UDC 612.017.1 https://doi.org/10.26577/2218-7979-2017-10-1-50-56

[1]Nuerbaheti Houwati,[2]Ydyrys Alibek, [2]Tuleuhanov S., [2]Ablaikhanova N.,
[3]Gulishayia D., [4]Muhemaiti Yueerlin,[4*]Asiya Baishanbo

[1]Xinjiang Kazakh Medicine Research Institute, Urumqi, China
[2]Al-Farabi Kazakh National University, Almaty, Kazakhstan
[3]Xinjiang Medical University Hou Bo institute Basic medicine faculty
[4]Pharmacy College of Xinjiang Medical University, Urumqi, China
[*]e-mail: 554623209@qq.com

Study on the effect of the Kazakh Traditional Medicine Kezimuk granules to the immunologic function of cyclophosphamide induced immunosuppressed mice

Abstract: The paper reports the study of the effect of the Kazakh mmedicine Kezimuk granules on himoral immunity, cell-medicated immunity and immunological function of cyclophosphamide immunosupressed mice. 60 SPF Kunming mice, half male and half female, were randomly divided into 6 groups. Each group contains 10 mice: Respectively the normal group (saline solution) comparing to the control group (Yupingfeng granules) and the model group (saline solution) comparing to high, medium and low dose Kezimuk granule groups. The high, medium and low dose ,2.72g/kg, 1.36g/kg, and 0.68g/kg, of Kezimuk were equivalent to 4, 9, and 18 times of it and the recommended dose was calculated through the basis of relative surface of mice, referring to humans. Doses were daily intaken by intragastric administration for 2 weeks. After drug administration from the seventh day to ninth day ,for 3 days, each group, except the normal control group, received intra-peritoneal injections,pyrophosphate,at a dose of 50mg/kg to induce the immunocompromise. The thymus index, spleen index, serum IL-2 content and the body weight of the mice, were measured to observe the effect of each dose of Kezimuk granules on pyrophosphate to induce immunosuppression of the cellular immunity and humoral immunity in the mice. Comparing to the model group Kezimuk granules significantly increase the thymus index and spleen coefficient and remarkably increase the index of B Lymphocyte proliferation, T Lymphocyte proliferation and the serum IL-2 content as well as body weight of immunocompromised mice ($P<0.05$); the results for the middle and high dose of Kezimuk granule groups were significantly higher than the model group ($P<0.05$). Kezimuk granule might have a protective effect on immunocompromised mice and improve immunologic function.
Key words: Kezimuk granules, pyrophosphate, cellular immunity; humoral immunity.

Introduction

Kezimuk granule is a kind of Kazakh traditional medicine. The formula consists of common bilberry or blue whortleberry (*Vaccinium myrtillus L.*), helichrysum arenarium, equisetum and other herbs. It recorded and clinically confirmed that Kezimuk granule has therapeutical effects on urinary tract infections, cystitis, pyelonephritis, nephrotic syndrome, hypertrophy of the prostate and impeded urination. It can also be used as a general tonic. Preliminarily researches proveed that this prescription plays positive roles on anti-inflammatory effect, as well as on increasing urine volume (1). This fundamental research is to further the study to the effects of the medicine on immunologic function.

Chinese herbal medicine includes many various components to effect immune system, such as polysaccharides, saponins, alkaloids, volatile substances and organic acid to improved immunity, to intensify macrophage activity and to enhance liver detoxification function (2; 3). Also it makes immune cells resist bacteria, fungi and viruses to help human body get against infection as well as to eradicate invasive bacteria and viruses (4; 5). Increased number of immune cells correspondingly induce resistance and activate of macrophages, NK cells and other immune effector cells and secrete various cytokines and other important media. This Kazakh traditional medicine markedly has been effective in enhancing the immune effect of the body, accelerating macrophage proliferation, increasing peritoneal macrophages,

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boosting phagocytosis, as well as improving the killing activity of NK cells and also has been evidently helpful to achieve the objective of inhibiting and get rid of pathogenic microorganisms.

