Potato freeze-thaw solution enhances immune function and antitumor activity *in vivo*

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Abstract. Although potato extract, derived from various methods, exhibits anticancer, antiviral and anti-parasite activities *in vitro* and *in vivo*, the bioactivity of potato solution remains unclear using the freeze-thaw extraction method granted by the State Intellectual Property Office of China. In the present study, a potato freeze-thaw solution (PFTS) was fed to mice with ascites tumor that were pre-treated with cyclophosphamide. The numbers of peripheral white blood cells (WBCs), macrophage phagocytosis, lymphocyte transformation and survival of mice were measured. While mice injected with cyclophosphamide exhibited decreased counts of peripheral WBCs, treatment of the cyclophosphamide-injected mice with PFTS for 10 days significantly increased the number of peripheral WBCs and reversed WBC counts to the normal level, a comparable effect to that of *Ganoderma lucidum*. In addition, treatment with PFTS for 20 days significantly enhanced peritoneal macrophage phagocytosis and lymphocyte transformation. Lastly, PFTS was noticed to prolong the survival of tumor-bearing mice when compared with that of control mice. Collectively, these data suggested that PFTS, at least in part, enhances immune function and possesses antitumor activity.

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Introduction

Although there have been various improvements in detection, diagnosis and treatment, cancer remains the number two cause of mortality in the world, and accounts for a higher number of mortalities than heart disease in those under 85 years of age (1). One of the biggest challenges currently in cancer treatment is to provide effective anticancer therapy without substantial adverse effects, while simultaneously minimizing toxicity. Considering the severe side effects of chemotherapy, studies have been increasingly focusing on the anticancer potential of various vegetables, including potatoes (2,3).

Potatoes are one of the most commonly consumed vegetables worldwide and are a good source of antioxidants, including phenolic compounds, vitamin C and carotenoids (4). Previous studies have demonstrated that potato extract (PE) exhibits anticancer, antiviral and anti-parasite activities *in vitro* and *in vivo* (5-7). Cheng *et al* (8) observed that rhamnogalacturonan I domain-rich pectin from potato inhibits the proliferation of human colon cancer HT-29 cells and induces significant G2/M cell cycle arrest. Additionally, Yan *et al* (9) and Gundala *et al* (10) have reported that chlorogenic acid, the predominant phenolic compound in potato, inhibits carcinogenesis in liver and prostate cancer cells *in vitro* and *in vivo*.

Studies on the anticancer properties of potato have utilized PEs derived from various methods (6,11,12). In a comparative analysis of eight phytoplankton chlorophyll-extraction methods (13), it has been shown that the freeze-thaw method produces high quality and stable phytoplankton chlorophyll, and is convenient to use. The authors of the present study successfully patented the 'potato freeze-thaw solution (PFTS)' in the national patented invention (grant

no. CN105211794A) (14). Our previous study demonstrated that PFTS exhibits an anti-inflammatory effect on the lung tissue of rats with chronic obstructive pulmonary disease induced by cigarette smoke (15). Considering the bioactivity of PFTS, it is unknown whether the bioactive constituents of PFTS possess anticancer properties. In the present study, the effect of PEs from PFTS on the immune function was investigated, including white blood cell (WBC) counts, macrophage phagocytosis and lymphocyte transformation in tumor-bearing mice. It was also examined whether PFTS has an antitumor property by measuring the survival time of tumor-bearing mice.

Materials and methods

Ethics. All experimental animal procedures were conducted according to the Institutional Animal Care and Use Committee at Inner Mongolia Medical University (Inner Mongolia Medical University, Jinshan Economic and Technological Development Zone, Hohhot, China). The protocol of the present study was approved by the Ethics Committee of Inner Mongolia Medical University prior to the initiation of the study, and permission was obtained to perform the study.

