Research on the enhancement effect of 6S-5-methyltetrahydrofolate calcium on the immunity of mice

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Abstract

Objective To observe the impacts of 6S-5-methyltetrahydrofolate calcium on the immunity of mice. Methods The mice were divided into the low dose group, the medium dose group, the high dose group and the negative control group, with 10 mice in each group. The low, medium and high dose groups were treated with 6S-5- methyltetrahydrofolate calcium of 0.8 mg/(kg·d), 1.7 mg/(kg·d) and 5.0 mg/(kg·d) by gavage respectively, while the negative control group was treated with the same volume of purified drinking water.

The impacts of 6S-5- methyltetrahydrofolate calcium on body weight, thymus/body weight ratio, spleen/body weight ratio, spleen lymphocyte transformation ability, delayed type hypersensitivity (DTH) reaction, hemolytic plaque number, serum hemolysin content, carbon clearance ability,macrophage phagocytosis of chicken red blood cells and natural killer (NK) cells activity in mice were observed. Results Compared with the negative control group, there was no significant difference in body weight among the three doses groups ($P > 0.05$), the ratios of thymus/body weight and spleen/body weight were relatively low ($P > 0.05$), and the degree of DTH in mice increased ($P > 0.05$). In the low, medium and high doses groups, the carbon clearance ability of mice increased in a dose-dependent manner ($P < 0.05$), and the NK cell activity increased significantly ($P < 0.05$). The ability of lymphocyte proliferation, hemolytic plaque number, antibody product number and macrophage phagocytic ability of chicken red blood cells increased in the medium and high doses groups ($P < 0.05$).

Conclusion

6S-5-methyltetrahydrofolate calcium can enhance the immunity of mice.

Key words

6S-5-methyltetrahydrofolate calcium; Enhance the immunity; Antibody producing cell detection test;
Determination of serum hemolysin; Carbon clearance test

Folate has become one of the most prevailing vitamins in recent decades due to its potential effects on the prevention of various diseases and disorder, such as neural tube defects (NTDs) in newborn, megaloblastic anemia, atherosclerosis, stroke, cancer, Alzheimer's diseases, cleft palate, migraine, and et al. [1-2] L-5-methyltetrahydrofolate calcium (L-5-MTHF-Ca) as a derivative of naturally occurring folate compounds, is the most bioavailable folate form. [3] Based on the fact that L-5-MTHF-Ca is unlikely to mask the symptoms of vitamin B12 deficiency, and thereby avoid the possible metabolic disorder of folate caused by the polymorphism of the gene encoding for 5,10-methylenetetrahydrofolate reductase, [4,5] which makes it compelling to the research

both in the medical and pharmaceutical field.
With the goal of exploring the ability of L-5-MTHF-Ca to enhance immunity, this article has investigated the action mechanism elaborately.

1. Materials and methods

1.1 Experimental Samples

The test objective L-5-MTHF-Ca, is an off-white crystallized powder with the batch number of 20141209.（Magnafolate® PRO made by Lianyungang Jinkang Pharmaceutical and Technology CO.,LTD） The powder was dissolved in purely potable water (all solvent in this work is water except for additional note) and diluted to an array of concentrations.

1.2 Experimental methods

1.2.1 The method of immunity enhancement [6]

1.2.1.1 The laboratory animal model

The SPF ICR mice were provided by B&K Universal Group Limited, (Shanghai, China) (male; weighing 16-20 g). The experimental animals were raised under aseptic conditions, at 20-24 °C and in a relative humidity of 40-60%. They began to acclimatize to the laboratory environment 4 days before the experiments. Other parameters: Production certificate NO. SCXK (Shanghai) 2013-0016, animal quality certificate No. 2008001646720, animal laboratory certificate NO. SYXK (Zhejiang) 2013-0816.

