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PROTECTIVE EFFECTS OF POLL GLAND SECRETION ON IMMUNOSUPPRESSED AND S180 TUMOUR-BEARING MICE

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ABSTRACT

The poll gland secretions (PGS) have been used traditionally for the treatment and prevention of many diseases for centuries in Inner Mongolia (China) and Mongolia. The present study was performed to evaluate the immunostimulatory activities and anti-tumour effects of PGS *in vivo* and *in vitro*. The concentration of TNF-α, IL-2, IL-6, IgG and IgM in the serum of experimental animals were measured by an enzyme-linked immunosorbent assay (ELISA) following the manufacturer's protocols and the spleen index and thymus index were calculated using the gravimetric method. The phagocytic activity of the macrophage monocytes was evaluated by a carbon clearance assay and the effect of PGS on the growth of S180 cells *in vitro* was examined by the determination of the IC50 of PGS. A moderate to high dose of PGS can elevate the spleen and thymus indices and significantly increase the serum concentrations of IL-6, IgG and TNF-α. Moreover, PGS can also enhance the phagocytic activities of macrophage monocytes in immunosuppressed experimental mice. On the other hand, PGS can significantly increase the concentration of serum TNF-α, IL-2 and IL-6 and directly inhibit the growth of solid tumours in mice. Additionally, PGS can also significantly inhibit the growth of S180 cells *in vitro*, with an IC50 of 15.63 $\mu g/ml^{-1} \pm 2.18$.PGS can significantly improve the inhibited immune function of mice induced by cyclophosphamide (CTX) and can reduce the growth of solid tumours *in vivo*. In addition, PGS can also directly inhibit the S180 cell's growth *in vitro*, as well as considerably enhance the immune function of tumour-bearing mice.

Key words: Anti-tumour effects, immunostimulatory activities, immunosuppressed mice, poll gland secretion, tumour-bearing mice

Drugs that are derived from animals have been widely used in traditional Mongolian medicine and play an important role in the treatment and prevention of certain diseases. Moreover, these drugs have historically made great contributions to mankind and some drugs still have pivotal medicinal values in modern medicine (Chen *et al,* 2004). Poll gland secretion (PGS), known as bokhi in Mongolia, is a drug of animal origin in traditional Mongolian medicine and has been commonly used in Inner Mongolia and Mongolia for the treatment and prevention of several diseases for centuries, such as uterine myoma and gastric cancer.

The poll glands are symmetrical bodies situated subcutaneously on the back of the neck and between the two ears of bull camels (Tingari and Rahma, 1981). They seem to get their name from their position in the poll region (Leese, 1927). They are present in male camels at birth and are mainly composed of sweat and sebaceous glands and no visible glands are observed in all of female camels at any age (Safwat *et al,* 2012). In addition, they are known to exhibit a cyclic activity, producing a yellowish watery secretion with a characteristics of offensive odour during the rutting season (Purohit and Singh, 1958; Lee and Schmidt, 1962; Singh and Bharadwaj, 1978; Yagil and Etzion, 1980; Taha *et al,* 1994). The glands then become atrophied during anestrus and completely shrinks in castrated bull camels. The PGS is composed of sexual hormones (e.g. progesterone, oestrogen and testosterone), as well as short chain fatty acids including acetic, propionic, isobutyric, butanoic and isopentanoic acids (Yagil and Etzion, 1980; Ayorinde *et al,* 1982; Tingari and George, 1984; Kumar and Agarwal, 1996; Rai *et al,* 1996; Rai *et al,* 1997; Yasuro *et al,* 1998). According to one report, PGS have a remarkable pheromone effect on the reproductive physiology of bull camels (Tingari and George, 1984) and attract females. The previous studies have primarily focused on the histological

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and histochemical characteristics of the poll glands, as well as ultrastructural features of the glands during the rutting and non-rutting seasons (Safwat *et al,* 2012; Tingari and George, 1984; Atoji *et al,* 1998). However, few scientific papers have been found regarding the pharmacological effects of PGS.

