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Bioactive Hepatic Peptide Improves Immune Function by Inducing Autophagy in Stomach Cancer-Bearing Nude Mice

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Abstract

Background: Bioactive Hepatic Peptide (BHP), a kind of bioactive peptide from goat liver, can regulate immune function in healthy mice. However, how the tumor-bearing mice? We investigated the effect of BHP on the immune function of gastric cancer-bearing nude mice and its possible underlying mechanism.

Methods: Nude mice were inoculated into the neck with human BGC-823 gastric cancer cells to establish the subcutaneous transplanted tumor model group. The percentage of peripheral blood lymphocytes was measured by flow cytometry. Hematoxylin-Eosin (HE) staining was used. The mRNA expression levels were measured by real-time fluorescence quantitative PCR and the protein expression of NF-κB, TNFα, Beclin1, LC3B, Akt1, COX-2 were determined by immunohistochemistry and Enzyme-Linked Immunosorbent Assay (ELISA).

Results: BHP could inhibit tumor cell proliferation, improve the percentage of NK cells/B lymphocytes, phagocytic index and phagocytic percentage and reduce the degree of inflammatory infiltration in tumor-bearing nude mice. BHP could significantly decrease the mRNA and protein expression of NF-κB, TNF-α in spleen and AKT1 and COX-2 in tumor tissue, but it could significantly increase the mRNA and protein expression of Beclin1 and LC3B (LC3) in tumor tissue ($P<0.05$). The levels of NF- κB, TNFα and COX-2 were significantly lower in the serum of nude mice treated with BHP ($P<0.05$). The level of Beclin1 increased significantly after BHP treatment ($P<0.05$).

Conclusion: BHP may down regulate NF-κB/AKT1 to inhibit inflammation and induce autophagy, thus enhancing the immune function and antitumor effect of tumor-bearing nude mice. These findings support BHP as an immune-boosting health food/drug for cancer patients.

Keywords: Bioactive Hepatic Peptide (BHP); Immunity; Inflammation; Autophagy

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Introduction

Gastric cancer is a malignant tumor of the digestive tract with high morbidity and mortality. At present, clinical treatment measures include surgical treatment, drug therapy, radiotherapy and chemotherapy, and targeted therapy, among others. However, the above treatment methods have their own advantages and disadvantages in the treatment process, and a gap persists between them and the expected value of clinical treatment effect. Chemotherapy is the main conventional treatment, and the side effects of treatment and production of drug resistance lead to a sharp decline in the immunity of patients, seriously affecting their quality of life. Therefore, it is urgent to develop new antineoplastic agents to improve patients' immune ability and quality of life. The BHP selected in this study was derived from bioactive peptides extracted from the liver of healthy goats. Its preparation process and quality control measured are highly developed, and previous studies have demonstrated that it not only has an anti-tumor effect by inhibiting proliferation and promoting apoptosis but also improves the immune function of mice without obvious side effects [1-4].

Humoral and cellular immunity make up the immune system. Because of immune deficiency caused by thymus atrophy, nude mice can undergo tissue transplantation from xenogeneic animals under certain conditions and become a good carrier of the malignant tumor. Consequently, we used nude mice to establish a model. The production of T cells in nude mice after thymus atrophy

is blocked, so humoral immunity mediated by B cells and cellular immunity mediated by NK cells and phagocytes (mononuclear phagocyte system and neutrophils) are the main route of immune function. The Mononuclear Phagocyte System (MPS) includes monocytes dissociated in the blood and macrophages that have developed after entering various tissues. Macrophages, which are also a kind of major antigen-presenting cell, have strong phagocytic ability and play a key role in the induction and regulation of specific immune response [5]. Neutrophils are a kind of small phagocyte with a nonspecific immune defense function that participates in the immune response and inflammatory injury, among others. TNF-α is an important bioactive cytokine from mononuclear macrophages, which can kill or inhibit tumor cells by destroying the stability of lysosomes and regulate immune function, enhance the killing effect of killer cells toward tumor cells, and improve the phagocytic ability of neutrophils [6-7]. TNF-α plays a dual role in gastric cancer. It not only promotes the inflammatory reaction, induces apoptosis and produces cytotoxicity but also promotes proliferation and inhibits apoptosis [8]. NF-κB is a transcription factor and can regulate TNF-α. Many genes transcribed by NF-κB promote gastric carcinogenesis, so NF-κB has become an important therapeutic target in chemotherapy [9]. Studies have shown that TNF-α participates in the immune response of many malignant tumors (such as ovarian cancer, breast cancer and endometrial cancer) by activating the NF-κB signal [10-12]. To explore the changes in immune function in nude mice during the occurrence and development of gastric cancer, we explored the expression of NF-κB and TNF-α. Autophagy is a mechanism of cell self protection as well as self-digestion. It has been described to play an indispensable role in the regulation of immune function [13,14]. Beclin 1 and LC3 are classical markers of autophagy. Autophagy is known to be a double-edged sword for tumors. What role does autophagy play in the changes in immune function of BHP-mediated gastric cancer nude mice? We studied the expression of Beclin1 and the classical autophagy marker protein LC3 in nude mice. AKT1 is a protein kinase, and its related PI3K-AKT1 pathway is involved in many biological phenomena, including autophagy, cell proliferation and angiogenesis [15]. Cox-2 is a key enzyme in the synthesis of prostaglandins. Cox-2 is enhanced after stimulation by carcinogens and inflammatory factors, and it has a binding site for NF-κB. COX-2 is not only an early tumor-related phenomenon but also is associated with the tumor stage and metastasis [16].

We studied the subcutaneous transplanted tumor model of human BGC-823 gastric cancer in nude mice, observed the anti-tumor effect of different doses of BHP and immune function of tumor-bearing nude mice, and preliminarily discussed the role and relative mechanisms of BHP in immunity, to provide a theoretical basis for further study of the BHP immune-related mechanism and development of BHP as a preparation to improve the quality of life of patients with gastric cancer.

Materials and Methods

Preparation of BHP

The preparation method of BHP refers to the preparation method of BHP used in the study by Chen et al. [4]. **4.2. Animal grouping administration and calculation of body weight, tumor volume, tumor inhibition rate, tumor rupture rate and spleen index**

The transplanted tumor model of gastric cancer was established in nude mice. A total of 63 SPF BALb/c female nude mice (Beijing Weitong Lihua Experimental Animal Technology Co., Ltd., Beijing, China) weighing 10 g to 14 g were randomly divided into 6 groups: Control (CON) group (n=12), (Model group) MG (n=11), Low dose of BHP (LBHP) group (n=10), Middle dose of BHP (MBHP) group (n=10), High dose of BHP (HBHP) group (n=10) and Calf Spleen Extractive Injection Group (CSEI) group (n=10). After 4 days of adaptive feeding (25°C, 60% humidity, light-dark cycle of 12 h), 2 mice died (possibly due to squeezing and bumping or a poor condition during transportation). There were 11 mice in the CON group and 10 mice in the other group. Excluding the CON group, the other 5 groups were inoculated in the neck with a suspension of 1×10^7/ml BGC-823 cells (logarithmic growth phase cells in the Clinical Experimental Center of affiliated Hospital of Inner Mongolia Medical University, China) to establish the subcutaneous transplanted tumor model. The rice-grained tumor was palpated on the neck of nude mice four days later, which indicated that the model was successful. An equal volume of 0.9% NaCl was administered by intragastric administration of CON group and MG. The above drugs are given once a day as follows: 0.094 mg/10 g in the MBHP group, 0.188 mg/10 g in the LBHP group, and 2.8 ng/10 g group intramuscular injection of CSEI (2 ml containing 5 mg peptide, standard words H22026121, Jilin Aodong Taonan Pharmaceutical Co., Ltd.). During this period, body weight and tumor diameter (a) short path (b) were measured every 2 days at a fixed time point (9:00 am), and the tumor volume TV (TV=ab2/2) was calculated. Tumor rupture in mice was observed and recorded to calculate the tumor rupture rate (number of mice with tumor collapse/total number of mice in each group × 100%) during the 14 days of administration. After the mice were anesthetized with ether, blood was collected from the eyeball vein, and then the mice were sacrificed with CO_2 and the spleen and tumor removed. The mice in each group were sacrificed and the spleen and tumor removed and subjected to the following calculation:

Tumor inhibition rate = (average tumor mass of the control group-average tumor mass of the experimental group)/average mass of the control group × 100%.