1.2.1.2 Dosage and grouping

The experiments were divided into four groups: the control group and three experimental groups， including low-, medium- and high-dose of L-5-MTHF-Ca groups, 10 mice in each group. Three experimental groups were at a dosage of 0.8, 1.7, and 5.0 mg/kg (mice weight), respectively, which were equivalent to 5, 10, and 30 times the recommended dosage of humans, respectively. The recommended dose of L-5-MTHF-Ca for humans is 10 mg/60 kg per day.

1.2.1.3 Gastric lavage

0.12 g of the test substance was dissolved into 240 mL of solvent, followed by homogenized to get a stock solution with a concentration of 0.5 mg/mL (high-dose). The medium-dose solution was prepared by diluting 70 mL of stock solution with 140 mL solvent to 0.17 mg/mL. The low-dose solution was prepared by diluting 70 mL of medium-dose solution with 70 mL solvent to 0.08 mg/mL. The mice were received either L-5-MTHF-Ca (n=10) or water (n=10) at 10 mL/kg by gastric lavage daily.

1.2.2 Concanavalin A (ConA) -induced spleen lymphocyte transformation test (MTT method)

40 mice were randomized to four groups by weight, followed by the gastric lavage treatment described in section 1.2.1.3 once per day over a period of 36 days. On the last day of tests, the mice were sacrificed by cervical dislocation and their spleens were harvested aseptically. The spleens were then placed in small Petri dishes containing sterile Hank’s buffers, respectively, and scratched into pieces with tweeze to obtain single-cell suspensions. The suspensions were filtered with 200-mesh screens and finally

dispersed into RPMI 1640 medium supplemented with 10% calf serum to a density of 3×10^6 cells/mL after rinsing with water for several times. 1 mL out of the purified cell suspension was seeded into two independent wells of the 24-well plate. For the experimental group, 75 μL of ConA solution (~7.5 μg/mL) was added to one of the wells and the other well was used as a control group. The plate was incubated at 37 °C, 5% CO_2 in humidified air in an incubator for 72 h. After incubation for 68 h, 700 μL of the supernatant was pipetted out and replenished with 700 μL of RPMI 1640 medium without calf serum and 50 μL of 5 mg/mL MTT to each well. After incubation for another 4 h, 1 mL of isopropanol acidified solution was added to each well and blowing the solution gently to dissolve the formazan crystals. Afterward, the solution was transferred to a 96-well plate and measured the optical density (OD) values at a wavelength of 570 nm, each group was conducted in triplicate wells. The capacity of lymphocyte proliferation was valued by the following equation:
The capacity of lymphocyte proliferation=The OD value of experimental group - The OD value of the control group.

1.2.3 Sheep red blood cells (SRBC)-induced delayed-type hypersensitivity (DTH) test (foot swelling method)

40 mice were randomized four groups by weight, followed by the gastric lavage treatment described in section 1.2.1.3 once per day over a period of 38 days. On the 34^{th} day of tests, the mice were immunized through intraperitoneal injection with 200 μL of 2% (v/v) packed SRBC suspension. The initial perimeter of the left hind footpads was measured 4 days after sensitization, hereafter the previously sensitized mice were challenged by subcutaneous injection with 20 μL of 20% (v/v) SRBC (~1×10^8 cells) into the left hind footpads. The DTH response was evaluated 24 h post-challenge by measuring the perimeter of footpads at the same part. Each measurement was repeated in triplicate to obtain a mean and the assessment of the DTH reaction in mice was represented by the difference of left hind footpad perimeter before and after the challenges.

