Effect of IDP® and Lactoferrin Whey Protein Powder on Immune Function in Mice

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Abstract: Immune Defense Proteins (IDP®) is a complex of milk-derived bioactive proteins with a variety of physiologically active functions. The objective of the study is to investigate the effects of IDP® and lactoferrin whey protein powder (which is commercially available as a registered health food) on immune function in mice, and compare the differences in effects, so as to provide evidence for further development and utilization of IDP®. SFP-grade ICR mice were randomly divided into 7 groups. IDP® dose groups of 0.41, 0.83, 2.50g/kg BW, lactoferrin whey protein powder groups of 0.17, 0.33, 1.00g/kg BW, and a negative control group were set up. After 30 days of continuous gastric perfusion, the immune organs index, cellular immune function, humoral immune function, monocyte-macrophage function, and NK cell activity were measured. The results showed that, compared with the control group, IDP® and lactoferrin whey protein powder in the medium and high dose group could significantly enhance SRBC-induced delayed type hypersensitivity and significantly enhance the ability of peritoneal macrophages to phagocytose chicken erythrocyte (P<0.05, P<0.01). The results showed that both samples had the function of enhancing immunity. The enhancement effect of IDP® on cellular immune function and monocyte-macrophage function was similar to that of lactoferrin whey protein powder which has been marketed as immune-enhancing product, with no statistical difference.

Keywords: IDP®; lactoferrin whey protein powder; immunoregulation; mice

CLC number: R151.2 Document code: A Article number:

Lactoferrin is a non-hematogenous glycoprotein, mainly distributed in exocrine secretion in human body and has a variety of physiologically active functions such as antimicrobial [1-2], antiviral [3-4], antioxidant [5-8], radioprotective [9], immunoregulation [10-12], anti-inflammatory [13] and tumor development inhibition [14-18]. In recent years, lactoferrin has been widely used in infant formula products, health food, drugs, etc. due to its many biological functions [19-22].

Immune Defense Proteins (IDP®), derived from milk and with a variety of active ingredients, is a patented and formulated protein complex composed of over 50 milk-based active proteins. The main protein is lactoferrin (minimum 40%), which supports the immune system through natural protection against inflammation and infection. Other key IDP® proteins include lactoperoxidase (minimum 18%), immunoglobulin G1, angiogenin, lysosomal alpha-mannosidase and ribonuclease-4. The objective of the study is, through the integral animal test, to investigate the effects of IDP® and lactoferrin whey protein powder (which is commercially available as a registered health food) on immunoregulation function in the body, and compare the differences in effects, so as to provide scientific evidence for rational development and utilization of IDP®.

1 Materials and Methods

1.1 Materials

IDP® blended into a milk powder base was produced and provided by Quantec Biotechnology (Hangzhou) Co., Ltd. The sample is milky white powder with a recommended intake of 5.0g/day for human body, i.e. 0.083g/kg BW/day for 60 kg body weight, and contains 100mg of IDP® that in turn contains 40mg lactoferrin.

The lactoferrin whey protein powder was bought from the market as a registered health food in China with the function of enhancing immunity. The sample is light pink powder with a recommended intake of 2.0g/day for human body, i.e. 0.033g/kg BW/day for 60 kg body weight, and contains 260mg of lactoferrin per daily intake.

Received date: 13588080482
Fund project:
First author introduction:

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1.2 Laboratory animals

ICR mice for test are provided by Zhejiang Laboratory Animal Center, SPF grade, female, 280 mice, weighing 18~22g. The quality certificate number of laboratory animals is 1808030006. The production license number of laboratory animals is SCXK (Zhejiang) 2014-0001, and the use license number of laboratory animals is SYXK (Zhejiang) 2014-0008. The experimental environmental conditions are: room temperature 20~25℃, relative humidity 40~70%.

