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 traditional medicine Kezimuk granules on humoral immunity, cell-mediated immunity and immunological function of cyclophosphamide immunosuppressed 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. Preliminary researches proved 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.
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 getting 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 Jiatisite 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 Biological 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 Biological Products (Beijing) Co., Ltd.) (6-12).

Instruments: precision electronic balance (Teraxs 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; CO2 incubator (RevecoUSA); Microscope (Olympus Corporation, Japan); UV VIS spectrophotometer (Shanghai Lengguang Technology Co., Ltd.).

Methods of research: Establishing the immuno-compromised 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:

\[ \text{Spleen index} = \frac{\text{spleen weight (mg)}}{\text{body weight (g)}} \times 100\% \]

\[ \text{Thymus index} = \frac{\text{thymus weight (mg)}}{\text{body weight (g)}} \times 100\% \]

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 transferred 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°C and 5% CO2 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:

\[
\text{The optical density (ABS) difference} = \text{optical density for wells with ConA} - \text{optical density for wells without ConA.}
\]

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. 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 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.

| 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<0.01.

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.

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. 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 saponshovia 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 in expression model. Compared to the control group, 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.

References

1. Kvinnland S., Anker G., Di rix L.Y., et al. High activity and tolerability demonst rated for exemestane in postmenopausal women with metastatic breast cancer who had previously falled on tamoxifen treatment // Eur J Cancer, 2000, 36(8):976-982.
2. Bottini A., Berruti A., Ber Siga A., et al. p53 but not bcl-2 immunostaining is predictive of poor clinical complete response to primary chemotherapy in breast cancer patients // J. Clinical cancer Res. 2000, 6(7):2751-2758.
3. Collins L.C., Schnitt S.J. HER2 protein overexpression in estrogen receptor-positive ducktail carcinoma in situ of the breast: frequency and implications for tamoxifen therapy // J. Med Pathol., 2005,18(5):615-620.
4. Guanpingsheng Luo, Teclegiorgis Gebremariam, Hongkyu Lee, Samuel W. French, Nathan P. Wiederhold, Thomas F. Patterson, Scott G. Filler, Ashraf S. Ibrahim. Efficacy of Liposomal Amphotericin B and Posaconazole in Intratracheal Models of Murine Mucormycosis. 2013, 57:3340-3346.
5. Hua-Sheng Chen, Jue Chen, De-Li Cui, Yuan-Yuan Zheng, Ai-Hua Xu, Gang Chen, Ling-Chang

International Journal of Biology and Chemistry 10, № 1, 50 (2017)
Jia. Effects of a Shuangling Fuzheng anticancer preparation on the proliferation of SGC-7901 cells and immune function in cyclophosphamide-treated murine model, 2007, 13(48): 6575-6580.

6. A. Tanioka, K. Tanabe, A. Hosono, H. Kawakami, S. Kaminogawa, K. Tsubaki, S. Hachimura. Enhancement of Intestinal Immune Function in Mice by b-D-Glucan from Aureobasidium Pullulans ADK-34, 2013. 78:61–68.

7. Thomas Secher, Stefanie Fas, Louis Fauchonner, Marieke Mathieu, Oliver Rutschi, Bernhard Ryffel, Michael Rudolf. The Anti-Pseudomonas aeruginosa Antibody Panobacumab Is Efficacious on Acute Pneumonia in Neutropenic Mice and Has Additive Effects with Meropenem, 2013. 8:1-12.

8. Chun-ji Han, Young K young Rhee, Young-Chul Lee, Kwang-Soon Shin, Hee-Do Hong. Immunostimulatory Effects of Polysaccharides Isolated from Makgeolli (Traditional Korean Rice Wine) Chang-Won Cho, 2014. 19:5266-5277

9. Shanshan Li, Naibin Yang, Xinyue Tang, Shengguo Zhang, Danping Hu, Mingqin Lu. Deregulation of Regulatory T Cells in Acute-on-Chronic Liver Failure: A Rat Model Shunlan Ni, 2017, 15:1-10.

10. Mingsan Miao, Bolin Cheng, Lin Guo, Jingjing Shi. Effects of Fuzheng Paidu tablet on peripheral blood T lymphocytes, intestinal mucosa T lymphocytes, and immune organs in cyclophosphamide-induced immunosuppressed mice. 2015, 11:2659–2663.

11. Jang, Se-Eun, Eun-Ha Joh, Ho-Yong Lee, Young-Tae Ahn, Jung-Hee Lee Chul-Sung Huh, Myung Joo Han, and Dong-Hyun Kim. Lactobacillus plantarum HY7712 Ameliorates Cyclophosphamide-Induced Immunosuppression in Mice. 2013. 23(3), 414–421.

12. Mohammad Rafiul Haque a, Shahid Hussain Ansari a and Azhar Rashikh , 2013. Coffea arabica Seed Extract Stimulate the Cellular Immune Function and Cyclophosphamide-induced Immunosuppression in Mice 12 (1): 101-108.

13. Ran Zhao, Geng Li, Xiujian Kong, Xiu-yan huang, Wei li, Yao-ying Zeng, Xiao-ping Iai, 2016. The improvement effects of edible bird’s nest on proliferation and activation of B lymphocyte and its antagonistic effects on immunosuppression induced by cyclophosphamide, 10: 371-381.

14. Swetha Manepalli, Jay A. Gandhi, Vaibhav V. Ekhar, Melissa B. Asplund, Carolina Coelho, Luis R. Martinez. Characterization of a cyclophosphamide-induced murine model of immunosuppression to study Acinetobacter baumannii pathogenesis. 2013 62:1747–1754.

15. Hong-ying Gao, Guo-yu Li, Jian Huang, Ying Han, Fu-zhou Sun, Xiao-wei Du, Li-juan An, Hang-yu Wang, Jin-hui Wang. Protective effects of Zhuyeqing liquor on the immune function of normal and immunosuppressed mice in vivo. 2013 13:252-259.