Preparation of PFTS and amino acid analysis. Fresh potato Kexing IV was developed by the Potato Research Institute of Heilongjiang Academy of Agricultural Sciences (Heilongjiang, China), and cultivated by Sheng Feng Potato Industry Planting Base in the Wuchua area of the Inner Mongolia Autonomous Region in China. The PE was isolated by PFTS as described in our previous study (15). Briefly, potatoes were first frozen at -30˚C for 12 h, and then thawed at 35˚C and disrupted. Following centrifugation at 1,000 x g for 30 min at 4˚C, the supernatant was collected. The extracted liquid was then purified by macroporous adsorptive resins (0.45 μm; Nantong FilterBio Technology, Jiangsu, China) at room temperature, and stored at 4˚C until used for experiments. Amino acid compositions of PFTS were described in our previous study (15).

Amino acid composition of PE was detected by an amino acid analyzer (L-8900; Hitachi, Ltd., Tokyo, Japan). Different amino acids were eluted sequentially based on their ionic strength (acidic amino acids were firstly obtained, neutral amino acids were then obtained, and finally, basic amino acids were obtained). Subsequently, these amino acids were reacted with ninhydrin at 135˚C. Finally, the concentrations of amino acids were quantified by an ultraviolet detector (VIS-7220N; Beijing Beifen-Ruili Analytical Instrument (Group) Co. Ltd., Beijing, China) at 570 and 440 nm.

Mice treatment. A total of 80 6-8-week-old Kunming mice (40 males and 40 females; weight, 18-22 g) were purchased from Beijing Weitong Lihua Experimental Animal Technology Co., Ltd. (Beijing, China). To suppress the immune function of mice, all mice were injected intramuscularly with cyclophosphamide (4 mg) 1 and 3 days prior to further treatment (16). In total, 20 mice were then each administered either 1 ml of PFTS or 1.8 mg of *Ganoderma lucidum (G. lucidum;* Research Center of Bioresource & Bioenergy, School of Biotechnology, Jiangnan University, Jiangsu, China) twice a day by gavage. Another 20 mice were treated with 1 ml of PBS. An additional 20 mice receiving no treatment served as the negative control group. On days 3 and 10, peripheral blood was collected from the tails and WBC count (neutrophils, eosinophils, basophils, monocytes and lymphocytes) was measured. The schema of mouse treatment is shown in Fig. 1A. All mice were housed at 25˚C with a 12-h light/12-h dark cycle with free access to pellet chow and water.

Peritoneal macrophage phagocytic index. To investigate whether PFTS could enhance innate immune functions, 20 mice were each treated with either 1 ml of PFTS or 1.8 mg of *G. lucidum* twice a day for 20 days by gavage. On day 20 these mice were injected intraperitoneally with 0.5 ml of 1% thioglycollate broth (Sigma-Aldrich; Merck KGaA) to induce the infiltration of macrophages. On the following day, these mice were injected intraperitoneally with 0.5 ml of 1% red blood cells from chicken (Bersee Biotechnology, Beijing, China). At 40 min post-injection of red blood cells, the peritoneal fluid was collected. Subsequent to centrifugation (1,500 x g), peritoneal cells were smeared and stained using the Giemsa stain (Sigma-Aldrich; Merck KGaA). In brief, cell films on slides were incubated in May-Grünwald stain at room temperature for 5 min. Subsequent to washing in phosphate buffer, cell films were then incubated in dilute Giemsa solution for 20 min at room temperature, followed by rinsing in deionized water. Morphological and morphometrical analyses were performed using a microscopy system at x20 magnification (BX 50; Olympus Corporation, Tokyo, Japan) connected to a digital camera (DP70; Olympus Corporation). Measurements of areas of interest were conducted using MetaMorph software (version NX2.5, Molecular Devices, LLC, Sunnyvale, CA, USA). The macrophage phagocytic index was calculated as a percentage of macrophages engulfing chicken red blood cells.