1.3 Instruments and reagents

1.3.1 Instruments

CO_2 incubator (Thermo Co., Ltd., USA); FilterMax F5 multi-mode microplate reader (Molecular Devices Co., Ltd., USA); CKX41 inverted microscope (Olympus Co., Ltd., Japan), etc.

Received date: 13588080482
Fund project:
First author introduction:

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1.3.2 Reagents

Calf serum, RPMI-1640 culture media, concanavalin A (ConA), SRBC, chicken erythrocyte, complement, Indian ink, isopropanol, LDH substrate solution, Hank's solution, MTT, etc.

1.4 Methods

Animals were randomly divided into 7 groups according to body weight, and ConA-induced lymphocyte transformation test (LTT) and NK cell activity determination; DTH test, antibody-producing cell test, mouse serum hemolysin determination and organs index determination; mouse carbon clearance test; chicken erythrocyte phagocytosis test by mouse peritoneal macrophage were conducted respectively. IDP® groups of 0.41, 0.83, 2.50g/kg BW, lactoferrin whey protein powder groups of 0.17, 0.33, 1.00g/kg BW, and a negative control group (distilled water) were set up in the test. The low, middle and high doses of IDP® and lactoferrin whey protein powder groups are respectively 5, 10 and 30 times of their recommended intake for human body. After 30 days of continuous gastric perfusion as per 0.1mL/10g BW, the tests were conducted respectively.

1.4.1 ConA-induced mouse lymphocyte transformation test

After each mouse was killed, the spleen was taken aseptically and placed in a small dish containing a proper amount of aseptic Hank's solution, which was then gently teared up with tweezer to prepare the single cell suspension. The concentration was adjusted to 3×10^6 cells/mL with RPMI1640 culture media. The cell suspension was added into two wells of the 24-well culture plate, with 1mL per well, to one of which 75μL ConA solution (equivalent to 7.5ug/mL) was added, and the other well was used as the control. The plate was then placed in an incubator with 5% CO_2 at 37℃ for 72h. Four hours before the end of the cultivation, 0.7mL of supernatant was gently pipetted from each well, and RPMI1640 culture media without calf serum as well as MTT (5 mg/ml) as per 50μL/well was added to continue cultivation for 4h. After the cultivation was completed, 1mL of acidic isopropyl alcohol was added to each well and blown evenly to completely dissolve the purple crystal. The optical density value (OD) was measured at 570nm wavelength. Finally, the value obtained by subtracting the optical density value of the well without ConA from that of the well with ConA shall represent the lymphocyte proliferation ability.

1.4.2 Delayed allergic reaction test

Five days before the end of the test, each mouse was injected intraperitoneally with 0.2 mL of 2% (v/v) packed sheep red blood cell (SRBC) suspension for immunization. Four days after immunization, the planta thickness of the left hindfoot was measured, then 20% (v/v) SRBC was injected subcutaneously at the measurement site as per 20μL for each mouse, the planta thickness of the left hindfoot was measured 24h after injection, and the planta thickness difference before and after injection (planta swelling degree) was calculated.

1.4.3 Antibody-producing cells test

Each mouse was immunized by intraperitoneal injection of 0.2mL 2% (v/v) SRBC. After 5 days, the mouse’s spleen was taken aseptically to prepare the single cell suspension, and the concentration was adjusted to 5×10^6 cells/mL with RPMI1640 culture media. The surface culture middle was heated and dissolved, then placed in a 45℃ water bath for heat preservation, and mixed with equal amount of Hank's solution with double concentration, which was split into small test tubes as per 0.5mL for each tube, then 50μL of 10% (v/v) SRBC and 20μL of spleen cell suspension were added into the tubes, which were quickly mixed evenly and poured on the glass slide brushed with thin layer of agarose. After the agarose was solidified, the glass slide was flatly placed on the glass slide rack upside down, which was placed in a carbon dioxide incubator to incubate for 1.5h, then complement was added, after continuing to incubate for 1.5h, the number of hemolytic plaques was counted.