Spleen weight index = spleen weight/body weight × 100%.

All operations were approved by the Institutional Animal Care and Use Committee (IACUC).

Percentage of NK cells and B lymphocytes

Prior to sacrifice, blood was collected from the eyeball vein into an EDTA anticoagulant tube. The percentages of NK cells and B lymphocytes in the venous blood were determined by flow cytometry (BD Accuri). Isotype control (mouse IgG1/IgG2a, cat. no 747288), CD3-PE (cat. no 555275), CD19-FITC (cat. no 553785) and APC mouse anti-Mouse NK-1.1 (cat. no 550627) were used. B lymphocytes were labeled with CD3-CD19+, and NK cells were labeled with CD3-/NK-1.1. The results were analyzed using CELLQuest Pro software (Figure 2).

Macrophage phagocytosis experiment

The 0.5% starch solution was injected intraperitoneally twelve hours before sacrifice. The 1% chicken red blood cells (Bio-Channel, cat. no. BC-RBC-C001) were intraperitoneally injected after 6 h, and the abdomen of the mouse was then gently dissected. The abdominal cavity was rinsed with physiological saline while continuing to gently manipulate the abdomen for 10 sec. A 1-ml needleless syringe was used to aspirate the abdominal cavity lavage fluid, and 1 to 2 drops were added to a slide, covered gently with a coverslip, and allowed to dry naturally at room temperature. Next, Wright-Giemsa stain

(Shanghai Yisheng Biotechnology Co., Ltd., cat. no. 60529ES01) was added for 1 minute (the dye solution cannot be dried) followed by an equal amount of distilled water and immediately mixing at 37°C for 5 min. Tap water was then slowly added for washing, followed by flushing to remove the color, drying of the surface of the slide glass with filter paper, and observation under an oil emersion microscope. The phagocytic index and percentage of phagocytosis were calculated as follows:

Percentage of phagocytosis (%) = number of macrophages that engulfed chicken red blood cells/100 phagocytes × 100,

Phagocytosis index = number of chicken erythrocytes engulfed/100 phagocytes × 100.

HE staining

The 4-μm-thick conventional paraffin-embedded sections were soaked in xylene for 10 min and dewaxed by repeating once. Afterwards, the sections were incubated with gradient alcohol (soaked twice with 100% alcohol for 10 min each time, twice with 95% alcohol for 5 min each time, once with 85% alcohol for 5 min, and once with distilled water for 5 min each) and stained with hematoxylin (Abcam) (spleen tissue for 5 min, tumor tissue for 6 min), followed by rinsing with tap water for 1 min, differentiation with hydrochloric acid for 10 sec, rinsing with tap water for 8 min and staining with eosin (Shanghai Yiyan Biotechnology Co., Ltd.) for 30 sec. After the incubation with gradient alcohol (80% alcohol rinse, 95% alcohol soak twice, 10 min each time), the section were treated with xylene until transparent, sealed with neutral gum, and observed under a microscope (100x and 400x).

Detection of TNF-α and NF-κB in spleen and Beclin1, LC3B, COX-2, and AKT1 mRNA expression in tumor tissue

The traditional TRIzol (CWBIO Beijing Kangwei Century Biotechnology Co., Ltd.) method was used to extract total spleen RNA according to the tissue RNA extraction instructions. Subsequently, the RNA concentration was measured using a protein nucleic acid detector (Nanodrop). 1 μg total RNA and the reverse transcription kit (KR103, Tiangen Biochemical Technology Beijing Co., Ltd.) was used. 1 × RT mix, 0.25 mM each dNTP, 1 μM Olig-dT18 and 1 μl Quant reverse transcriptase was used in 20 μl reaction system, then performed 60 min at 37°C using the gene amplification instrument (TaKaRa Corporation of Japan). The fluorescence quantitative kit (FP205, Tiangen Biochemical Technology Beijing Co., Ltd.) with the 7500 real-time PCR instrument (USA Applied Biosystems) and a 20 μl reaction system in accordance with the instructions in which the final concentration of primers is 0.3 μM, cDNA is 2 μl, and the Superreal Premix plus is 1x. Pre-deformation at 95°C for 15 min, denaturation at 95°C for 10 sec, annealing at 55°C for 30 sec, and extension at 72°C for 32 sec, a total of 40 cycles.

Forward primers (TNF-α: C C C T C A C A C T C A G A T C A T C T T C T, NF-κB: A T G G C A G A C G A T G A T C C C T A C, Beclin1: A T G G A G G G G T C T A A G G C G T C, LC3B: G G A C T G A A C C C A G C A T A G, COX2: C C G A G T C G T T C T G C C A A T A G, COX2: C C G A G T C G T T C T G C C A A T A G, AKT1: A T G A A C G A C G T A G C C A T T G T G) and reverse primers (TNF-α: G C T A C G A C G T G G G C T A C A G, NF-κB: C G G A A T C G A A A T C C C C T C T G T T, Beclin1: T G G G C T G T G G T A A G T A A T G G A, LC3B: A T C C T T A C T G A T C G C A C C, COX2: C T T G A T T T A G T C G G C C T G G G A, COX2: C C G A G T C G T T C T G C C A A T A G, AKT1: T T G T A G C C A A T A A A G G T G C C A T) were synthesized by Biotechnology Shanghai Engineering Co., Ltd. Three replicate wells for each sample were evaluated on the same 96-well plate. The housekeeping gene used in this experiment was GAPDH. The relative quantitative value of each sample was determined according to $2^{-\Delta\Delta Ct}$ ($\Delta\Delta Ct = \Delta Ct$ experimental group-ΔCt control group), which was processed using SPSS23.0 statistical software. The data were the average with the added or subtracted deviation (mean ± SD), and if the data followed a normal distribution, single-factor analysis of variance was used. If the data were non-normally distributed, a nonparametric rank sum test was performed. $P<0.05$ was considered statistically significant.

Immunohistochemical determination of TNF-α and NF-κB in spleen and LC3, AKT1, and COX-2 protein expression in tumors

The expression levels of TNF-α and NF-κB in spleen and LC3, AKT1, and COX-2 protein in spleen and the staining result score were evaluated according to the immunohistochemical method previously reported by our group [4]. The primary antibodies were as follows: Rabbit anti-TNF-alpha antibody (cat. no. bs-0078R, dilution of 1:500, Bioss), rabbit anti-NF-κB p65 polyclonal antibody (cat. no. bs-0465R, dilution of 1:800, Bioss), LC3A/B polyclonal antibody (cat. no. E-AB-70053, dilution of 1:500, Elabscience), AKT1 polyclonal antibody (cat. no. E-AB-63821, dilution of 1:800, Elabscience), and COX-2 polyclonal antibody (cat. no. E-AB-70030, dilution of 1:500, Elabscience). The samples were incubated with primary antibody for 1 hour at room temperature, followed by conjugated secondary antibody (cat. no. SP-0023). DAB staining was performed for 5 min, followed by sealing of the glass slides with neutral gum and observation under a microscope (100x).