When PGS is used as an alternative medicine, it has a particularly important role in the treatment and prevention of many diseases, especially for certain types of tumours. These effects have been described for over 200 years in traditional Mongolian medicine, with precise therapeutic effects. Since decreased immune function is closely related to tumourigenesis and development, the immunosuppressive and tumour-bearing animal models will be established for this study. In addition, we will elucidate whether PGS possesses any positive effects on these animal models and whether it can directly inhibit the growth of solid tumour *in vivo* and S180 cells *in vitro*.

Materials and Methods

Chemicals and Reagents

Indian Ink (Xizhong, China); Cyclophosphamide (CTX, CPA) (Pude, China); ELISA kits for TNF-α, IL-2, IL-6, IgG and IgM (Boster Bioscience, China); thiazolyl blue (MTT) cell growth assay kits (Sigma); RPMI-1640 (Sigma); foetal bovine serum (TBD, China); and dimethyl sulfoxide (DMSO) (Gayload Slidell, USA).

Instruments

The primary instruments used throughout this study include, an ultraviolet–visible spectrophotometer (TU-1800PC, Persee, China); electronic analytical balance (Sartorius); Labconco Freeze Dry System/Freezone 2.5 (USA); cell counter (Cyt-1000, Japan); inverted microscope (ZXT1, Olympus); carbon dioxide incubator (Thermo forma371); automatic high pressure sterilising pot (HVE-50, Israel); multi-mode microplate reader (Synergy 4); and a refrigerated high-speed centrifuge (3-30K, Sigma).

Samples and preparation of the poll gland secretion extract

The PGS samples were collected from rutting bull camels in West Sonid, Inner Mongolia, China. The sample collection procedure was approved by the camel protection association (CPA) of Inner Mongolia for the control and supervision of experimental camels. Firstly, we sheared the hairs surrounding the poll gland, then held the poll gland with prepared gauze. When the secretion fully penetrated into the gauze, we collected the gauze and packed into sealed bags and sent them to the laboratory by cooler box. The gauze containing the secretion was steeped in 200 ml of distilled water at 37°C 3 times and then filtered. The filtration was lyophilised using the Labconco Freeze Dry System and the lyophilised PGS powder was collected.

Animals

All animal experimental procedures were performed in accordance with the guidelines of the Ethical Committee for the experimental use of animals at Inner Mongolia Agricultural University (Huhhot, Inner Mongolia, China).

Kunming mice (half male and half female, weighing 20.0±2.0 g) were provided by the Experimental Animal Centre of the Chinese Academy of Military Medical Science. The standard conditions for temperature and humidity along with the exposure to a 12h:12h light and dark cycle were maintained throughout the study. All mice were fed a standard rodent diet and were allowed to drink water *ad libitum*. Moreover, all animals were allowed to acclimatise to the experimental conditions for one week before beginning the study to minimise animal stress.

Cell lines

Mouse sarcoma S180 cell lines (ATCC-TIB66) were provided by the Chinese Academy of Military Medical Science. The S180 cell lines were maintained in the logarithmic phase of growth in the peritoneal cavity of the mice, as well as in RPMI 1640 medium supplemented with 100 IU/ml penicillin and 100 IU/ml streptomycin and 10% foetal bovine serum at 37°C under humidified air with 5% CO_2.

Induction of immunosuppressed mouse model and treatment with PGS

A total of 60 mice were randomly divided into 5 groups (n=12), with an equal number of males and females. Specific grouping and treatment protocols are presented in table 1.

Blood was collected from each animal *via* a retro-orbital puncture 24 h after the last administration of drugs and the serum was separated to detect the concentration of TNF-α, IL-6, IgM and IgG. After the blood collection, all of the mice were sacrificed, the spleen and thymus were harvested and weighed and their indices were calculated according to the following formula: the organ index = weight of organ (mg) /average body weight (g).

Table 1. Grouping of experimental mice and treatment protocols.

