Supporting Information

Changes in the Serum and Tissue Levels of Free and Conjugated Sialic Acids, Neu5Ac, Neu5Gc, and KDN in Mice after the Oral Administration of Edible Bird's Nests: an LC–MS/MS Quantitative Analysis

Meng-Hua Wang [1], Zhi-Fan Wang [1], Man Yuan [2,3], Chun-Guo Yang [4], Dong-Liang Wang [2,3,*] and Shu-Qi Wang [1,*]

1.School of Pharmaceutical Science, Shandong University, Jinan 250012, Shandong, China;

2. Beijing Rongshutang Biotechnology Co., Ltd., Beijing 100020, China;

3. Hebei Edible Bird's Nest Fresh Stew Technology Innovation Center, Langfang 065700, Hebei, China;

4. Shandong Yifang Pharmaceutical Co. Ltd., Jinan 250012, China;

1. Immunomodulatory functions of low-temperature stewed edible bird's nests.

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Table S1. Effect of stewed edible bird's nest on organ/body weight ratio in mice

Table S2. Effect of stewed edible bird's nest on DTH and CONA-induced lymphocyte transformation in mice

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Correspondence:

Dr. Dong-Liang Wang (dongliang.wang@xxdun.com)

Dr. Shu-Qi Wang (wangsq@sdu.edu.cn)

1.1 Experimental

1.1.1 Chemicals

Low-temperature stewed edible bird's nest, provided by Beijing Xiaoxian Stew Biotechnology Co., Ltd. (Shanghai, China), the freeze-dried powder is obtained after freeze-dried treatment. Clean bench (Suzhou Antai Airtech Co., Ltd., Jiangsu, China), carbon dioxide incubator (Thermo Fisher Scientific Co., Ltd., Shanghai, China),, centrifuge (Sigma-Aldrich, Shanghai, China), constant temperature water bath (Shanghai Lichen Instrument Technology Co., Ltd., Shanghai, China), enzyme marker (Thermo Fisher Scientific Co., Ltd., Shanghai, China). Sheep red blood cells (SRBC), chicken red blood cells, normal saline, Hank's solution (PH7.2), RPMI-1640 culture solution, calf serum, actinomycin, Concanavalin A (ConA), 1% glacial acetic acid, 1mol L^{-1} HCL, isopropyl alcohol, Agarose, YAC-1 cells, MTT, PBS buffer, complement (guinea pig serum), SA buffer, lithium lactate, iodine-nitro tetrazolium chloride, phenazine dimethyl sulfate, oxidized Coenzyme I, Tris-HCL, Venzi's reagent, 1% NP40, Indian ink, 0.1% Na2CO3, Giemsa dye, etc : All reagents was purchased from Shanghai Macklin Biochemical Co., Ltd. (Shanghai, China).

1.1.2 Animals

Female ICR mice were used in testing for oral administration of bird's nest immunological effect. The experiment used SPF-grade female ICR mice (18−20 g)

supplied by the Beijing vital river laboratory animal technology Co., Ltd. Lab (No. SYXK 2022-0006). The animals were housed in a temperature- and humidity-controlled facility (temperature 20–26 ˚C and 40–70% humidity) maintained on a 12-h light/12-h dark cycle and given a standard laboratory rodent diet**.**

The experiment set up blank control group and low, medium, and high doses of low-temperature stewed edible bird's nest group (n=12). The method of oral gavage was used. The low, medium and high doses were set as 5 times, 10 times and 30 times of the recommended consumption of low-temperature stewed edible bird's nest, namely 0.188 g kg^{-1}, 0.375 g kg^{-1}, and 1.125 g kg^{-1}. Gavage was administered at 0.2 mL /(10 g·BW). The amount of gavage was adjusted weekly according to body weight for 30 days.

1.1.3 The organ/body weight ratio of mice was measured

After weighing, the cervical vertebra was dislocated and killed. Spleen and thymus were removed, fascia was removed, and blood on the surface of organs was drained with filter paper. Spleen/body weight ratio and thymus/body weight ratio were calculated.

1.1.4 Delayed allergic reaction was determined by the plantar thickening method

Fresh defibrillated sheep blood was taken, washed with normal saline for 3 times, and each mouse was intraperitoneally injected with 2% (v/v, prepared with normal saline) SRBC 0.2 mL. The thickness of the left hind foot was measured 4 days after sensitization, the same part was measured three times, and the average value was taken.