Materials and methods

Test medicine Kezimuk granule is formulated from 3 medicinal herbs: *Vaccinium myrtillus L*, 115g, *Helichrysum arenarium*, 384g, and *Equisetum hyemale L*. 192g. The prescription materials in the formula were provided by the Autonomous Region Kazakh Medicine Institute. Kezimuk granules are dark brown course particles prepared at Xinjiang Jiasite Pharmaceutical Co. Ltd., in strict accordance with the preparation process formulated in the preliminary study; batch number: 20130308. When used it was prepared according to the requirements of the experiment using double distilled water.

Animals: 60 Kunming mice, 8-12 weeks of age, 18-22 g body weight, half male and half female, purchased from the experimental animal center of Xinjiang Medical University. Experimental animal production license number: SYXK(新) 2011-0001.

Reagents: injectable pyrophosphate (CAT. No. C8650); DMSO (MP Biomedicals, LLC); Yupingfeng granules (Guangdong Global Pharmaceutical Co. Ltd., Product batch number: 130735); Mouse serum IL-2 Kit (Joyee Biotechnics Co., Ltd); RPMI1640 culture solution (Thermo Fisher Biochemical Products (Beijing) Co., Ltd.); MTT (MP Biomedicals, LLC); saline solution (Xi'an Jingxi Shuanghe Pharmaceutical Co. Ltd.); serum (Gibco Brand LOT 41G5532K); Concanavalin A (MP Biomedicals, LLC); PBS buffer (Thermo Fisher Biochemical Products (Beijing) Co., Ltd.) (6-12).

Instruments: precision electronic balance (Sartorius scientific instruments (Beijing) Co., Ltd.); Multiskan Spectrum (Benchmark Plus); Syringe (Jiangsu Shenli Medical Products Co., Ltd.); Petri dish (Corning Incorporated); 96 well plates (Corning Incorporated); Centrifuge; Clean bench; CO_2 incubator (RevcoUSA); Microscope (Olympus Corporation, Japan); UV VIS spectrophotometer (Shanghai Lengguang Technology Co., Ltd.).

Methods of research: Establishing the immunocompromised mice model: 60 SPF Kunming mice, half male and half female, were randomly divided into 6 groups. Each group contains 10 mice Respectively the normal group (saline solution) comparing to the control group (Yupingfeng granules) and the model group (saline solution) comparing to high, medium and low dose Kezimuk granule groups. The high, medium and low dosage of 2.72g/kg, 1.36g/kg and 0.68g/kg of Kezimuk was equivalent to 4, 9 and 18 times of it and the recommended dosage of animal model was calculated through the basis of its relative surface, according to the corresponding dosage (13). Doses were daily intaken by intragastric administration for 2 weeks. After drug administration from the seventh day to ninth day, for 3 consecutive days, each group, except the normal control group, received intra-peritoneal injections,pyrophosphate,at a dose of 50mg/kg to induce the immunocompromise.

Test indexes: Determination of the immune organ weight and the body weight of the mice: the day after the last administration of the medicine, the body weights of the mice were accurately weighed with an electronic balance, and after blood was taken by the eye removal technique, the weight of the thymus and spleen were measured under aseptic conditions and the spleen and thymus index calculated according to the formula:

$$\text{Spleen index} = \text{spleen weight (mg)}/\text{body weight (g)} \times 100\%$$

$$\text{Thymus index} = \text{thymus weight (mg)}/\text{body weight (g)} \times 100\%$$

Thymus and spleen lymphocyte proliferation: on the 14th day of treatment, the mice were put to death. The thymus and spleen were removed under sterile conditions and placed in a Petri dish containing PBS buffer with the fat and connective tissue removed. RPMI1640 culture medium was added and the inner core of the thymus and spleen were gently crushed using a sterile syringe. The single cells went through a nylon mesh (200 mesh) into the centrifuge tube, the cells were rinsed with RPMI1640 culture medium, then the separation medium containing lymphocytes was transfered to 15ml centrifuge tubes, centrifuged at 1000rpm for 5 min and the supernatant removed. After mixture of red cell lysate (200ul) and a constant volume of RPMI1640 culture medium, they were centrifuged and its supernatant must be removed. Then add (1ml) PBS buffer and centrifuged them twice with removing its supernatant. Then (1ml) 10%RPMI1640 was added, it was divided into two groups of (500ul) and centrifuged also remove its supernatant. 10% RPMI1640 culture medium (2ml) was added to one group and ConA solution (2ml) was added to the other group. Then they were incubated at 37^{0}C and 5% CO_2 for 24h in an incubation box in 96 well plates. 4 hours before the end of the incubation period they were taken out and 20ul MTT[14](5mg/