1.2.4 Quantification of antibody-secreting cells (modified Jerne's method)

40 mice were randomized four groups by weight, followed by the gastric lavage treatment described in section 1.2.1.3 once per day over a period of 35 days. On the 31^{st} day of tests, each of the mice was immunized through intraperitoneal injection with 200 μL of 2% (v/v) packed SRBC suspension. All the mice were sacrificed by cervical dislocation post 4 days sensitization and their spleens were harvested by the described procedures in section 1.2.2, through which suspensions at a density of 5×10^6 cells/mL were obtained, respectively. The top layer medium (1g of agarose mixing with 100 mL of ddH_2O) was heated to melt and kept in a water bath at 45 °C, and then mixed with the same amount of double concentration of Hank's buffer. An aliquot of the solution was divided into tubes at a volume of 0.5 mL, respectively, and followed by adding 50 μL of 10% (v/v in SA solution) SRBC and 20 μL of spleen cell suspension (5×10^6 cells/mL). The mixture was quickly well-mixed and poured on the glass slides with a thin layer of agarose coating. All the slides were placed on the slide tray facing down at a horizontal position after the agarose solidified and incubated in CO_2 incubator for 1 h. The complement was diluted with a balanced salt solution at a ratio of 1:8, and then was added to the groove of slide tray, followed by incubation for another 1 h. The antibody-secreting cells were identified and

counted with a hemolytic plaque assay.

1.2.5 The determination of serum hemolysin in mice (Coagulation method)

40 mice were randomized four groups by weight, followed by the gastric lavage treatment described in section 1.2.1.3 once per day over a period of 39 days. On the 34th day of tests, each of the mice was immunized through intraperitoneal injection with 200 μL of 2% (v/v) packed SRBC suspension. The blood collection was then done post 5 days sensitization and the serum was collected by centrifugation. The serum was diluted with saline to an array of concentrations and subsequently deposited on a micro-sized coagulation plate with a volume of 100 μL to each well. Another 100 μL of 0.5% SRBC suspension was added to each well and then transferred to a wet flat plate, followed by covering with a lid and incubation at 37 °C incubator for 3 h. The degree of hemagglutination of each group was recorded to calculate the antibody level as the following method:
Antibody Level = (S_1+2S_2+3S_3...nS_n),
in which 1, 2, 3...n represents the exponent of double dilution, S represents the classification of hemagglutination.

1.2.6 Carbon granular clearance tests in mice

40 mice were randomized four groups by weight, followed by the gastric lavage treatment described in section 1.2.1.3 once per day over a period of 38 days. On the last day of tests, each mouse was administered with India carbon ink suspension (diluted with NaCl) through the tail vein at a dosage of 0.1 mL/kg. Retro-orbital blood collection was done at 2 and 10 min immediately after injection of carbon suspension. 20 μL of blood was added to 2 mL 0.1% $NaHCO_3$ solution and the UV-vis absorbance of the mixture was measured at a wavelength of 600 nm. The OD value of 0.1% $NaHCO_3$ without blood was measured as blank control. The mice were sacrificed to harvest the livers and spleens immediately post-second blood collection. The organs were cleaned with filter paper and weighed, respectively. The rate of carbon clearance, termed as the phagocytic index, was calculated by the following equations:

$$\mathrm{K} = \frac{logOD_2 - logOD_{10}}{10 - 2}$$

$$\alpha = \frac{weight}{liver\ mass + spleen\ mass} \times \sqrt[3]{K}$$

1.2.7 Phagocytic function of peritoneal macrophage

40 mice were randomized four groups by weight, followed by the gastric lavage treatment described in section 1.2.1.3 once per day over a period of 37 days. On the last day of tests, each mouse was administered with 1 mL of 20% (v/v) chicken red blood cell suspension through intraperitoneal injection. The mice were sacrificed post 30 min injection. 2 mL of saline was further intraperitoneally injected. After turning over the mice body for 1 min, 1 mL of the lotion was pipetted out to two glass slides. The slides were then transferred to Petri dishes with a lid covering and incubated at 37 °C in electro-heating standing-temperature cultivator for 30 min. The cells were fixed and stained with Giemsa in PBS. Macrophages were counted 100 on each piece with an oil-immersion objective. The percentage of phagocytosis was calculated as the following equation:

The percentage of phagocytosis (%) $= \frac{The\ number\ of\ macrophage\ that\ swallow\ chicken\ red\ blood\ cells}{100\ macrophages} \times 100\%$