Groups	N	Treatments	
		Day 1~10	Day 11~14
Normal Control	12	PO NS 0.2ml $10g^{-1}$	IP NS 0.2ml $10g^{-1}$ + PO NS 0.2ml $10g^{-1}$
CTX Model	12	PO NS 0.2ml $10g^{-1}$	IP CTX 80 mg kg^{-1} + PO NS 0.2ml $10g^{-1}$
PGS Low	12	PO PGS 2.5 mg kg^{-1}	IP CTX 80 mg kg^{-1} + PO PGS 2.5 mg kg^{-1}
PGS Meddle	12	PO PGS 25 mg kg^{-1}	IP CTX 80 mg kg^{-1} + PO PGS 25 mg kg^{-1}
PGS High	12	PO PGS 250 mg kg^{-1}	IP CTX 80 mg kg^{-1} + PO PGS 250 mg kg^{-1}

Establishment of tumour-bearing mouse model and treatment with PGS

A mouse sarcoma S180 cell line was harvested and washed three times with sterilised normal saline (NS), then diluted with sterilised saline to a concentration of 1×10^7 cells/ml. Each mouse was subcutaneously inoculated into the right armpit region with 0.2 ml of the cell suspension on the first day of the experiment and the mice were randomly divided into 5 groups (n=12). The tumour model group was treated only with normal saline at 0.1ml/10 g; and the CTX-treated group was administered 20mg/kg body weight (bw) CTX. In addition, three experimental groups were treated with, i.e. a low-dose of 2.5mg/kg bw PGS; a middle-dose of 25mg/kg bw PGS; and a high-dose of 250mg/kg.bw PGS, respectively. Following 14 days of consecutive treatment of once per day, peripheral blood was collected from each mouse by a retro-orbital puncture 1h after the last administration and the serum was separated for the detection of TNF-α, IL-6, IL-6, IgM and IgG concentrations. Finally, all of the mice were sacrificed, the solid tumour was harvested from each mouse and weighed and the tumour inhibition rate was calculated according to following formula: the tumour inhibition rate = [(average tumour weight of tumour model group— average tumour weight of the experimental group) ÷ average tumour weight of the tumour model group]×100%.

Concentration of cytokines and immunoglobulins in the serum

The concentrations of TNF-α, IL-2, IL-6, IgG and IgM in the serum were determined by ELISA kits according to the manufacturer's instructions. The analytic sensitivities for these assays were 7.8 pg/ml for TNF-α, 15.6 pg/ml for IL-2 and IL-6), 0.3 µg/ml for IgG and 0.6 µg/ml for IgM, respectively.

The phagocytic activity of macrophage monocytes

Animal groups, drug delivery and the immunosuppressed mouse model are similar to mentioned above. The phagocytic activity of the macrophage monocytes was determined by the carbon clearance test. Briefly, 1h after the last dose of the drug administration, all of the mice were injected with 20% Indian ink via the coccygeal vein at a dose of 0.1ml/10 g.bw. A 20µl blood sample was collected at 2min and 20min, respectively following the injection of Indian ink, then mixed with 2 ml of 0.1% sodium carbonate solution. The absorbance of this solution was determined at 600nm by a UV spectrophotometer. The carbon clearance index k and the phagocytic index α of the macrophage monocytes were calculated using the following equation (Hafiz *et al,* 2016) :

$$k= (\lg OD_2 - \lg OD_{20}) / (T_{20} - T_2)$$

$$\alpha=\sqrt[3]{k} \times \text{body weight}/ (\text{spleen weight}+\text{liver weight})$$

Direct inhibitory effect of PGS on the in vitro growth of tumour cells

The inhibition rate of the tumour cells *in vitro* following PGS treatments was performed using the MTT method (Wanpeng *et al,* 2013). The S180 cells during logarithmic growth were resuspended in serum-free complete RPMI-1640 medium to prepare a 1×10^7 cells/ml cell suspension and seeded into 96-well culture plates with 90 µl/well. All treatments were divided into 5 groups with 6 parallels in each group. A different concentration of the PGS solution was added into each experimental well to a final concentration of 1 µg ml^{-1}, 0.1 µg ml^{-1}, 0.01 µg ml^{-1}, 0.001 µg ml^{-1} and 0.0001 µg ml^{-1}, respectively. The same volume of serum-free complete RPMI-1640 medium containing the S180 cells was used as a control. The plates were then incubated for 48 h at 37°C under 5% CO_2. Next, the culture media was removed and 10 µl of the MTT solution (5 mg/ml) was added to each well. After an additional 4 h incubation, the formazan crystals were solubilised with 100 µl DMSO for 15 min. The absorbance at 570 nm was determined using a multi-mode microplate reader. The inhibition rate was calculated using the following formula:

