and 5% CO_2 for 48 h. The final concentrations of LLE were 0, 5, 25, 50 or 100 mg/L per well, and the total volume of solution in each well was 200 μl. The culture supernatants were collected and stored at −20 °C for measurement of the NO content. NO in the culture medium was determined by Griess reaction [20]. A total 100 μl/well of culture supernatants was mixed with equal volume of Griess solution (1% sulfanilamide, 0.1% napthyl ethyl diamine dihydrochloride in 2.5% phosphoric acid) at room temperature for 10 min. The absorbance was read at 550 nm, and the concentrations of NO_2^- were determined from a least squares linear regression analysis of a sodium nitrite standard curve.

2.8. Statistical analysis

The data were analyzed using SPSS 17.0 statistical software. The results were statistically analyzed using one-way ANOVA. Duncan's multiple range test was used to compare the differences among the treatment groups. A P-value of less than 0.05 was taken to indicate statistical significance.