1.2.8 Natural killer (NK) cell cytotoxicity tests (Lactate dehydrogenase, LDH, assay)

40 mice were randomized four groups by weight, followed by the gastric lavage treatment described in section 1.2.1.3 once per day over a period of 36 days. On the last day of tests, the mice were sacrificed and obtaining the spleen cell suspensions at a density of 2×10^7 cells/mL, respectively, as the procedures described in section 1.2.2. In this work, the YAC-1 cell line was set as the target cells and spleen cells were the effector cells. YAC-1 cells were subcultured 24 h before tests and then rinsing with Hank's buffer for three times, followed by dispersed into RPMI 1640 medium supplemented with 10% calf serum to a density of 4×10^5 cells/mL in stock.

100 μL of effector cells and 100 μL of target cells were added to a round bottom 96-well plate (effector/target ratio of 50:1). In the target cell spontaneous release well, 100 μL of target cells and 100 μL of RPMI 1640 medium were added. In the target cell maximal release well, 100 μL of target cells and 100 μL of 1% NP40 cell lysis buffer were added. Each group was set in triplicate wells. The 96-well plate was then incubated in a humidified chamber incubator at 37 °C, 5% CO_2 for 4 h to allow the ample contact between target and effector cells. After the incubation completed, harvesting the supernatants by centrifugation and then adding them to a flat bottom 96-well plate, followed by adding another 100 μL of LDH matrix solution to each well. The plate was left to allow the reaction for 7 min and then adding 30 μL of 1 mol/L HCl solution to each well. The OD values of each well were measured at a wavelength of 490 nm. The NK cell cytotoxicity was calculated as the following equation:

$$\text{NK cototoxicity}\ (\%) = \frac{OD\ value\ in\ experimental\ release - OD\ value\ in\ spontaneous\ release}{OD\ value\ in\ maximal\ release - OD\ value\ in\ spontaneous\ release} \times 100\%$$

1.2.9 The ratio of organs to weight

40 mice were randomized four groups by weight, followed by the gastric lavage treatment described in section 1.2.1.3 once per day over a period of 37 days. On the last day of tests, the mice were sacrificed by cervical dislocation to harvest the thymus and spleens. The organs were weighed and the ratio of thymus mass to weight, as well as the ratio of spleen mass to weight, were calculated, respectively.

1.2.10 The effect on the weight of mice tests

52 mice were randomized four groups by weight, 13 mice for each group, followed by the gastric lavage treatment described in section 1.2.1.3 once per day over a period of 30 days. The mice were weighed at the initial day, 15th day and 30th day, respectively.

1.3 The statistical methods

All the experimental data was displayed as a mean±standard deviation ($\bar{x} \pm \mathrm{SD}$). The analysis of variance and the statistical test was done by adopting Sun Ruiyuan et al. [7]: Multi-group mean analysis in DAS statistical software.

2. The results

2.1 The animal experiments

2.1.1 ConA-induced splenic lymphocyte transformation test (MTT method)

The results are shown in Table 1. The original data meets the requirement of homogeneity of variance. Compared with the control group, the difference of OD value in both high- and medium-dose of L-5-MTHF-Ca groups increased significantly, indicating the enhancement of lymphocyte proliferation ability in mice. The differences were statistical significance (p<0.01).

Table 1. The results of ConA-induced splenic lymphocyte transformation test.

Group (mg/kg)	Animal Number	Difference of OD Value
Control	10	0.251±0.053
Low-dose / 0.8	10	0.247±0.042
Medium-dose / 1.7	10	0.369±0.048**
High-dose / 5.0	10	0.534±0.050**
F Statistic		77.535
P-Value		0.000

t-test: *p<0.05, **p<0.01 compared with the control group.