Lymphocyte transformation rate. To investigate whether PFTS enhances the rate of lymphocyte transformation, 20 mice were each treated with either PFTS or *G. lucidum* twice a day for 20 days by gavage. On day 20, these mice were injected intramuscularly with 0.4 mg of phytohaemagglutinin (Ziqi Biotechology, Shanghai, China). Blood was collected from tails, and smeared and stained using the aforementioned Giemsa stain method. Morphological and morphometrical analyses were performed using a microscopy system (BX 50; Olympus Corporation) connected to a digital camera (DP70; Olympus Corporation). Measurements of areas of interest were conducted using MetaMorph software (version NX2.5, Molecular Devices, LLC). The percentage of lymphocyte transformation was calculated by dividing the number of lymphoblasts by the total number of lymphocytes and lymphoblasts.

Ascites tumor model. To investigate whether PFTS extends the survival time of tumor-bearing mice, 20 mice were inoculated intraperitoneally with 1 ml of $2.3x10^9$ S-180 ascites tumor cells (purchased from X-Y Biotechnology, Shanghai, China). A total of 10 mice were treated with 1 ml of PFTS three times per day by gavage and another 10 mice were treated with PBS (serving as the control). The growth of ascites tumor was monitored by body weight and the appearance of the abdomen. The survival time of tumor-bearing mice was measured.

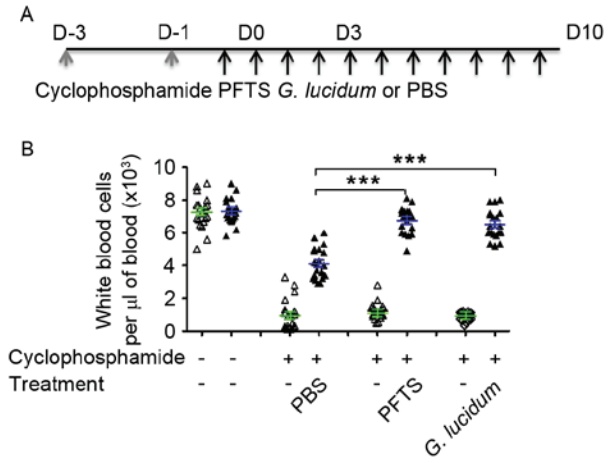

Figure 1. PFTS increases the peripheral white blood cell counts suppressed by cyclophosphamide. (A) Schema of treatment of tumor-bearing mice. (B) Quantitative analysis showing that the white blood cell counts were significantly higher in mice treated with either PFTS or *G. Lucidum* for 10 days than in PBS-treated mice. ***P<0.001 by the Mann-Whitney test. D, day; PFTS, potato freeze-thaw solution; *G. lucidum*, *Ganoderma lucidum*.

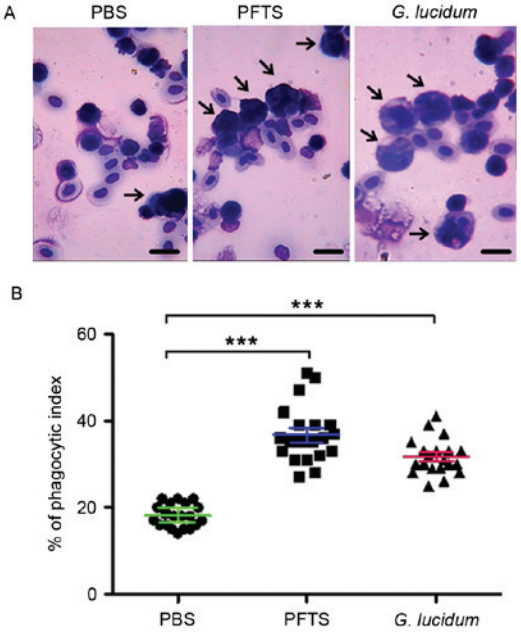

Figure 2. PFTS enhances peritoneal macrophage phagocytosis. (A) Images of macrophages engulfing chicken red blood cells in the PBS, PFTS and *G. lucidum* groups (magnification, x400). Arrows indicate macrophages engulfing chicken red blood cells. Scale bar, 50 μm. (B) Quantitative analyses revealed the significantly higher phagocytic index in mice treated with either PFTS or *G. lucidum* compared with that of PBS-treated mice. ***P<0.001 by the Mann-Whitney test. PFTS, potato freeze-thaw solution; *G. lucidum*, *Ganoderma lucidum*.