1.4.4 Mouse serum hemolysin determination

Each group received the test substance by gastric perfusion once a day for one month. Five days before the end of the test, each mouse was injected intraperitoneally with 0.2 mL of 2% (v/v) hematocrit sheep red blood cell (SRBC) suspension for immunization. Five days later, blood was collected by removing eyeball and centrifuged to collect serum. The serum was serially diluted with physiological saline. The different dilutions of serum were placed in the micro blood coagulation plate, as per 100μL for each well, and then 100μL of 0.5% SRBC suspension was added. After mixing, the plate was placed in a wet flat plate, capped,

and incubated for 3h in a 37℃ incubator. The hemagglutination degree was observed and the value of volume resistance was calculated.

1.4.5 Mouse carbon clearance test

Indian ink (0.1 mL per 10g body weight) was injected into the caudal vein of each rat. 20μL of blood was taken from the inner canthus venous plexus 2min and 10min after the injection of ink respectively, and added into 2mL of 0.1% Na_2CO_3 solution. The optical density (OD) value was measured at 600nm wavelength. The mouse was killed immediately after the second blood collection, the liver and spleen were weighed to calculate the phagocytic index a.

1.4.6 Chicken erythrocyte phagocytosis test by mouse peritoneal macrophages

Each mouse was immunized by intraperitoneal injection of 0.2 mL of 2% (v/v) SRBC suspension. Four days later, each mouse was intraperitoneally injected with 4mL of Hank's solution added with calf serum. The mice were killed by cervical dislocation and the abdomen was gently rubbed 20 times to wash out peritoneal macrophages. Pipette and add 0.5mL of peritoneal washing liquid into a test tube containing 0.5 mL of 1% (v/v) chicken erythrocyte suspension, and mix well. Pipette and add 0.5mL of the mixed solution into the agar circle on the slide. After incubation at 37℃ for 15min in the incubator, wash away the non-adherent cells quickly with physiological saline, fix in methanol solution for 1 min, dye in Giemsa solution for 15min, then rinse clean with distilled water and dry in the air. The phagocytic rate and index were calculated by counting under microscope.

1.4.7 NK cell activity determination

Each mouse's spleen was taken aseptically to prepare single cell suspension, and RPMI1640 culture media was used to adjust the concentration to 2×10^7 cells/mL, which was taken as the effector cell. YAC-1 cells were subcultured 24h before the test, and washed 3 times with Hank's solution before application, and the concentration was adjusted to 4×10^5 cells/mL with RPMI1640 complete culture media, which was taken as the target cell. Pipette and add 100μL of target cell and 100μL of effector cell (the ratio of effector cell to target cell is 50:1) into the U-shaped 96-well culture plate; add 100μL of target cell and 100μL of culture media to the natural release well of target cell, and add 100μL of target cell and 100μL of 1% NP40 to the maximum release well of target cell; prepare three parallel wells for each well above, incubate in an incubator with 5% CO_2 at 37℃ for 4h, then centrifuge the 96-well culture plate at 1500r/min for 5m, pipette 100μL of supernatant from each well into the flat bottom 96-well culture plate, then add 100μL of LDH matrix liquid for reaction for 8min, add 30μL of 1mol/L HCl to each well, measure the optical density value (OD) at 490nm of the enzyme reader, and calculate the NK cell activity.

1.4.8 Visceral organ/body weight ratio

After the animals were killed by cervical dislocation, the thymus and spleen were taken out and weighed, and the thymus/body weight and spleen/body weight ratio were calculated.

2 Results and Analysis

2.1 Effect of two samples on cellular immune function

MTT ConA-induced mouse spleen lymphocyte transformation test results showed that there was no significant difference in lymphocyte proliferation ability (OD difference) between each dose group of the two tested samples and the negative control group (P>0.05). DTH test results conducted by the planta swelling method showed that the planta swelling degree in the middle and high dose groups of the two samples were both higher than that in the control group and with statistical difference (P<0.05, P<0.01). There was no significant difference in the planta swelling degree between IDP® and lactoferrin whey protein powder (P>0.05). See Table 1.