The inhibition rate (%) = $(1 - A_{control}/A_{treated}) \times 100\%$.

Statistical analysis

All of the data in this study are expressed as the mean±standard deviation (SD). The data were evaluated by SAS 9.0 software using a one-way analysis of variance (ANOVA). The results were regarded to be statistically significant if the P value was < 0.05.

Results

The effect of PGS on the thymus index, spleen index and the phagocytic activity of macrophage monocytes in immunosuppressed mice

As shown in table 2, compared with the normal control group, the spleen index, thymus index and the phagocytic activities of the macrophage monocytes were remarkably decreased in the CTX model group ($P < 0.05$). However, a middle and a high dose of PGS were associated with a marked elevation of both spleen and thymus indices and the phagocytic activity of macrophage monocytes was also enhanced in the immunosuppressed mice. Therefore, a moderate to high dose of PGS can restore the suppressed immune function of the animals by promoting the growth of immune organs and enhancing the phagocytic function of macrophage monocytes.

Effect of PGS on serum cytokines and immunoglobulin concentrations in immunosuppressed mice

In table 3, the concentrations of IL-6, TNF-α, IgM and IgG were significantly decreased in the CTX model control group compared with the normal control group ($p < 0.05$). PGS can promote the production of IL-6 and TNF-α and their serum concentrations in immunosuppressed mice treated with middle dose and high dose of PGS reached the same or higher levels compared to the normal control group especially, the concentrations of IL-6. Additionally, the middle and high dose of PGS markedly increased the concentration of IgG in the immunosuppressed groups, while the effect of PGS on IgM levels was not statistically different. Therefore, an adequate amount of PGS can improve the suppressed immune function by promoting both cellular and humoral immune functions.

Table 2. Effect of PGS on thymus and spleen indices and macrophages phagocytic activity ($\bar{x}$±s, n=12).

Groups	Thymus Index (mg/g)	Spleen Index (mg/g)	Phagocytic Index (α)
Normal Control	2.86±0.34	3.95±0.59	6.278±0.485
CTX Model Control	1.36±0.26*	1.05±0.22*	5.540 ±0.701*
PGS Low	1.27±0.16*	0.95±0.14**	6.151±0.314
PGS Middle	1.47±0.19*	1.42±0.11$^{*\#}$	6.260 ±0.419$^{\#}$
PGS High	1.63±0.07$^{*\#}$	1.26±0.18$^{\#}$	6.651 ±0.562$^{\#}$

** Significant difference at $p < 0.01$ compared with the normal control group.
* Significant difference at $p < 0.05$ compared with the normal control group.
Significant difference at $p < 0.01$ compared with the CTX model control.
Significant difference at $p < 0.05$ compared with the CTX model control.

The effect of PGS on serum cytokines and immunoglobulin concentrations in tumour-bearing mice

The effect of PGS on the serum concentrations of IL-2, IL-6, TNF-α, IgM and IgG in tumour-bearing mice is presented in table 4 and Fig 1. Compared with the tumour model control, the IL-6, TNF-α and IL-2 concentrations in the serum of mice that received a high and middle dose of PGS were significantly increased. Additionally, some of these cytokines reached almost the same level as the CTX-treated group. These results indicate that a moderate to high dose of PGS can exert anti-tumour effects by significantly elevating pro-inflammatory cytokine and immunoglobulin concentrations in tumour-bearing animals.