Statistical analysis. All data are expressed as the mean ± standard error of mean. Statistical analysis was performed by SPSS version 17.0 (SPSS, Inc., Chicago, IL, USA). For the analysis of WBC counts, macrophage phagocytic index and lymphocyte transformation rate, the Mann-Whitney test was used. The log-rank test was performed to analyze difference of survival between tumor-bearing and control mice. P<0.05 was considered to indicate a statistically significant difference.

Results

Amino acid compositions of PFTS. Total content of amino acids was 648.3 mg/ml in PE. It was identified to be composed by various amino acids, including aspartic acid (227.348 mg/100 ml), glutamic acid (127.686 mg/100 ml), valine (44.407 mg/100 ml), alanine (25.295 mg/100 ml), threonine (23.350 mg/100 ml), leucine (22.354 mg/100 ml), isoleucine (21.290 mg/100 ml), phenylalanine (21.909 mg/100 ml), glycine (15.796 mg/100 ml), serine (15.620 mg/100 ml), cystine (9.005 mg/100 ml), methionine (12.986 mg/100 ml), lysine (24.309 mg/100 ml), arginine (20.140 mg/100 ml), proline (21.162 mg/100 ml) and histidine (8.583 mg/100 ml).

PFTS increases peripheral WBCs suppressed by cyclophosphamide comparably to G. lucidum. To test the effect of PFTS on peripheral WBC counts, pre-treatment with cyclophosphamide, a well-established immunosuppressant was used to suppress immune function of mice. Following two doses of cyclophosphamide treatment, WBC counts dropped 10-fold from the value detected in the untreated mice group ($7.2x10^3$ cells/μl) to that observed in the cyclophosphamide-treated mice groups ($0.7x10^3$ cells/μl) (Fig. 1B). Cyclophosphamide-treated mice were then fed with PFTS or *G. lucidum* for 3 or 10 days and WBC counts were measured in these mice. As shown in Fig. 1B, WBC counts were significantly higher in mice treated with either PFTS or *G. lucidum* for 10 days compared with those exhibited by PBS-treated mice.

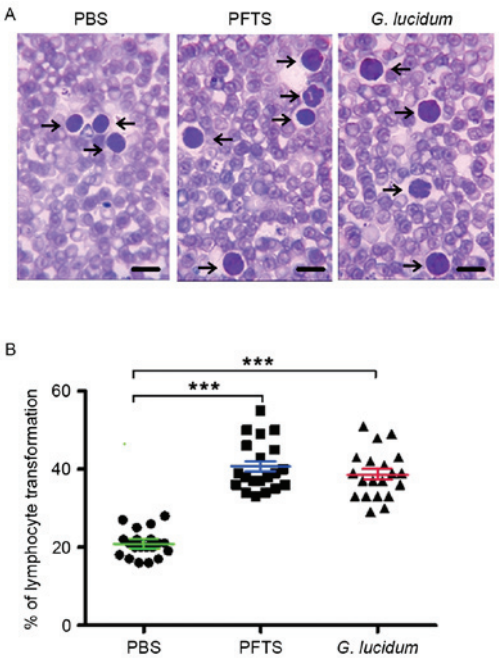

Figure 3. PFTS enhances the lymphocyte transformation rate. (A) Images of blood smear from the control, PFTS and *G. lucidum* groups (magnification, x400). Arrows indicate lymphoblasts. Scale bar, 50 μm (B) Quantitative analyses revealed a significantly higher percentage of lymphocyte transformation in mice treated with either PFTS or *G. lucidum* compared with that in PBS-treated mice. ***P<0.001 by the Mann-Whitney test. PFTS, potato freeze-thaw solution; *G. lucidum*, *Ganoderma lucidum*.