Table 1 Comparison of ConA-induced lymphocyte proliferation ability and DTH results in mice ($\bar{x}\pm s$)

Samples	Group	Optical density difference	Planta swelling degree (cm)
Distilled water	Negative control	0.034±0.014	0.066±0.033

IDP®	Low dose group	0.032±0.021	0.071±0.028
	Middle dose group	0.033±0.019	0.107±0.015*
	High dose group	0.036±0.026	0.121±0.046**
Lactoferrin whey protein powder	Low dose group	0.039±0.031	0.092±0.036
	Middle dose group	0.046±0.020	0.119±0.022*
	High dose group	0.037±0.023	0.124±0.065*

Compared with the negative control group, *P<0.05, **P<0.01

2.2 Effect of two samples on mouse humoral immune function

The results of antibody-producing cell test by Jerne modified slide method showed that there was no significant difference in the number of hemolytic plaques between each dose group of the two tested samples and the negative control group (P>0.05).The results of mouse serum hemolysin determination by hemagglutination method showed that there was no significant difference between the value of volume resistance of mouse serum hemolysin in each dose group of the two samples and the negative control group (P>0.05). See Table 2.

Table 2 Effect of two samples on mouse antibody-producing cell test results and serum hemolysin test results ($\bar{x}\pm s$)

Samples	Group	Number of hemolytic plaques	Value of volume resistance
Distilled water	Negative control	29±3	113.7±12.5
IDP®	Low dose group	30±4	121.0±21.2
	Middle dose group	31±3	114.3±11.9
	High dose group	32±4	124.0±9.5
Lactoferrin whey protein powder	Low dose group	30±2	118.7±8.1
	Middle dose group	31±2	116.9±7.0
	High dose group	31±3	119.0±6.7

2.3 Determination of monocyte-macrophage function

The results of mouse carbon clearance test showed that there was no significant difference in the phagocytic index a of mice between each dose group of the two tested samples and the negative control group (P>0.05). The results of chicken erythrocyte phagocytosis test by mouse peritoneal macrophages showed that the phagocytic rate and phagocytic index of chicken erythrocyte by mouse peritoneal macrophages in middle and high dose groups of the two samples were both higher than those in control group (P<0.05, P<0.01). There was no significant difference in the phagocytic rate and phagocytic index between IDP® and lactoferrin whey protein powder (P>0.01).See Table 3.

Table 3 Effect of two samples on the results of mouse carbon clearance test and macrophage phagocytosis test ($\bar{x}\pm s$)

Samples	Group	Phagocytic index a	Phagocytic rate (%)	Phagocytic index
Distilled water	Negative control	4.379±1.923	28.3±3.1	0.37±0.04
IDP®	Low dose	6.036±1.778	29.2±3.2	0.38±0.04
	Middle dose	5.976±1.850	33.9±3.2*	0.42±0.03*
	High dose	6.134±1.612	34.9±3.1**	0.43±0.03**
Lactoferrin whey protein powder	Low dose	4.651±1.639	28.2±3.2	0.37±0.03
	Middle dose	5.461±1.586	32.0±2.1*	0.43±0.04*

	High dose	6.106±1.053	32.5±3.5*	0.44±0.03**

Compared with negative control group, *P<0.05, **P<0.01

2.4 Effect of two samples on NK cell activity and organ weight ratio

The results of NK cell activity determination showed that there was no significant difference in the NK cell activity of mice between each dose group of the two tested samples and the negative control group (P>0.05). There was no significant difference in thymus/body weight ratio and spleen/body weight ratio between each dose group of the two tested samples and the control group (P>0.05).