In Fig 1, compared with the tumour model control, the concentrations of serum IgG in each group was not statistically different, except that

Table 3. The effect of PGS on cytokine and immunoglobulin concentrations in immunosuppressed mice ($\bar{x}$±s, n=12).

Groups	TNF-α (pg.ml^{-1})	IL-6 (pg.ml^{-1})	IgM (μg.ml^{-1})	IgG (μg.ml^{-1})
Normal Control	47.98±1.83	75.33±6.02	3.36±0.29	5.64±1.16
CTX Model Control	31.68±3.08*	63.25±5.59	2.82±0.32*	3.78±0.56*
PGS Low	35.88±8.09*	84.75±7.85$^{*\#}$	2.92±0.46*	3.24±0.77*
PGS Middle	36.68±7.04*	102.58±18.72$^{**\#\#}$	3.18±0.36	4.94±0.16$^{\#}$
PGS High	45.53±3.17$^{\#}$	100.5±9.49$^{**\#\#}$	3.10±0.13	5.42±0.82$^{\#}$

** Significant difference at $p < 0.01$ compared with the normal control group.
* Significant difference at $p < 0.05$ compared with the normal control group.
Significant difference at $p < 0.01$ compared with the CTX model control.
Significant difference at $p < 0.05$ compared with the CTX model control.

Table 4. The effect of PGS on serum TNF-α, IL-2 and IL-6 concentrations in tumour-bearing mice.

Groups	IL-6 (pg ml^{-1})	TNF-a (pg ml^{-1})	IL-2 (pg ml^{-1})
Tumour Model Control	91.83±2.56	98.56±2.80	104±19.15
CTX Treated	125.41±4.42**	196.33±10.33**	180.11±26.59**
PGS Low	99.75±4.08$^{\#}$	118.55±14.16$^{\#}$	114.72±16.03$^{\#}$
PGS Middle	115.33±7.01**	126.33±18.55$^{*\#}$	142.88±22.34*
PGS High	122.08±7.43**	165.22±21.35**	175.11±15.41**

** Significant difference at $p < 0.01$ compared with the normal control group.

* Significant difference at $p < 0.05$ compared with the normal control group.

Significant difference at $p < 0.01$ compared with the CTX model control.

Significant difference at $p < 0.05$ compared with the CTX model control.

the serum IgG content was significantly increased in the CTX treated group. However, the serum IgM concentration in the low dose PGS group was remarkably higher than that of tumour model control. Therefore, an adequate amount of PGS can promote IgM generation in tumour-bearing animals.

The effect of PGS on the growth of solid tumour in mice

The results of the effect of PGS on solid tumour weight gain and the inhibition rate are shown in fig 2. Compared with the tumour model control, the high dose of PGS remarkably inhibited the growth of solid tumour. The tumour weight decreased significantly, with a tumour inhibition rate of 48.5%. Therefore, these results suggest that PGS has a notable inhibitory effect on solid tumour in S180 sarcoma tumour-bearing mice.

Inhibitory effect of PGS on the growth of tumour cells in vitro

The cell culture time and concentration of the cell suspension was determined to be 48 h and 1×10^7/ml, respectively, according to the results of the preliminary test. The results indicated that PGS can inhibit the growth of S180 sarcoma cells *in vitro* in a dose-dependent manner (Fig 3). In addition, the half maximal inhibitory concentration (IC_{50}) was 15.63±2.18 (μg ml^{-1}), less than 30 μg ml^{-1}. Therefore, PGS has a significant direct inhibitory effect on the growth of S180 sarcoma cells *in vitro*.