Then 20% (v/v, prepared with normal saline) SRBC 20 μL was injected subcutaneously at the measurement site, and the left posterior plantar thickness was measured 24 h after injection. The average value of three measurements at the same site was taken, and the difference in plantar thickness before and after the attack was used to indicate the degree of delayed allergic reaction.

1.1.5 Mouse lymphocyte transformation induced by ConA

After the mice were killed, the spleen was sterilized in a 75% alcohol beater for 10 min, and the spleen was taken aseptically and placed in a sterile plate of 3 cm×3 cm four-layer gauze with an autoclave. The appropriate amount of sterile Hank's solution was added, the spleen was wrapped in the gauze, and the spleen was gently ground by bending tweezers to make a single-cell suspension, which was washed with Hank's solution three times. Centrifuge at 1000 rpm for 10 min each time, count the number of viable cells, adjust the cell concentration to 2×10^7 /mL, take 0.4 mL cell suspension into two holes, and add it into the 24-well plate, the total volume of each hole is 1mL, one hole is added with 75 μL ConA solution, the other hole as the control, and culture it in 5% CO_2 at 37 ℃ for 72 h. 4 h before the end of culture, 0.7 mL of supernatant was gently absorbed from each well, 0.7 mL of RPMI1640 culture medium without calf serum was added, and 50 μL of 5 mg mL^{-1} MTT was added to each well, and the culture was continued for 4 h. After the end of culture, add 1 mL of acid isopropyl alcohol per well, blow evenly so that the purple crystal is completely dissolved. It was transferred into a 96-well plate and measured on an enzyme-labeled instrument with a wavelength of 570 nm. Where the proliferation capacity of lymphocytes = the optical density OD value of the ConA hole - the optical density OD value of the ConA hole is not added.

1.1.6 Antibody-producing cell detection experiment

Fresh defibrillated sheep blood was taken, and washed with normal saline three times, and each mouse was intraperitoneally injected with 2% (v/v, prepared with normal saline) SRBC 0.2 mL. The mice were killed 4 days after sensitization, and the whole spleen was taken to make cell suspension and 8 mL of Hank's solution was added. After the surface medium was dissolved by heating, it was mixed with an equal amount of Hank's solution, divided into small test tubes, 0.5 mL each, and then added 10% (V/V, prepared with SA buffer) into the tube to accumulate SRBC 50 μL and spleen cell suspension 20 μL, quickly mixed, and poured onto the slide with AGAR thin layer. After the AGAR was solidified, the slides were placed horizontally on the rack and incubated in a CO_2 incubator for 1.5 h, and then the complement diluted with SA buffer (1:8) was placed in the groove of the slide rack for 2.0 h, and the number of hemolytic plaques was counted.

1.1.7 Determination of serum hemolysin half hemolysis value (HC_{50})

Fresh defibrillated sheep blood was taken and washed with normal saline 3 times (2000 rpm, 10 min). Each mouse was intraperitoneally injected with 2% (v/v, prepared with normal saline) to collect 0.2 ml SRBC.4 days after sensitization, the eyeball was removed and blood was taken into 1.5 mL centrifuge tube and placed for about 1 h to fully precipitate the serum. Centrifuge at 2000 rpm for 10 min and collect serum. The diluted serum was diluted 300 times with SA buffer, 0.1 mL was placed in 96-well plates, followed by 0.05 mL of 10%(v/v) SRBC and 0.1 mL of complement (diluted with SA buffer at 1:8), and kept in a constant temperature water bath at 37 ℃ for 30

min. The reaction was terminated in an ice bath. Centrifuge at 1500 rpm for 10 min, take 0.05 mL supernatant and 0.15 mL Garvenzi's reagent; meanwhile, take 10% (v/v, prepared with SA buffer) to compacted 0.0125 mL SRBC, and place Garvenzi's reagent to 0.2 mL on another 96-well plate, and mix thoroughly. After being placed for 10 min, the optical density of each hole was measured at 540 nm wavelength by an enzyme-labeled instrument. The amount of hemolysin is expressed as a half hemolysis value (HC50) and calculated by the following formula: sample HC_{50}= (sample optical density value /SRBC optical density value at half hemolysis) × dilution ratio

1.1.8 Statistical Analysis

All the data obtained in this experiment were statistically analyzed by GraphPad Prism 9.4 data processing system. The data is expressed as "Mean ± SD". LSD test was used for comparison between groups.