WBC counts in the PFTS-treated mice reached a similar level to those in the cyclophosphamide-untreated mice (6.76±0.77

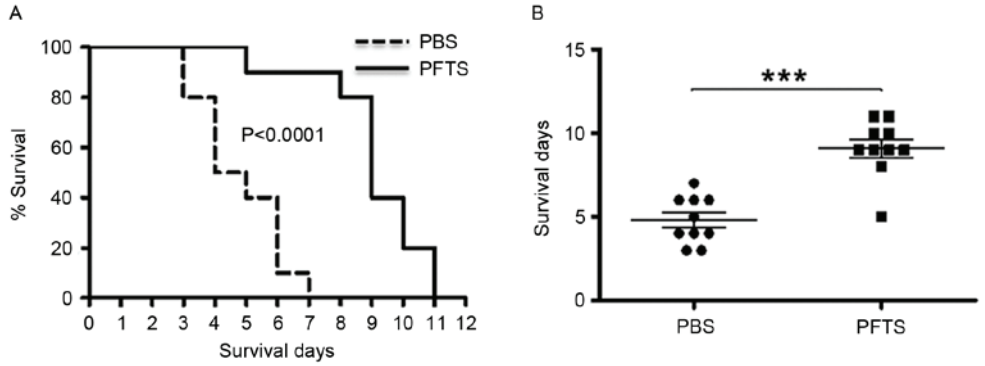

Figure 4. PFTS extends the survival time of tumor-bearing mice. (A) Percentage of survival of tumor-bearing mice in the PBS and PFTS groups. P<0.0001 by the log-rank test. (B) The survival time of tumor-bearing mice in the PFTS group was significantly longer than that in the PBS group. ***P<0.001 by the log-rank test. PFTS, potato freeze-thaw solution.

vs. 7.28±0.76, respectively; P=0.0978 by the Mann-Whitney test). Notably, the PFTS-mediated effect was comparable to that caused by *G. lucidum*, a well-known agent that increases WBC counts (17).

In addition, it was observed that the proportion of subsets of WBCs (neutrophils, eosinophils, basophils, monocytes and lymphocytes) was unchanged (data not shown) in PFTS-treated mice, which was comparable to the results obtained in cyclophosphamide-untreated mice. Thus, as food nutrition, PFTS may serve as a dietary supplement to increase the WBC count of patients receiving chemotherapy.

PFTS enhances peritoneal macrophage phagocytosis. To investigate whether PFTS enhances innate immune function, the capacity of macrophages engulfing chicken red blood cells in mice treated with PFTS was determined. By Giemsa staining, it was revealed that macrophages engulfed more chicken red blood cells in the PFTS and *G. lucidum* groups than in the PBS-treated group (Fig. 2A). The phagocytic index was significantly higher in PFTS-treated mice (36.8±4.1%) compared with that in PBS-treated mice (18.1±2.8%), and was comparable to that in *G. lucidum*-treated mice (31.6±4.7%) (Fig. 2B). These results indicated that PFTS promotes innate immune function by enhancing peritoneal macrophage phagocytosis.

PFTS improves the lymphocyte transformation rate. It was then investigated whether PFTS improves the lymphocyte transformation rate. By Giemsa staining, it was revealed that treatment with phytohaemagglutinin transformed more lymphocytes in PFTS- or *G. lucidum*-treated mice than in PBS-treated mice (Fig. 3A). The lymphocyte transformation rate was 40.7±5.1 and 38.6±5.7% in the PFTS and *G. lucidum* groups, respectively, which was significantly higher than that in the PBS-treated group (20.9±3.9%) (Fig. 3B).

PFTS prolongs the survival of tumor-bearing mice. The present study demonstrated that PFTS enhances the immune response in mice, and then aimed to test whether PFTS possesses antitumor properties. A tumor-bearing mouse model was established by inoculating mice intraperitoneally with S-180 tumor cells, and the survival of these mice with either PFTS or PBS treatment was then measured. As shown in Fig. 4A, the median survival time was 9.1 and 4.8 days in PFTS- and PBS-treated mice, respectively. Using the log-rank test analysis, PFTS-treated mice had a significantly longer survival time compared with that exhibited by PBS-treated mice (P<0.0001; Fig. 4B).