Determination of TNFα, NF-κB, COX-2 and Beclin1 in serum by ELISA

The venous blood from the eyeball was collected into a blood collection tube equipped with PRP separation gel, gently inverted 3 to 5 times, and allowed to stand at room temperature for 15 min. It was then centrifuged at 1060 g for 15 min at room temperature, and the supernatant was placed in a refrigerator at 4°C. The Mouse TNF-α ELISA KIT (cat. no. CSB-E04741m, Huameishengwu in Wuhan, China), Mouse NF-κB ELISA Kit (cat. no. CSB-E12108m, Huameishengwu in Wuhan, China), Mouse Cyclooxygenase-2 (COX-2) ELISA Kit (cat. no. CSB-E12910m, Huameishengwu in Wuhan, China) and Mouse Autophagy Gene Beclin1 ELISA Kit (cat. no.TX27331, Yingxin Lab, Shanghai, China) were applied according to the manufacturers' instructions. The OD value was measured at 450 nm using a microplate reader (RT-6100, Rayto, Shenzhen), and three wells were reread for each sample. SPSS 23.0 was used for the statistical analysis. Data were statistically analyzed using SPSS version 23.0 and expressed as the mean ± standard deviation. Comparison between multiple groups was performed using one-way ANOVA followed by Turkey's multiple comparison tests to detect intergroup differences. $P<0.05$ was considered to be statistically significant.

Results

Mouse body weight, tumor volume, tumor inhibition rate, tumor rupture rate and spleen index

The body weight of MG nude mice increased slowly compared with the CON group (Figure 1a) after BHP and CSEI treatment, and it recovered or even showed an increasing trend compared with MG. In the MG and LBHP groups, the tumor volume increased significantly

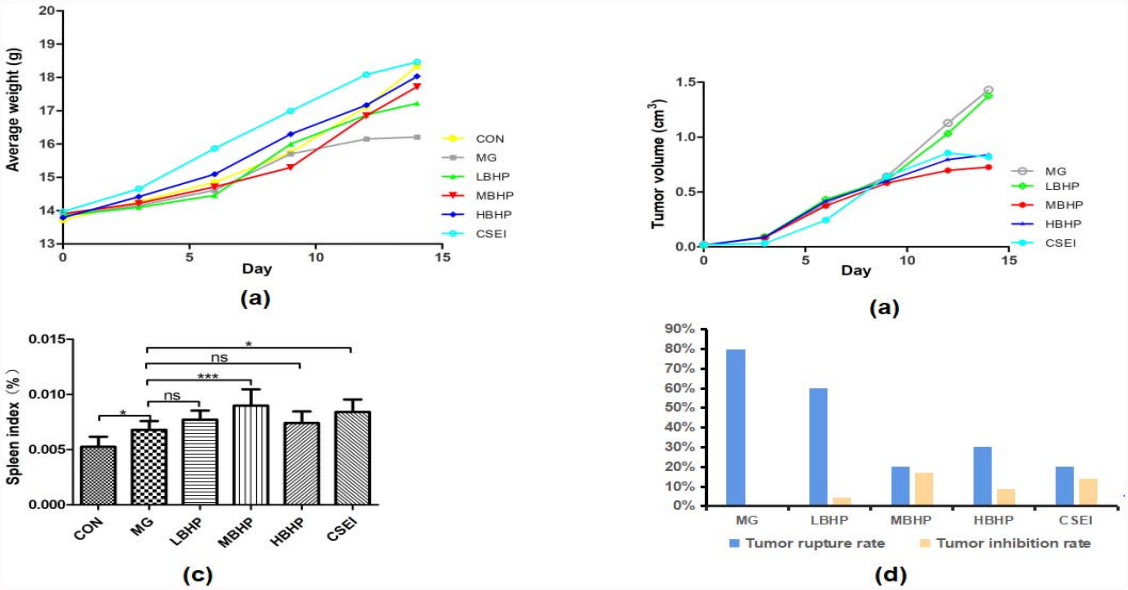

Figure 1: The average body weight (1a), tumor volume (1b), spleen index (1c) and tumor rupture rate (1d) and tumor inhibition rate (1d) of nude mice in each group. The body weight of CSEI nude mice increased the fastest, and the average body weight was the highest, while that in the MG and LBHP groups increased slowly, as shown in Figure 1a. The volume of MG and LBHP tumors in Figure 1b increased rapidly, and after 12 days of administration, growth in the MBHP, HBHP and CSEI groups decreased significantly. The spleen index showed an increasing trend in all groups, as shown in Figure 1c. The increase in the spleen index in MG nude mice may be related to the enhancement of stress immunity. Figure 1d shows the tumor rupture rate (number of nude mice with tumor rupture/total number of nude mice in each group ×100%) and tumor inhibition rate [(average mass of tumor in experimental group-average mass of tumor in relative control group)/average mass of tumor in relative control group ×100%].
"ns" P>0.05; "*" P=0.01-0.05; "**" P<0.01; "***" P<0.001. All data were from two independent experiments, each repeated in parallel twice.

and rapidly (Figure 1b). Figure 1c shows that the spleen index was significantly increased in MG mice than in the CON group (P<0.05). An increasing trend was apparent after BHP and CSEI treatment compared with MG. In particular, the increase in MBHP and CSEI was significant (P<0.05). The tumor rupture rate reached 80% in the MG group, and the tumor inhibition rate reached 16.83%, while the MBHP and CSEI group showed the lowest rate of rupture of 20% and tumor inhibition rate of 13.92% (Figure 1d). We can conclude that BHP could improve the quality of life of nude mice bearing tumors.

Percentage of NK cells and B lymphocytes

The percentage of NK cells and B lymphocytes was significantly lower in MG than CON (P<0.01 and P<0.001). The percentages of NK cells and B lymphocytes were significantly higher in the MBHP, HBHP and CSEI groups than the MG mice (NK cell percentage in HPHP was P<0.01, others were P<0.001) (Figure 2). B lymphocytes play an important role in humoral immunity, and NK cells mainly mediate cellular immunity, which indicates that BHP treatment could improve humoral and cellular immunity in nude mice.

Determination of the phagocytic ability of macrophages

Macrophage phagocytosis is generally expressed as the phagocytosis percentage and phagocytosis index. Macrophages are an important part of cellular immunity. Our results showed that the phagocytic percentage and phagocytic index of macrophages were significantly lower in MG than CON mice (Figure 2), but they were increased after treatment with BHP and CSEI and significantly increased in the HBHP and CSEI groups (P<0.05 and P<0.01). These results indicated that BHP could improve the phagocytic ability of macrophages and subsequently improve cellular immune function mediated by macrophages.

HE staining

The HE staining results for the spleen were observed at low magnification (Figure 3a), and high magnification (Figure 3b). The splenic trabeculae, capsule, megakaryocyte, marginal zone, septum, red pulp and white pulp were observed. Different degrees of lymphocyte infiltration were observed in the red pulp, indicating the presence of inflammatory infiltration, and BHP could reduce the degree of infiltration. Germinal centers appeared in all other groups except the CON group. The germinal center is essentially a place where lymphocytes are produced, and the appearance of the germinal center indicated that immune function was enhanced. Among all the staining results, the MBHP group had the most germinal centers. The cancer nests, mucus pools, mitotic phases, multinucleated heterotypic cells and vascular cavities could be observed in the tumor tissue (Figure 3c, 3d). The nuclei were deeply colored and aggregated to form a beaded shape. The nucleus-cytoplasm ratio was severely disorganized. The MG and LBHP groups exhibited the most obvious changes.

Expression of NF-κB, TNF- α, Beclin1, LC3B, AKT1, and COX-2 mRNA

The qPCR results (Figure 4c) showed that the expression levels of NF-κB and TNF-α mRNA were significantly elevated in the spleen of MG nude mice compared with the CON group (P<0.001) and significantly decreased after treatment with a certain dose of BHP and CSEI (P<0.05 and P<0.001). The mRNA expression levels of Beclin1 and LC3B were significantly higher in tumor tissues of nude mice treated with BHP and CSEI than MG (P<0.001). Beclin1 is a classical autophagy factor, which indicates that the level of autophagy was decreased in MG nude mice, while BHP can mediate the regulation of immune function in nude mice by inducing autophagy and increasing the level of autophagy. The expression levels of AKT1 and COX-2 were relatively increased in the BHP and CSEI groups. These results suggested that BHP could down regulate the expression of AKT1 and COX-2 mRNA.