1.2 Results and Discussion

1.2.1 Effects of low-temperature stewed edible bird's nest on immune organ/body weight ratio of mice

The effects of low-temperature stewed edible bird's nest on the weight ratio of thymus and spleen in mice are shown in Table S1. Thymus and spleen are important immune organs of the body, which can play an important role in the immune function of the body by regulating the immune response, and their weight ratio indicates the innate immune ability of the body to a certain extent. In this experiment, when different doses of low-temperature stewed edible bird's nest were administered orally for 30 days, there was no significant difference in the thymus/body weight ratio and spleen/body

weight ratio of mice compared with the blank control group (P>0.05), indicating that low-temperature stewed edible bird's nest had no effect on immune organ index.

1.2.2 Effects of low-temperature stewed bird's nest on cellular immune function of mice

Delayed allergic reaction is due to the antigen entering the body, the cell receives the antigen information locally and transforms into sensitized lymphocytes. When the sensitized lymphocytes come into contact with the same antigen again, they release a variety of lymphatic factors, prompting the monocytes in the bloodstream to gather locally and phagocytose the antigen. A delayed allergic reaction is an exudative inflammation dominated by infiltration of lymphocytes and mononuclear macrophages, so the strength of cellular immune function can be reflected by measuring the swelling thickness of the skin after being stimulated by the same antigen again. As shown in Table 2, there was no significant difference in the degree of the swelling between each dose group and the control group.

Lymphocytes are key participants in the adaptive immune response, primarily responsible for activating antigen presentation and mitogen stimulation processes. Splenic lymphocyte proliferation is an important biomarker of activation of cellular and humoral immune responses, and this activity is induced by mitogen Con A, which is commonly used to assess T or B lymphocyte activity. The test results of low-temperature stewed edible bird's nest on the proliferation of mouse lymphocytes were shown in Table S2. The low-temperature stewed edible bird's nest medium dose group could significantly promote the proliferation of spleen lymphocytes stimulated by

ConA ($P<0.01$).

1.2.3 Effects of low-temperature stewed edible bird's nest on humoral immune function of mice

Hemolytic plaque test is a method to quantitatively measure samples to enhance humoral immune response, which can be used to detect and count antibody-producing cells (plasma cells) that produce IgM and other types of immunoglobulins. The number of hemolytic plaques is the number of antibody-producing cells, which can be used to characterize the strength of humoral immune function. As shown in Table S3, the number of antibody-producing cells in mice induced by low-temperature stewed edible bird's nest can be significantly increased in each dose group ($P<0.01$). In addition, serum hemolysin level, as an important indicator of humoral immunity, can reflect the ability of B cells to secrete hemolysin after contact with specific antigens. There was no significant difference in median hemolytic value (HC_{50}) between the high, medium, and low doses and the control group ($P>0.05$).

Table S1 Effect of stewed edible bird's nest on organ/body weight ratio in mice ($\overline{X} \pm$ SD, n=12)

Group	weight ratio of thymus（mg g^{-1}）	weight ratio of spleen (mg g^{-1})
Control	2.12 ± 0.44	5.48 ± 1.25
Low dose	2.31 ± 0.42	5.37 ± 1.16
Medium dose	2.22 ± 0.47	6.10 ± 1.10
High dose	2.05 ± 0.38	5.78 ± 0.95

Table S2 Effect of stewed edible bird's nest on DTH and CONA-induced lymphocyte transformation in mice ($\bar{X} \pm$ SD, n=12)

Group	Toe swelling（mm）	Lymphocyte proliferation capacity (OD difference)
Control	0.37 ± 0.19	0.067 ± 0.043
Low dose	0.39 ± 0.19	0.199 ± 0.154
Medium dose	0.36 ± 0.18	0.346 ± 0.202**
High dose	0.36 ± 0.15	0.119 ± 0.056