Discussion

In the present study, PFTS was demonstrated to enhance peripheral WBCs suppressed by cyclophosphamide, improve peritoneal macrophage phagocytosis and increase the rate of lymphocyte transformation in mice. Furthermore, PFTS extended the survival time of tumor-bearing mice. To the best of our knowledge, the present study is the first to clarify the effects of the freeze-thaw-extracted PFTS on immune function and antitumor activity *in vivo*.

Leukocytes or WBCs are a major component of the immune system and perform a crucial role in defending the body against infectious organisms and carcinogenesis (18). Abrogation of leukocytes with chemotherapy drugs is life-threatening for patients with cancer (19). Although granulocyte macrophage-colony-stimulating factor is usually effective in raising leukocyte count following chemotherapy, its cost and side effects pose a challenge for patients (20). In a previous study, PFTS significantly enhanced peripheral leukocytes and almost completely reversed the leukopenia in mice induced by cyclophosphamide, to a similar level to that mediated by *G. lucidum*, a well-known immune activator (21). These findings are compatible with a notion that dietary supplementation of purple sweet potato extract attenuates the suppression of T cell and B cell proliferation and T helper 1/T helper 2 cytokine imbalance in immunodeficient mice, partly contributing to ameliorate immune dysfunction (22). Collectively, it was speculated that stimulation of leukocyte proliferation or counteraction of bone marrow suppression may contribute to PFTS-mediated reversal of leukopenia by cyclophosphamide (23).

It is of interest to know whether PFTS has the potential to increase immune functions. This question was addressed by measuring the peritoneal macrophage phagocytosis and lymphocyte transformation in mice treated with PFTS. It has been well established that macrophage phagocytosis characterizes the activation of macrophages and reflects innate immune responses, and that lymphocyte transformation reflects the

ability of lymphocytes to respond to antigen stimulation and represents adaptive immunity (24). Innate and adaptive immunity serve important roles in preventing infections and surveying tumorigenesis (25,26). The present data demonstrated that, as with *G. lucidum*, PFTS increased peritoneal macrophage phagocytosis and the rate of lymphocyte transformation. In this line, a dose-dependent manner of purified sweet potato polysaccharide has been demonstrated to affect the phagocytic function (27).

The finding that PFTS is able to promote immune functions led us to test directly whether PFTS has antitumor properties. Using a mouse tumor model, it was observed that mice fed with PFTS had significantly longer survival times compared with those of mice fed with PBS, demonstrating its antitumor property. These results were supported by a previous study showing that polyphenol-rich sweet potato greens extract extends survival time by inhibiting proliferation and inducing apoptosis in prostate cancer cells *in vitro* and *in vivo* (28). The mechanisms underlying the antitumor function of PFTS remain to be elucidated.

There are four caveats to the current study that require brief mention. First, only potato cultivated in the Wuchuan area of Inner Mongolia, mid-west region of China was tested, where the climate is dry and sunshine is abundant. It is unknown whether potatoes cultivated in other geographical regions are similar to those used in the current study. Second, the freeze-thaw method causes cells to swell and shrink and break up ultimately due to the ice crystals formed in the freezing process. Several freeze-thaw cycles may be required to facilitate cell membrane breakage and the release of cell components (29). Methodological modification is ongoing for maximizing the PFTS bioactivity. Third, following the routine drug discovery approach, PFTS bioactivity was tested but not observed *in vitro*. This may be a result of physical or chemical cytotoxicity. Lastly, the effect of PFTS on macrophage phagocytosis and lymphocyte transformation was assessed at only one time point (day 20), but its long-term effects were not evaluated. These assessments from multiple doses and time points are required in future studies.

In conclusion, the present study demonstrated for the first time that PFTS improves immune functions and extends the survival time of mice with ascite tumors. With its ease of production and dietary administration path, PFTS may possess the potential to become a clinical option for the prevention and treatment of cancer. While the present study is only a first step towards uncovering the anticancer properties of PFTS, further investigation is warranted to assess the bioactivity and clinical potential of PFTS comprehensively.

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