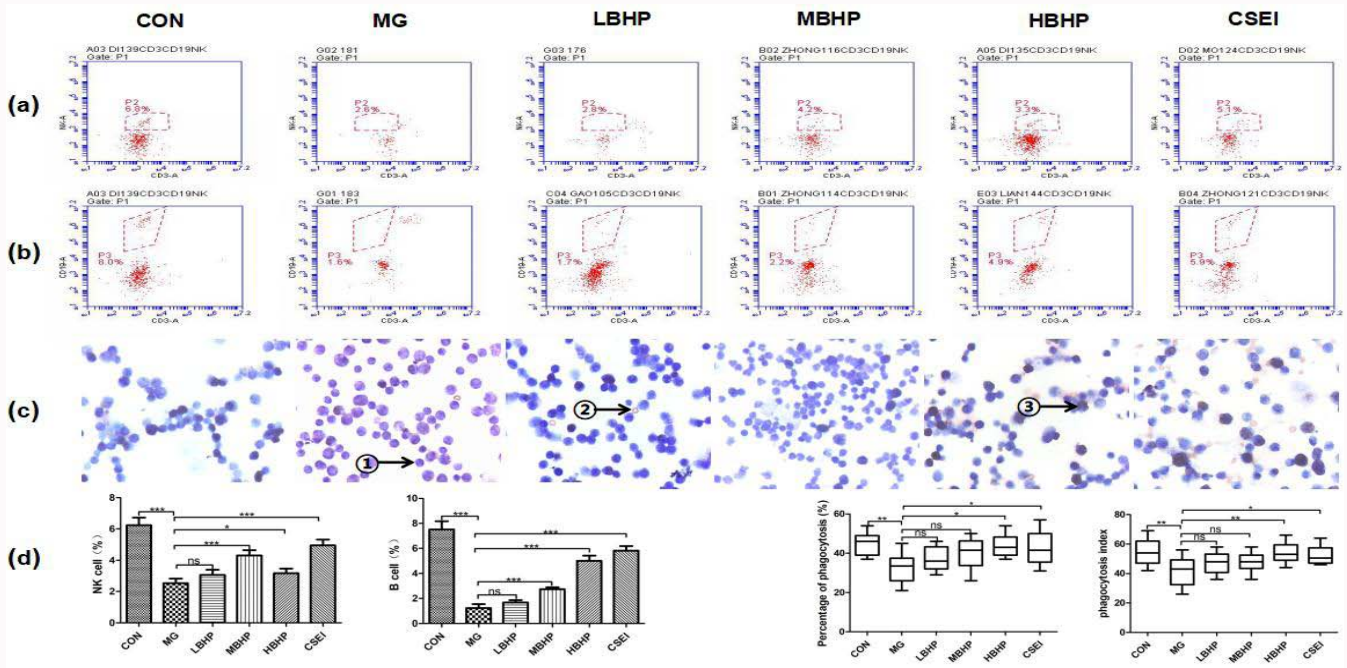

Figure 2: The percentage of NK cells (2a, 2d) and B lymphocytes (2b, 2d) in peripheral blood and the Wright-Giemsa staining results (2c), macrophage phagocytic percentage (2d) and phagocytic index (2d).

The percentage of NK cells and B lymphocytes in MG nude mice was significantly lower than in the CON group. The percentage of NK cells (2a, 2d) and B lymphocytes (2b, 2d) was significantly higher in the BHP and CSEI group than the MG group. BHP may increase the lymphocyte percentage by stimulating the proliferation and differentiation of lymphocytes in tumor-bearing nude mice, which in turn can improve the immune function of tumor-bearing nude mice. 2c shows 1: Nonphagocytic macrophages, 2: noninternalized chicken erythrocytes, and 3: chicken erythrocytes internalized by macrophages. The phagocytic ability of macrophages can be used to measure the cellular immune function to some extent. The phagocytic percentage (2d) and phagocytic index (2d) of MG nude mice was significantly lower in the CON group. After BHP and CSEI treatment, these two indexes showed an increasing trend (compared with MG), and there were significant changes in the HBHP and CSEI groups.

"ns" P>0.05; "*" P=0.01-0.05; "**" P<0.01; "***" P<0.001. All data were from two independent experiments, each repeated in parallel twice.

Determination of TNF- α, NF- κB, LC3, AKT1, and COX-2 protein expression

As Figure 4a and Figure 5 shows, the expression of TNF-α and NF-κB protein was significantly higher in spleen of MG nude than the CON group (P<0.001), while it was significantly lower in the BHP and CSEI groups than in MG nude mice (TNF-α, NF-κB after BHP treatment, P<0.05). The expression of TNF-α and NF-κB was significantly lower after CSEI treatment in comparison to MG nude mice (P<0.001). The expression of LC3 protein increased in tumor tissues after treatment with BHP and CSEI, and the enhancement of BHP and CSEI at certain doses was significant (P<0.001). These results suggested that BHP could induce autophagy. The expression trend of AKT1 and COX-2 protein was opposite to that of LC3 and decreased in the BHP and CSEI-treated groups. Our results indicated that BHP induced autophagy; down regulated the expression of NF-κB/TNF-α/AKT1/COX-2 protein and participated in the regulation of immune function in nude mice.

Determination of TNF-α, NF-κB, COX-2 and Beclin1 in serum

The content of TNF-α, NF-κB and COX-2 was significantly higher in the serum of MG nude than in the CON group (P<0.05 and P<0.001). The BHP and CSEI treatments showed a decreasing trend, and the degree of reduction was related to the dose (Figure 4b). The content of Beclin1 was significantly lower in the serum of MG nude mice than in the normal group (P<0.001). After CSEI treatment, the serum level of Beclin1 has recovered, but the degree of recovery was dose-dependent, especially in the MBHP and HBHP groups (Figure 4b). The above results showed that BHP could induce autophagy enhancement in tumor-bearing nude mice, thereby improving immune function.

Discussion

Gastric cancer is a stubborn and grave disease. Conventional treatment has many negative effects, such as postoperative complications and a high recurrence rate. Traditional drug treatments include steroids to improve the appetite of cancer patients, non-steroidal anti-inflammatory drugs to inhibit systemic inflammation, and multidrug combination with multitarget therapy, leading to drug deposition causing a variety of side effects in patients. During the course of disease, radiotherapy and chemotherapy not only have a certain killing effect on tumor cells but also cause damage to normal human cells and tissues, resulting in multiple organ failure, hair loss, myelosuppression, immunosuppression and many other side effects [17]. The above treatment methods treat the disease, yet they also reduce the quality of life of patients. It has become difficult for the medical profession to find a drug that can deliver a therapeutic effect and improve the quality of life of patients during the course of their disease, and BHP seems to provide little hope.