Table 4 Effect of two samples on NK cell activity and organ weight ratio ($\bar{x}\pm s$)

Samples	Group	NK cell activity (%)	Spleen/body weight (mg/g)	Thymus/body weight (mg/g)
Distilled water	Negative control	27.9±9.7	3.074±0.533	1.858±0.386
IDP®	Low dose	30.7±11.3	2.817±0.518	1.659±0.553
	Middle dose	28.5±3.0	2.851±0.578	1.919±0.457
	High dose	32.1±7.2	2.986±0.727	1.853±0.562
Lactoferrin whey protein powder	Low dose	28.1±5.8	2.949±0.993	1.724±0.267
	Middle dose	31.9±5.2	3.340±0.812	1.997±0.523
	High dose	29.6±8.8	2.974±0.654	1.820±0.532

3 Discussion

The immune system plays a vital role in balancing health and protecting human body from pathogens. The constituent cells of innate immunity are mainly macrophages, eosinophils, basophils, NK cells, etc., and the acquired immune response cells are mainly T cells and B cells. Related studies show that lactoferrin has a variety of immunoregulation functions such as promoting the maturation and activation of T cells and macrophages, lymphocyte proliferation, activating NK cells, etc. Zong, X, et al. have established the mouse model of lipopolysaccharide-induced systemic inflammatory response, and studied the effect of LFP-20 (an antibacterial peptide containing 20 amino acids at the N end of porcine lactoferrin) on immune homeostasis. The results showed that LFP-20 can regulate the secretion of IL-12p70, interferon-γ and TNF-α which are related to the activation of Th1 and the secretion of IL-4, IL-5 and IL-6 which are related to activation of Th2, through promoting cell defense mechanism and inducing B cells to produce some conditioned antibodies belonging to IgG subclass to prevent LPS stimulation [23]. Li Zhaoxia et al. have established the immunosuppressive animal model with cyclophosphamide, and discussed the immunoregulation effect of lactoferrin on immunosuppressive mice. The results showed that lactoferrin can repair the immune imbalance state of mice caused by cyclophosphamide to varying degrees and has immunoprotective effect [24]. Pi Bingbing et al. have studied the effects of lactoferrin and lactoferricin from different sources on the proliferation of mouse spleen lymphocytes. The results showed that both can improve the proliferation of concanavalin A-induced T lymphocytes and the proliferation of lipopolysaccharide-induced B lymphocytes within a certain mass concentration range [25]. Chang H et al. found that, in macrophage RAW264 cell line, lactoferrin can stimulate macrophages to express human B cell activating factor (BAFF) by mediation through Smad3 and Smad4 signaling pathways, thus stimulating B cell differentiation [26]. The delayed type hypersensitivity (DTH) is an in vivo detection method of cellular immunity, i.e., when sensitized T cells contact antigen again, T cells are activated, releasing a variety of cytokines, causing monocytes-based inflammatory reaction in local tissue.

The results of this study showed that, the increase of mouse planta swelling degree in middle and high dose groups of IDP® and lactoferrin whey protein powder, as shown in the results of mouse DTH test induced by sheep red blood cells (SRBC), suggested that both the two test substances can promote delayed type hypersensitivity and have the effect of enhancing cellular immune function. The test results of chicken erythrocyte phagocytosis by mouse peritoneal macrophages showed that the phagocytic rate and phagocytic index of chicken erythrocyte phagocytized by peritoneal macrophages increased in the middle and high dose groups of IDP® and lactoferrin whey protein powder, suggesting that both the two test substances could improve the phagocytic ability of monocyte-macrophage cells. According to the judgment criteria on

the function of enhancing immunity, i.e. the results of any two of cellular immunity function, humoral immunity function, monocyte-macrophage function and NK cell activity are positive, it can be judged that both IDP® and lactoferrin whey protein powder have the function of enhancing immunity. In this study, the enhancement effect of IDP® on cellular immune function and monocyte-macrophage function was similar to that of the lactoferrin whey protein powder (a registered health food with the function of enhancing immunity), and the difference was not statistically significant. The results of this study provide basic data for the further development and application of IDP®, but the mechanism of its function of enhancing immunity still needs further study.

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