Discussion

A large number of therapeutic agents derived from animal sources in traditional medicine have been used to prevent and treat various types of disease and to improve the immune functions (Choi *et al,* 2006). The poll gland is a special gland only seen in male camels and the secretions of the poll gland play a critical role in camel reproductive physiology with respect to pheromone and hormone production. Previous studies have primarily focused on the histological and histochemical characteristics of the poll glands, as well as on the ultrastructural changes of the poll glands during the rutting and non-rutting seasons of bull camels (Safwat *et al,* 2012; Tingari and George, 1984; Rai *et al,* 1996; Atoji *et al,* 1998) and the chemical components of the secretions (Ayorinde *et al,* 1982; Kumar and Agarwal, 1996). But no report has elucidated the immunostimulatory activities and anti-tumour properties of PGS. However, PGS has not only historically used in traditional Mongolian medicine for the treatment of many diseases, but is also used in modern Mongolian medicine for adjuvant therapy of immunosuppressed patients with certain types of

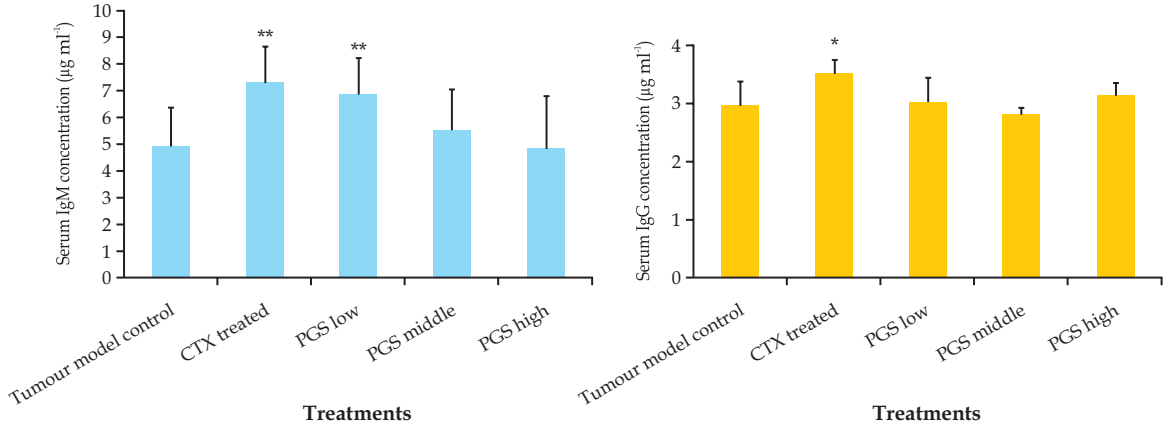

Fig 1. Effect of PGS on serum IgG and IgM concentrations in tumour-bearing mice (Note:** Significant differences at $p < 0.01$ compared with the tumour model group; * Significant differences at $p < 0.05$ levels compared with the tumour model group).

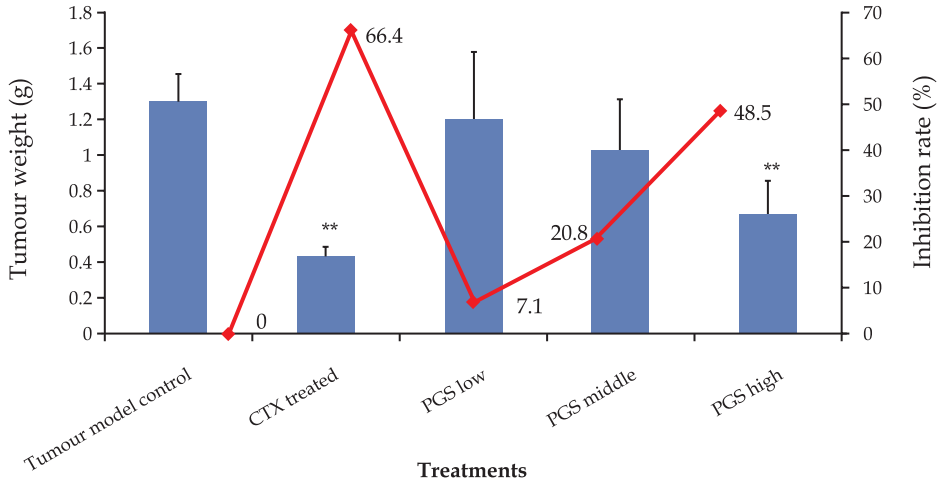

Fig 2. The effect of PGS on tumour weight and tumour inhibition rate (Note: ** Significant difference at $p < 0.01$ levels compared with the tumour model group; blue column chart indicate tumour weight; red line chart indicate tumour inhibition rate)