The spleen, as the largest immune organ in tumor-bearing nude mice, contains a large number of lymphocytes, which is the main location and source of humoral immunity. A change in the spleen index can reflect the change in spleen immune function to some extent. We found that the spleen index was significantly increased in nude MG mice than in the CON group, which might be due to the

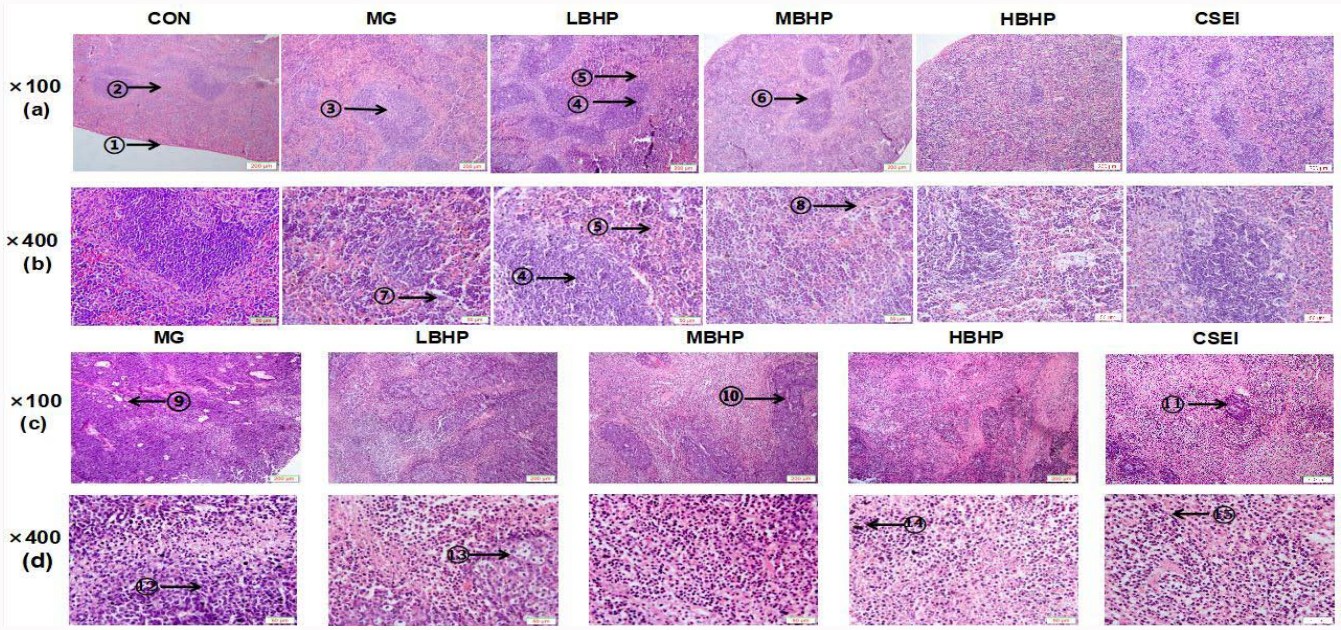

Figure 3: HE staining of spleens of tumor-bearing nude mice under a low-power microscope (3a) and a high-power microscope (3b), and tumor tissue HE staining under a low-power microscope (3c) and a high-power microscope (3d).

Figure 3 shows 1: Spleen capsule, 2: splenic trabecula, 3: germinal center, 4: white pulp, 5: red pulp, 6: marginal zone, 7: megakaryocyte, 8: septum, 9: blood vessel, 10: mucus pond, 11: cancer nest, 12: division phase cells, 13: multinuclear heterotypic nucleus, 14: deeply stained nucleus, and 15: nucleus arranged in a beaded shape. Germinal centers appear in spleen tissue. The spleen cells were neatly arranged in the CON group, and the spleen lymphocytes in the remaining groups were relatively disordered and displayed inflammatory infiltration. However, the red and white pulp structures were clear. The HE staining of tumor tissue revealed cancer nests, irregularly shaped cancer cells, large and deeply stained nuclei, and a nucleocytoplasmic ratio imbalance. A large number of atypical nuclei and pathological split images could be observed, and occasionally microvessels and a mucus cavity. The reduced content and irregular cavity shape indicated that the five groups all had subcutaneously transplanted tumor tissues. Taken together, these conditions were more common and obvious in the MG and LBHP groups. All the data were from two independent experiments, each repeated in parallel three times.

stress caused by splenomegaly due to the implantation of tumor cells. Despite the splenomegaly in MG nude mice, the percentage of NK and B lymphocytes (B cells) was lower than in the CON group, which might indicate that splenomegaly was not necessarily associated with a strong immune function. BHP could significantly increase the spleen index in tumor-bearing nude mice, indicating that it might enhance immune function in the spleen. NK cells play a cytotoxic and anti-tumor role mainly through immune clearance and immune surveillance [18], while B lymphocytes mainly play a regulatory role in tumors by secreting cytokines from regulatory B cells (Bregs) [19]. We found that BHP could significantly increase the percentage of NK cells and B lymphocytes in tumor-bearing nude mice, as well as enhance the immune function of tumor-bearing nude mice to achieve tumor suppression. Another study has also found that B cells will accumulate in large amounts in tumor tissue and play a dual role in anti-tumor immunity [20]. The upregulation of deacetylase Sirtuin1 (SIRT1) has been shown to lead to an increase in immunosuppressive factors (such as IL-10, PD-L1, and TGF- β) secreted by B cells and an inhibition of SIRT1-enhanced IgG secreted by B cells in colorectal cancer [21]. We know that B cells can stimulate anti-tumor immunity by producing antibody-dependent cytotoxicity mediated by IgG. These immunosuppressive molecules secreted by B cells can inhibit the proliferation of CD8+ T cells and thus inhibit anti-tumor activity and promote tumor progression. In addition, whether B cells play a positive or negative role in tumor immunity may be related to the tumor type, tumor stage and tumor development. On the one hand, NK cells can release cytotoxic particles such as perforin and granzyme through exocytosis, activate the caspase pathway and induce tumor cell apoptosis. On the other hand, they can synthesize and secrete cytokines (including TNFα) to mediate the killing effect of tumor cells [22]. The percentage of NK cells was reduced but the level of TNFα at the protein and mRNA levels was higher in MG nude mice than in the CON group, which indicated that the level of TNFα from other pathways had markedly changed. In addition, TNFα also has a dual role in tumors. On the one hand, it plays a role in anti-tumor immunity as a cytokine that kills tumors, but on the other hand, it promotes the development of tumors as an inflammatory factor. It has been found that the structural changes of TNFα affect its effect on tumorigenesis [23]. Additionally, research by Yu et al. has shown that a gene polymorphism of TNFα is also associated with the occurrence of gastric cancer [24]. These two findings may help explain why TNFα plays a dual role in tumorigenesis. Sun et al. found that cosilencing of TNFα and IL-1β could inhibit the proliferation and migration of gastric cancer [25]. These results show that TNFα has a greater contribution in promoting the development of gastric cancer. There are also reports in the literature that various stimuli such as trauma, ischemia, hypoxia and tumors can induce activation of NF-κB and then promote the large-scale synthesis and release of inflammatory factors such as TNF-α and IL-6, which causes a large amount of NF-κB expression and induces systemic inflammatory response syndrome to reduce the phagocytic function of phagocytes and suppress immune function [3,26]. In the macrophage phagocytosis experiment, starch was used as a stimulus for macrophages, and chicken erythrocytes were injected into mice as non-self foreign matter. We found that the expression of TNFα and NF-κB in spleen and serum were higher in nude MG mice than in the CON group, but the macrophage phagocytosis ability was lower. BHP largely improved this phenomenon, which is consistent with the above research results,