ml) was added per well and incubation was continued for 4 hours. After this 4 hours the supernatant was sucked out and discarded, 150ul of DMSO was added per well. They were mixed at low speed vibration in an oscillator, and then the optical density value was determined in a Microplate Reader at a wavelength of 490nm. The value of optical density was calculated according to the following formula:

The optical density (ABS) difference = optical density for wells with ConA – optical density for wells without ConA.

The determination of serum IL – 2 content in the mice: after treatment for each group of mice having finished, blood was taken via eye removal technique and the serum was obtained after centrifugation. Analysis was carried out according to the ELISA kit user guide. Standard diluent (1ml) was added to the ELISA kit standard product and left to stand for 10~15 minutes during which time suction was applied several times to ensure it was completely dissolved. In this way a concentration gradient of (1000pg/ul, 500pg/ul, 250pg/ul, 125pg/ul, 62.5pg/ul, 31.25pg/ul,0) was achieved. A 96 well plate preparation was used with 100ul/ well of the standard product in the replicate wells; serum samples (50ul/ well) and sample analysis buffer (50ul/ well). After incubation at room temperature for 120min, the plates were washed 5 times and on the final wash were placed on thick absorbent paper and patted dry.

Biotinylated antibody working fluid (100ul/well) was added, and then after incubation at room temperature for 1hr, the plates were washed 5 times and on the final wash were placed on thick absorbent paper and patted dry. HRP enzyme conjugate working fluid (100ul/well) was added, and then after incubation at room temperature protected from light for 20min, the plates were washed 5 times and on the final wash were placed on thick absorbent paper and patted dry. Chromogenic reagent TMB (100ul/well) was added, and then incubated at room temperature protected from light for 20min. Finally the suspension liquid was added (50ul/ well) and the OD450 values measured immediately after mixing, to detect the content of IL-2 in serum of each group.

Results and discussion

The effect of different doses of Kezimuk granules on the body weight was measured by electronic balance. This table below shows the difference between the initial weight and the final weight of the mice. See Table 1.

Table 1 – The effect of Kezimuk granules at different doses on the bodyweight of immunocompromised mice

Group	Dose, g/kg	Animal No.	Body Weight Gain
Normal group	NS	10	16.74 ± 5.08
Model group	NS	10	11.11 ± 5.07▲*
Yupingfeng granules	2.25	10	10.65 ± 4.64▲
Kezimuk granule low dose group	0.68	10	15.06 ± 3.61
Kezimuk granule med dose group	1.35	10	18.45 ± 3.57▲
Kezimuk granule high dose group	2.70	10	15.07 ± 4.24▲

Note: compared with the model group, ▲P<0.05; compared with the normal group, *P<0.05.

Analysis: Table 1 shows that the Kezimuk granule high and medium dose groups along with the Yupingfeng granule group were significantly different (▲P<0.05) from the model group. The Kezimuk granule low dose group along with the model group were significantly different (* P<0.05) from the normal group. This shows that Kezimuk granules have a significant effect on the weight gain of mice.

The effect of different doses of Kezimuk granules on the spleen index of immunocompromised mice was determined by weighing its spleens under aseptic conditions and by calculating the spleen index according to the formula. See Table 2.

Table 2 – The effect of Kezimuk granules at different doses on the spleen/body weight ratio of immunocompromised mice.

Group	Dose, g/kg	Animal No.	Spleen Index, mg/g
Normal group	NS	10	0.009 ± 0.001
Model group	NS	10	0.005 ± 0.003*▲
Yupingfeng granules	2.25	10	0.007± 0.002
low dose group	0.68	10	0.013 ± 0.002
med dose group	1.35	10	0.012 ± 0.004▲
high dose group	2.70	10	0.013 ± 0.003▲

Note: compared with the model group, ▲P<0.05; compared with the normal group, *P<0.05.