2.1.2 SRBC-induced DTH tests in mice (foot swelling method)

The results are shown in Table 2. The original data meets the requirement of homogeneity of variance. Compared with the control group, the perimeter difference of the left hind footpad of mice in all the experimental groups had no significant differences before and post 24 h SRBC injection treatment. The differences were no statistical significance.

Table 2. The results of SRBC-induced DTH tests in mice.

Group (mg/kg)	Animal Number	Difference of Left Hind Footpad Perimeter (cm)
Control	10	0.071±0.023
Low-dose / 0.8	10	0.095±0.046
Medium-dose / 1.7	10	0.093±0.019
High-dose / 5.0	10	0.105±0.042
F Statistic		1.740
P-Value		0.176

2.1.3 Quantification of antibody-secreting cells (modified Jerne's method)

The results are shown in Table 3. The original data meets the requirement of homogeneity of variance. Compared with the control group, the number of plaques to total spleen cells in both high- and medium-dose of L-5-MTHF-Ca groups climbed remarkably. The differences were statistical significance (p<0.01).

Table 3. The results of the quantification of antibody-secreting cells.

Group (mg/kg)	Animal Number	Number of Plaques /Total

		Spleen Cells
Control	10	28.3±3.2
Low-dose / 0.8	10	29.6±3.5
Medium-dose / 1.7	10	37.0±2.8**
High-dose / 5.0	10	41.2±2.1**
F Statistic		43.553
P-Value		0.000

t-test: *p<0.05, **p<0.01 compared with the control group.

2.1.4 The determination of serum hemolysin (Coagulation method)

As shown in Table 4, the original data meets the requirement of homogeneity of variance. In comparison with the control group, the antibody level in both high- and medium-dose of L-5-MTHF-Ca groups increased notably. The differences were statistical significance (p<0.05 or p<0.01).

Table 4. The results of the serum hemolysin determination (Coagulation method).

Group (mg/kg)	Animal Number	Antibody level
Control	10	54.3±8.8
Low-dose / 0.8	10	64.3±19.3
Medium-dose / 1.7	10	86.6±29.8*
High-dose / 5.0	10	91.0±29.7**
F Statistic		5.574
P-Value		0.003

t-test: *p<0.05, **p<0.01 compared with the control group.

2.1.5 Carbon granular clearance tests in mice

As shown in Table 5, the original data meets the requirement of homogeneity of variance. In comparison with the control group, the phagocytic index in all the experimental groups increased. The differences were statistical significance (p<0.05 or p<0.01).

Table 5. The results of carbon granular clearance tests.

Group (mg/kg)	Animal Number	Phagocytic Index (%)
Control	10	5.79±0.68
Low-dose / 0.8	10	6.63±0.71*
Medium-dose / 1.7	10	6.88±0.91**
High-dose / 5.0	10	7.10±0.74**
F Statistic		5.553
P-Value		0.003

t-test: *p<0.05, **p<0.01 compared with the control group.

2.1.6 Phagocytic function of peritoneal macrophage

As shown in Table 6, the original data and the values converted via the equation $X =$

$\sin^{-1} P^{1/2}$ meet the requirement of homogeneity of variance. In comparison with the control group, the phagocytic rate (P%) and X values in both high- and medium-dose of L-5-MTHF-Ca treated mice increased. The differences were statistical significance (p<0.05).

Table 6. The results of the phagocytic function of peritoneal macrophage.

Group (mg/kg)	Animal Number	P(%)	X
Control	10	35.5±1.8	0.638±0.019
Low-dose / 0.8	10	38.7±3.3	0.671±0.034
Medium-dose / 1.7	10	40.0±3.2*	0.684±0.033*
High-dose / 5.0	10	39.6±4.9*	0.680±0.050*
F Statistic		3.398	3.407
P-Value		0.028	0.028

t-test: *p<0.05, **p<0.01 compared with the control group.