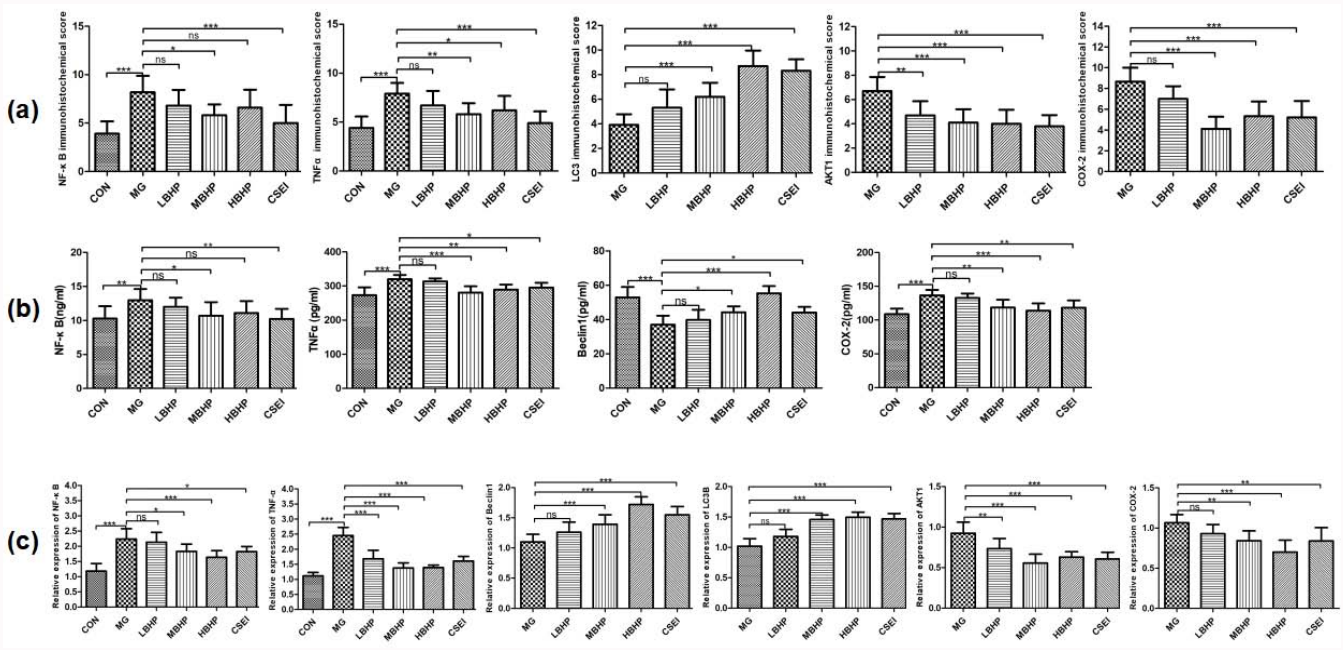

Figure 4: Immunohistochemical scores of NF-κB, TNF-α, LC3, AKT1 and COX2, the contents of TNF-α, NF-κB, COX-2 and Beclin1 in serum and the expression of related factors at the mRNA level.

The expression of NF-κB and TNF-α protein in spleen was significantly higher in MG mice than in the CON group (4a). Some doses of BHP and CSEI groups resulted in a significant decrease compared with MG mice. BHP and CSEI significantly increased the expression of LC3 protein and down regulated the expression of AKT1 and COX2 protein (4a) in tumor-bearing nude mice, indicating that BHP could induce autophagy to mediate the regulation of immune function in nude mice. 4b shows that the contents of NF-κB, TNF-α and COX-2 in serum were significantly higher in the MG group the CON group. After treatment with BHP and CSEI, the serum contents decreased compared with MG. The content of serum Beclin1 was significantly lower in MG nude mice than in the CON group, while it was higher in the BHP and CSEI groups than in MG mice, showing that BHP could enhance immune function by inducing autophagy in tumor-bearing nude mice. 4c shows that the relative expression levels of NF-κB and TNF-α mRNA in spleen tissue were significantly higher in MG than the CON group. The levels in BHP and CSEI were significantly lower than in MG. These findings confirmed that the immune function of MG nude mice might be increased under stress in the early stage of tumor development, but BHP could reduce the levels of NF-κB and TNF-α related to immune function. The mRNA expression level of Beclin1 and LC3B were increased in tumor tissues of nude mice in a dose-dependent manner with an increasing BHP dose, and this increase was significant in the MBHP and HBHP groups. These results showed that BHP could induce autophagy in tumor-bearing nude mice. The mRNA expression levels of AKT1 and COX2 in tumor tissues decreased after treatment with BHP and CSEI, and a certain dose of BHP could significantly down regulate the expression of AKT1 and COX2 in tumor-bearing nude mice.

"ns" P>0.05; "*" P=0.01-0.05; "**" P<0.01; "***" P<0.001. All data were from two independent experiments, each repeated in parallel three times.

indicating that BHP enhanced the phagocytic function of macrophages by inhibiting TNFα/NF-κB. Helicobacter pylori infection is a major factor that induces/promotes the formation of gastric cancer. Many researchers have studied the role of TNFα and NF-κB in the development of *Helicobacter pylori* infection in gastric cancer and found that inhibiting TNFα/NF-κB helps to inhibit the progress of gastric cancer [27-29]. NF-κB is activated by inflammatory factors such as TNFα, which contributes to tumorigenesis. In contrast, NF-κB can regulate the expression of TNFα [30]. Our results showed that TNFa and NF-κB were higher in MG than the CON group, which might be related to inflammation in the tumor microenvironment. The HE staining results showed different degrees of inflammatory cell infiltration in the red pulp, and BHP could effectively reduce the degree of infiltration. Inflammation can aggravate the occurrence and development of tumors, which indicates that BHP can inhibit the occurrence and development of cancer. COX-2, a member of the COX family, is rarely found in normal tissues and usually exists in inflammatory tissues and tumor tissues. COX-2 is also called an inflammatory mediator. Inflammation promotes tumor development by inducing COX-2 and activating NF-κB. We found that BHP could down regulate COX-2 in tumor tissue and serum of tumor-bearing nude mice, indicating that BHP might have an anti-tumor immune effect by reducing inflammatory mediators. Some people have observed changes in the tumor inflammatory microenvironment in a model of gastric cancer induced by COX-2 [31]. These findings show that inflammation induced by upregulation of COX-2 has a positive effect on tumorigenesis. A similar situation has been observed not only in animal models but also clinically in patients with gastric cancer. Some research groups have shown that COX-2 is promising as a prognostic marker for patients with gastric cancer, especially for first-stage clinical patients [16]. In addition, it has been shown that the progression of gastric cancer can be inhibited by regulating the immune response and apoptosis after inhibition of COX-2 [32]. These findings confirm once again that COX-2 is involved in anti-tumor immunity. The tumor microenvironment plays a key role in the occurrence and development of tumors. Inflammatory factors, immune cells and other factors in the tumor microenvironment may promote or inhibit tumor growth. Studies have shown that COX-2 in human breast cancer helps tumors escape the surveillance of the immune system by regulating the activity of T lymphocytes and macrophages [33-36]. COX-2 regulates T lymphocytes and macrophages in the tumor microenvironment, which can promote tumor angiogenesis and tumor cell movement by escaping immune system surveillance, thus aggravating the development of malignant tumor. In addition, COX-2 may also contribute to tumor escape by changing the structure and morphology of immune cells [37]. Inhibition of COX-2 can promote anti-glioma immune surveillance and inhibit the occurrence and development of glioma [38]. Garlic extract enhances the chemotherapeutic effect of doxorubicin by down regulating the expression of COX-2 in gastric cancer [39]. It has