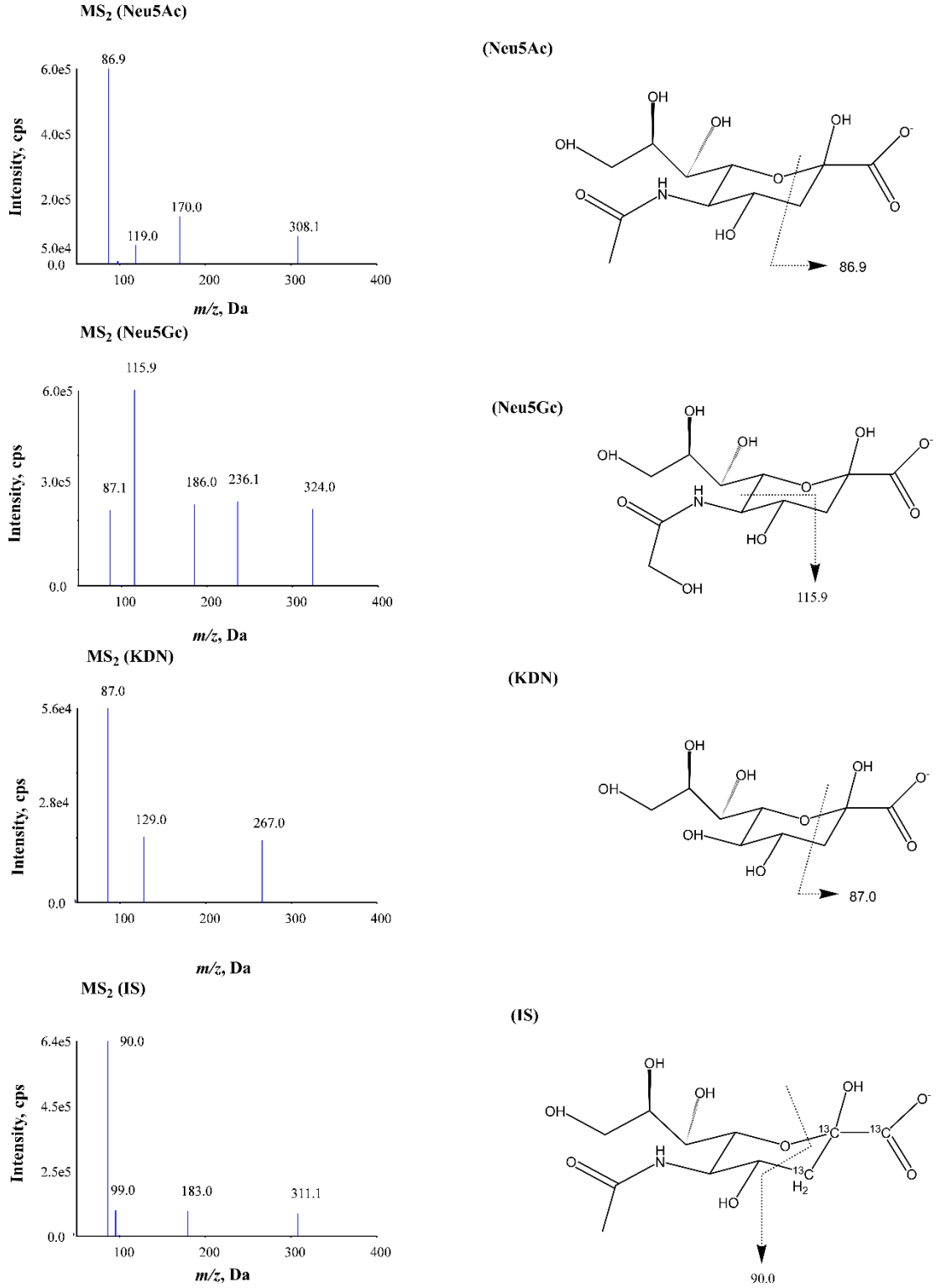
Note：** $p < 0.01$, compared with the control

Table S3 Effect of fresh stewed edible bird's nest on the number of antibody-producing cells and HC 50 ($\bar{X} \pm$ SD, n=12)

Group	Number of hemolytic plaques ($\times 10^3$)	Half the hemolytic value HC_{50}
Control	108.67 ± 13.28	75.33 ± 8.98
Low dose	138.00 ± 15.36**	79.13 ± 12.46
Medium dose	130.08 ± 9.97**	78.56 ± 8.78
High dose	174.17 ± 15.79**	80.79 ± 12.51

Note：** $p < 0.01$, compared with the control

2. Figure S1. Product ion mass spectra of the $[M-H]^-$ ions of Neu5Gc, Neu5Ac, KDN, and IS.



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