2.1.7 NK cell cytotoxicity tests (LDH assay)

The results are shown in Table 7. The values of NK cell cytotoxicity were converted via the equation $\mathrm{X} = \sin^{-1} P^{1/2}$, and the results before and after the conversion meet the requirement of homogeneity of variance. In comparison with the control group, the NK cell cytotoxicity in all the experimental groups increased. The differences were statistical significance (p<0.01).

Table 7. The results of NK cell cytotoxicity tests.

Group (mg/kg)	Animal Number	NK cell cytotoxicity (%)	X
Control	10	3.44±2.72	0.171±0.082
Low-dose / 0.8	10	14.35±4.59**	0.384±0.067**
Medium-dose / 1.7	10	22.03±5.80**	0.486±0.067**
High-dose / 5.0	10	20.97±4.03**	0.474±0.048**
F Statistic		37.229	47.167
P-Value		0.000	0.000

t-test: *p<0.05, **p<0.01 compared with the control group.

2.1.8 The ratio of organs to weight

As shown in Table 8, the original data of the ratio of thymus mass to weight, as well as the ratio of spleen mass to weight meet the requirement of homogeneity of variance. In comparison with the control group, the differences of the ratio of thymus mass to weight and the ratio of spleen mass to the weight of mice in all the experimental groups were non statistical significance (p>0.5).

Table 8. The results of the ratio of organs to weight.

Group (mg/kg)	Animal Number	Thymus mass/weight (mg/g)	Spleen mass/weight (mg/g)

Control	10	1.58±0.22	4.56±0.91
Low-dose / 0.8	10	1.55±0.20	4.12±0.32
Medium-dose / 1.7	10	1.52±0.16	4.04±0.40
High-dose / 5.0	10	1.50±0.20	4.29±0.48
F Statistic		0.327	1.592
P-Value		0.806	0.208

2.1.9 The effect on the weight of mice

As shown in Table 9, the original data meets the requirement of homogeneity of variance. In comparison with the control group, the differences of mice weight at the intial and intermediate, as well as the final periods in all the experimental groups were no statistical significance (p>0.5).

Table 9. The results of the effect on the weight of mice.

Group (mg/kg)	Animal Number	Initial Weight (g)	Intermediate Period Weight (g)	Final Weight (g)
Control	13	18.8±0.4	26.4±0.9	37.2±1.0
Low-dose / 0.8	13	18.8±0.4	27.0±0.8	37.2±1.3
Medium-dose / 1.7	13	18.8±0.5	26.1±1.1	36.9±1.0
High-dose / 5.0	13	18.8±0.5	26.9±1.4	36.7±1.6
F Statistic		0.090	1.893	0.675
P-Value		0.965	0.143	0.572

2.1.10 Conclusion

According to the criteria of “Technical Standards for Testing & Assessment of Health Food” (2003 Edition), L-5-MTHF-Ca possess the ability to enhance the immunity of the organism.

3. Discussion

Folate is the essential vitamin in the human body, which is involved in the synthesis process of protein and DNA, as well as the critical donor of the methyl group in the one-carbon unit metabolism. The deficiency of folate in body circulation is possible to result in the generation of NTDs in fetuses, as well as increasing the incidence of diverse diseases, including neurological disorders, cardiovascular disease and cancer. [8-10] Therefore, it is imperative to have a scientific dietary supplement of folate.
L-5-MTHF-Ca is the most active form of the folate compounds in the plasma circulation. Compared with folate, the absorption of L-5-MTHF-Ca is faster and its distribution is more uniform, which endows it with higher bioavailability and bioactivation. [11] Up to now, the research of L-5-MTHF-Ca in both domestic and international institutes focuses on the synthesis method, the detection of content and the stability tests, [12-14] while its effects on immunity enhancement are rarely reported. In this work, we comprehensively investigated the effectiveness of L-5-MTHF-Ca on enhancing the immunity of mice. The results have indicated that L-5-MTHF-Ca has a positive enhancement ability to the immunity of mice,

which lay us a good foundation for the proceeding study.

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