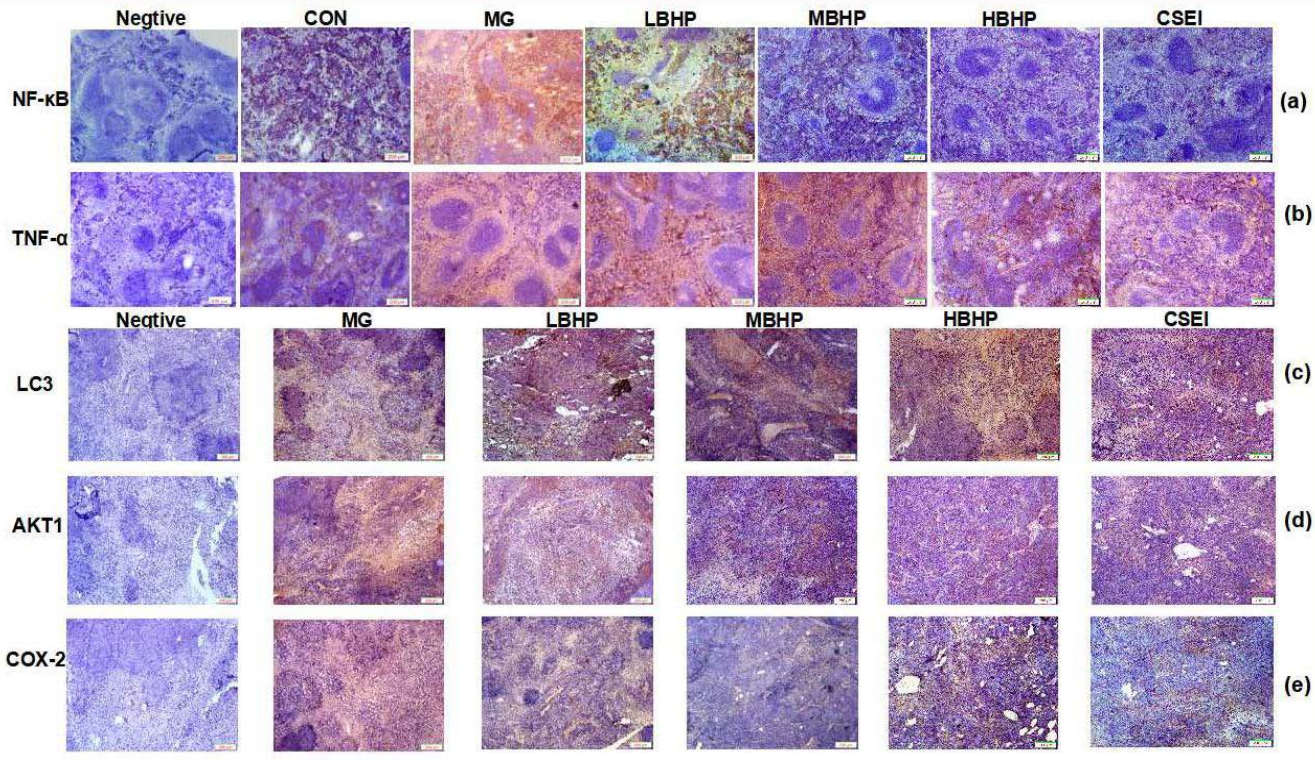

Figure 5: Immunohistochemical staining results for NF-κB, TNF-α, LC3, AKT1 and COX2.
The brownish yellow staining area and staining depth represent the protein expression. The positive expression of NF-κB (5a) and TNF-α (5b) was significantly elevated in the MG group than the CON group, and both BHP and CSEI showed a decreasing trend compared with MG. The expression of LC3 protein (5c) was higher in tumor tissue of nude mice treated with BHP and CSEI than in MG mice. The positive expression of AKT1 (5d) and COX2 (5e) was lower in tumor tissue in the BHP and CSEI-treated group compared with MG. These findings showed that BHP could down regulate the expression of AKT1 and COX2.

potential application value in chemoprophylaxis and treatment support of gastric cancer. Interestingly, hemorrhage is triggered during the process of high-speed proliferation and metabolism of tumor cells, resulting in an extracellular acidic environment. An extracellular acid environment can regulate the expression of many oncogenes and EMT-related transcription factors (including NF-κB and COX-2), and acidity plays a role in the migration of tumor cells from the acidic tumor environment to non-tumor environment [40-42]. It has been confirmed that ellagic acid can inhibit the invasion and metastasis of gastric cancer cells by inhibiting COX-2 induced by extracellular acid [43]. BHP may play an anti-tumor immune effect by down regulating the expression of COX-2. Based on these findings, we know that BHP may aid in changing the extracellular acid environment, which requires further study.

Autophagy is a phenomenon of self-phagocytosis, which can turn waste into treasure, that is, the process by which misfolded proteins, damaged organelles and other components (including inflammatory factors and oxygen free radicals) that are useless to cells (including inflammatory factors and oxygen free radicals) are internalized and converted into energy. It is a kind of immunogenic-stimulated cell death, also known as autophagy-induced death, which has good application prospects in the anti-tumor immunity of chemotherapy. In recent years, the relationship between autophagy and tumors has attracted wide attention, including the expression of autophagy in tumors and its role in tumorigenesis, as well as in tumors of different organs or different types of tumors in the same organs. The expression of autophagy in different stages of the same tumor is even different. In short, autophagy is a double-edged sword for tumors [44]. Autophagy in tumorigenesis is mainly reflected as follows: The maintenance of internal environment stability and inhibition of tumor formation by regulating the intracellular peroxide concentration and changing the disorder of protein metabolism, while decreased autophagy increases oxidative stress and the accumulation of tumorigenic mutations [45-47]. The process of autophagy includes autophagy induction, vesicle nucleation, autophagosome membrane elongation, autophagosome maturation, autophagosome degradation and reuse. LC3 participates in autophagy membrane elongation. BHP can enhance the expression of LC3 in tumor tissue, which shows that it plays a role in promoting the occurrence of autophagy. Beclin1 is not only a homologue of yeast ATG6 but also a specific gene that is indispensable during the vesicle nucleation phase for materials involved in autophagy [48]. The Beclin1 gene mainly regulates the localization of other ATG proteins in the structure of autophagy precursors and autophagy activity by forming a complex with type III PI3K (Phosphatidylinositol 3 Kinases). It has been shown that autophagy can be stimulated by up regulating the expression of Beclin1 in mammalian cells [49]. Other groups have found that Beclin1 deletion is associated with a poor prognosis in patients with ovarian cancer [50]. Knockout of Beclin1 can promote the growth of tumor cells and inhibit apoptosis [51]. These findings show that Beclin1 plays an important role in anti-tumor. BHP can upregulate LC3 and Beclin1 in tumor tissue of tumor-bearing nude mice, which may promote an anti-tumor immune response by inducing autophagy. Of course, contradictory findings have been obtained. A research group has reported that knockout of the Beclin1 autophagy-related genes Beclin1 and LC3 can inhibit tumor cell growth, invasion and metastasis, potentially due to the dual role of autophagy in both inhibiting and promoting tumor development