Analysis: Table 2 shows that all three Kezimuk granule dosage groups along with the Yupingfeng granule group were significantly different (▲P<0.05) from the model group. The model group was significantly different (*P<0.05) from the normal group. The spleen index of the model group was lower than that of the normal group. Compared with model group the Yupingfeng group and Kezimuk groups indicated improved spleen index for immunocompromised mice.

The effect of different doses of Kezimuk granules on the spleen lymphocyte (B) proliferation ability of immunocompromised mice. The preparation of the spleen lymphocyte suspension was conducted according to the steps in 2.2.2, and the absorbance of each well was determined in a microplate reader at a wavelength of 490n. See Table 3.

Table 3 – The effect of different doses of Kezimuk granules on the spleen lymphocyte proliferation ability in immunocompromised mice

Group	Dose, g/kg	Animal No.	Spleen lymphocyte proliferation ability,A
Normal group	NS	10	2.45 ± 0.12
Model group	NS	10	1.44 ± 0.12▲*
Yupingfeng granules	2.25	10	2.20 ± 0.41
low dose group	0.68	10	2.16 ± 0.24▲
med dose group	1.35	10	2.20 ± 0.15
high dose group	2.70	10	2.30 ± 0.40▲

Note: compared with the model group, ▲P<0.05; compared with the normal group, *P<0.05.

Analysis: Table 3 shows that all three Kezimuk dosage groups and the Yupingfeng granule group were significantly different (▲P<0.05) from the model group. The model group was significantly different (*P<0.05) from the normal group. The spleen lymphocyte proliferation in the model group was significantly lower than in the normal group (P<0.05). Compared with the model group, Kezimuk granules showed a significant improvement in the spleen lymphocyte proliferation ability in immunosuppressed mice, and Yupingfeng particles also showed a significant improvement in spleen lymphocyte proliferation ability (P<0.05).

The effect of different doses of Kezimuk granules on the thymus cell (T) proliferation ability of immunocompromised mice. The preparation of the thymus cell suspension was conducted according to the steps in 2.2.2, and the absorbance of each well was determined in a microplate reader at a wavelength of 490n. See Table 4.

Table 4 – The effect of different doses of Kezimuk granules on the thymus cell proliferation ability in immunocompromised mice

Group	Dose, g/kg	Animal No.	Thymus cell proliferation ability, A
Normal group	NS	10	2.47 ± 0.26
Model group	NS	10	1.32 ± 0.16▲*
Yupingfeng granules	2.25	10	2.72 ± 0.27
low dose group	0.68	10	2.22 ± 0.41
med dose group	1.35	10	2.01 ± 0.40▲
high dose group	2.70	10	2.30 ± 0.31▲

Note: compared with the model group, ▲P<0.05; compared with the normal group, *P<0.05.

Analysis: Table 4 shows that all three Kezimuk dosage groups and the Yupingfeng granule group were significantly different (▲P<0.05) from the model group. The Model group and the Yupingfeng granule group were significantly different (*P<0.05) from the normal group. The thymus cell proliferation in the model group was significantly lower than in the normal group (P<0.05). Compared with the model group, Kezimuk granules showed a significant improvement in the thymus cell proliferation ability of immunosuppressed mice, and Yupingfeng particles also showed a significant improvement in thymus cell proliferation ability (P <0.05).

The effect of different doses of Kezimuk granules on the Serum IL-2 in immunocompromised mice. Serum was acquired by taking blood from the eye, which was then stood for 30 minutes and centrifuged according to the steps in 2.2.3, and the optical density was determined in a microplate reader at a wavelength of 450nm.

Table 5 – The effect of different doses of Kezimuk granules on serum IL – 2 in immunocompromised mice

Group	Dose, g/kg	Animal No.	Serum IL-2
Normal group	NS	10	4.17 ± 0.69
Model group	NS	10	2.94 ± 0.21▲*
Yupingfeng granules	2.25	10	3.81 ± 0.27
low dose group	0.68	10	3.98 ± 0.18
med dose group	1.35	10	4.00 ± 0.13▲
high dose group	2.70	10	3.72 ± 0.13▲

Note: compared with the model group, ▲P<0.05; compared with the normal group, *P<0.05.