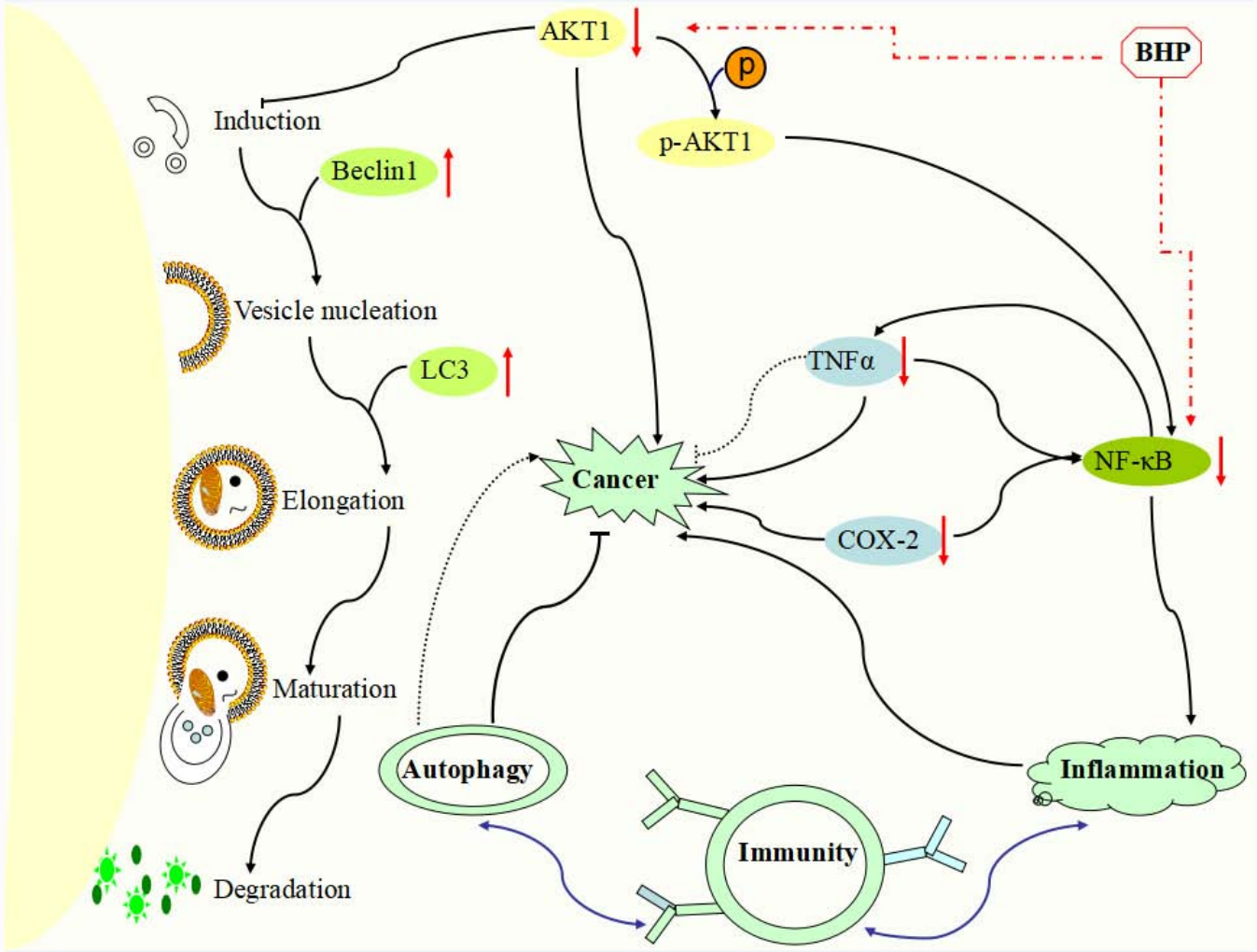

Figure 6: BHP enhances the immune function of nude mice by inducing autophagy and inhibiting inflammation.
The occurrence of autophagy mainly includes the induction and formation of autophagy, autophagosome membrane extension, autophagosome maturation and autophagosome lysis. AKT1 plays an important role in the formation of autophagy. LC3 participates in the induction of autophagy, and Beclin1 participates in the extension of the autophagosome membrane. BHP inhibits tumorigenesis and development by inhibiting AKT1 expression and promotes autophagy. There is a certain relationship between autophagy and immunity. In addition, the suppression of inflammation is a manifestation of the role of the immune system. BHP improves the immune function of tumor-bearing nude mice by inhibiting inflammation factors TNFα, COX2 and transcription factor NF-κB to suppress inflammation.

[52]. AKT1, one of the main members of the AKT family (including AKT1, AKT2 and AKT3), mainly encodes serine/threonine protein kinases. Activation of AKT1 can alter many functions in cells, including motility, adhesion, participation in cell cycle regulation, promotion of cell proliferation and tumor neovascularization, leading to the malignant transformation of cells [53]. AKT1 is also recruited to participate in anti-tumor immunity during the process of tumorigenesis and development [54]. For example, AKT1 also plays a role in promoting tumorigenesis and malignant transformation in breast cancer [55]. AKT1 not only plays a role alone but also plays a role in anti-tumor immunity through some signaling pathways. AKT can be activated by an extracellular signal through a mechanism that is dependent on Phosphatidylinositol 3 kinase (PI3K). Activated AKT (p-AKT) m which plays a significant role in the induction of autophagy, is relocated to the cytoplasm, nucleus or other parts of the cell, phosphorylates a large number of substrate proteins, and then regulates cell function. Mis-expression or mutation of proteins regulated by the PI3K/Akt pathway may lead to uncontrollable cell proliferation, growth and angiogenesis [56-58]. The PI3K/Akt pathway is activated in gastric cancer, which may become a target for treatment and prognosis of patients with gastric cancer [59]. Normally, p-AKT originates from the phosphorylation of AKT. Our results showed that BHP could down regulate the expression of AKT1 in tumor tissue, after which p-AKT1 was decreased indirectly, which could induce further autophagy. Novel AKT1 inhibitors can induce autophagy-related death in hepatocellular carcinoma cells [60]. However, some research results indicate that overexpression of Akt1 can inhibit autophagy and increase the anti-tumor effect, in contrast to our findings [61]. These researchers believe that overexpression of AKT1 can inhibit autophagy, which is consistent with our results, but the increase in anti-tumor activity is inconsistent with our results, which is also a sign of the positive and negative role of autophagy in tumors. In addition, p-AKT can also promote the expression of NF-κB [62]. Activation of NF-κB and the production of pro-inflammatory factors can induce autophagy. Abnormal autophagy leads to highly activated NF-κB, and the accumulation of pro-inflammatory factors stimulates inflammation. The inflammatory microenvironment can aggravate the development and deterioration of tumor. Therefore,

the continuous activation of NF-κB plays an important role in tumorigenesis and development. For example, persistent activation of NF-κB has been detected in breast, ovarian, colon, thyroid, and prostate cancers [63-67]. In endometrial carcinoma expressing p-AKT, the activity of NF-κB was upregulated, and p-akt could increase the expression of the COX-2 gene through NF-κB, which could promote the deterioration of endometrial carcinoma [68,69]. Our results showed that BHP could down regulate not only AKT1 but also NF-κB, although in different tissues of the spleen and tumor, p-AKT might play a role in regulating the expression of NF-κB. Simultaneously, PI3K/Akt is a classic autophagy pathway. A relationship has been found between the PI3K/Akt pathway and autophagy in gastric cancer [70]. Oshima and Masuda also showed that up regulation of the PI3K/Akt1 downstream pathway is associated with a poor prognosis in gastric cancer, which may contribute to the resolution of chemotherapy resistance [71]. *Helicobacter pylori* are an important cause of chronic gastritis and one of the risk factors for the formation of gastric cancer. The factor induced by autophagy may be a new antibiotic for the treatment of this kind of gastritis [72]. Some research groups have confirmed that the method of inducing autophagy can improve the effect of anticancer therapy [73]. In addition, BHP has the characteristics of most bioactive peptides. After entering the digestive system, it is broken down into short peptides, which can be more easily absorbed and utilized by the body than individual amino acids. Therefore, BHP may be a potential biological agent for the prevention and treatment of gastric cancer, and it may also be developed as a health food/drug to enhance the immunity of patients with gastric cancer. In addition, BHP may also play a role in inflammation and/or other diseases involved in autophagy.

There are still some limitations in the design of this study. For example, there was no autophagy inhibitor group, and there was no systematic verification of the p-AKT1 and its PI3K/AKT pathway. Due to limited experimental conditions and funding, electron microscopes and Western relatively advanced experimental instruments and technical methods were not used. The mechanism by which BHP improves immune function requires further in-depth study to provide a theoretical basis, experimental data support, and facilitate new ideas for its application in the prevention and treatment of GC and its development as a preparation dedicated to GC patients to enhance immunity. In addition to inflammation and autophagy, the pathogenesis of gastric cancer also has very complicated components, and certain difficulties are associated with its treatment. Since it is impossible to destroy all tumor cells at present, the enhancement of the autoimmune function to inhibit the proliferation and spread of tumor cells, delay the progress of gastric cancer, and control its development within the body can represent other strategies to overcome tumors.

Conclusion

BHP may inhibit inflammation through the NF-κB pathway and down regulate AKT1 to induce autophagy, and it may have an anti-tumor and immune-enhancing role in nude mice bearing tumors (Figure 6).

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Authors' Contribution

CC performed the experiments and wrote the manuscript. HG participated in the experiments, drew and edited the figures. XL designed the research and analyzed the experimental data. All authors read and approved the final manuscript.

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