Analysis: Table 5 showed that all three Kezimuk dosage groups and the Yupingfeng granule group were significantly different (▲P<0.05) from the model group. The model group was significantly different (*P<0.05) from the normal group. Compared with the model group, Kezimuk granules showed a significant improvement in the serum IL – 2 proliferation ability in immunosuppressed mice, and Yupingfeng particles also showed a significant improvement in serum IL – 2 proliferation ability (P <0.05).

Discussion

This study showed that Chinese medicine plays an important role in regulating body's immune system by rectifying immune suppression, and because of its unique asset, people accept and use it generally.[15] The functions of disease prevention and health care of traditional Chinese Medicine is mostly achieved through immunoregulation. This special effect of compound traditional Chinese medicine in

improving immunity system is mainly reflected in promoting the development of immune organs, increasing the weight of immune organs, enhancing immune response, and increasing the concentration of immune cells, thereby improving the ability of resistance to disease. T lymphocytes are important components of cellular immunity as they, along with other cells and cytokines, interact together to achieve immune functions such as recognition, activation and killing. Yupingfeng powder is a well known part in the "Fuzheng Gubiao" treatment principle in traditional Chinese medicine, originating from the ancient medical medical works; "Shi Yi De Xiao Fang". It's comprised of 3 medicinal herbs: milkvetch root (Astragalus membranaceus (Fisch.) Bunge.), Atractylodes macrocephala Koidz., and divaricate saposhniovia root, (Saposhnikovia divaricata (Trucz.) Schischk.). Of these milkvetch root and Atractylodes macrocephala combine to fill important roles in traditional Chinese medicine treatment principles.

Cyclophosphamide is commonly used alkylating agent for the treatment of malignant tumors. With broad-spectrum anti-cancer effect, and being one of the drugs which has been commonly used for chemotherapy, surgery, radiotherapy and adjuvant chemotherapy, and its main side effect is immunosuppression. Because it takes longer to the working period of healthy foods and needs larger doses which would lead to excessive animal immune suppression beyond the regulating ability of the healthy foods concealing its effectiveness. In this research our team took low various doses to establish a more stable immune suppression model. Compared to the control group, the pyrophosphate group showed significant differences indicating the success of the model.

The thymus and the spleen are vital central as well as peripheral immune organs. The spleen generates lymphocytes, and also the place where settles and the proliferates lymphocytes, which makes spleen a the basic organ for producing specific immunity. The thymus can produce a large number of thymus cells, and is a backup and supplement of T lymphocytes. Increasing weight of immune organs to a certain extent can reflect the body's immune ability. The T lymphocytes, which carry out a cellular immune function, develop and mature in the thymus, reside in the spleen, and proliferate during an immune response. The spleen and thymus index level is a response to the T lymphocyte proliferation ability and the cell numbers[16-18] and is one of the parameters that directly reflects the immune function level. The thymus and spleen are, respectively, the central as well as the peripheral immune organs; the thymus makes T cells mature, and the spleen is where the lymphocytes mature and B cells reside. The thymus coefficient and spleen coefficient can directly reflect the function of the immune (19; 20).

In this study Kezimuk granules significantly improved the control model`s spleen index. The concanavalin A (ConA) induced lymphocyte transformation in the experiment carried out by the MTT method and the results of the mice serum IL-2 values show that the increase in immune organs of normal mice is related to the spleen and thymus weight. This is due to response of immune organs, in turn to enhance immune function in the mice expressed in the enhanced lymphocyte transformation and elevated values of serum IL-2 value. The immune regulation network is very complex, and it still needs further research in order to explain the mechanism and function of Kezimuk granules in improving the body's immunity, and to provide reference for more scientific use of this distinctive resource of Xinjiang traditional medicine.

Acknowledgments

Funding Program:Xinjiang real estate of Chinese national drug research and development project.

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