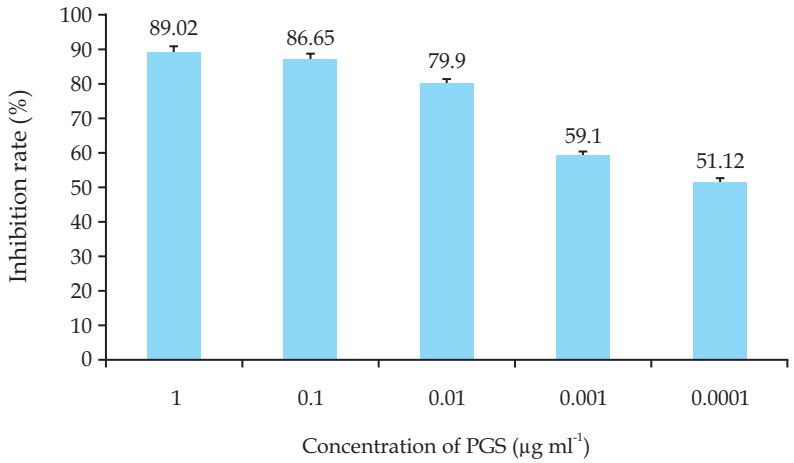

Fig 3. Effect of different concentrations of PGS on the growth of S180 cells *in vitro*

cancer. To identify and prove the pharmacological activities and potential mechanisms of PGS, we focused on the immune-potentiating activities and anti-tumour activities of PGS in present study.

The thymus and the spleen are primary and secondary lymphoid organs, respectively and the thymus index and spleen index directly reflect the nonspecific immunity of the organism (Cesta, 2006). Moreover, immunopotentiators could increase the relative weights of the thymus and the spleen (Zhang *et al,* 2013). On the other hand, macrophages are the primary phagocytes of the immune system. These cells reside in every tissue of the body (e.g., microglia, Kupffer cells and osteoclasts according to the location in the body) where they engulf apoptotic cells and pathogens, as well as produce immune effector molecules. Among these effector responses, the phagocytic function of macrophage monocytes is one of the most important indexes of nonspecific immune function and is commonly used in evaluating the non-specific immune status of animals (Andrew *et al,* 2011). Compared with the CTX model control group, the middle and high dose of PGS markedly increased both the spleen and thymus indices, as well as the phagocytic function of the monocyte-derived macrophages in our study. Therefore, we believe that PGS can restore the suppressed non-specific immune function by promoting the growth of immune organs and activating monocyte-derived macrophages.

The cytokines, including IL-2, IL-6 and TNF-α, can regulate both the cellular and humoral immune responses by affecting immune cell proliferation, differentiation and functionality, which play a critical role in combating tumour growth. IL-2 is one of the most important immune factors secreted primarily by T cells, which promotes immune cell

proliferation and differentiation. Moreover, IL-2 has been approved by the FDA for the treatment of metastatic renal cell carcinoma and metastatic melanoma (Geok and Laszlo, 2014). In addition, IL-6 is another important immune mediator that regulates diverse cellular functions, including the proliferation and differentiation of B-cells and T-cells (Sobota *et al*, 2008). TNF-α is produced at the highest levels by activated macrophages, T lymphocytes and NK cells and plays a pivotal role in the cellular immune process by aiding in the activation of macrophages following the phagocytosis of pathogens or abnormal cells (Shiro, 2011). The ability to enhance the production of these cytokines has been widely used for the evaluation of the immunostimulatory activity of immunopotentiators. Therefore, we studied the effects of PGS on the serum concentrations of IL-2, IL-6 and TNF-α, as well as, IgM and IgG in both immunosuppressed mice and tumour-bearing mice in the current study. Compared with the immunosuppressed or tumour control models, moderate to high doses of PGS could elevate the serum concentrations of IL-6 and TNF-α in an immunosuppressed mice, but could also increase the serum concentration of IL-2, IL-6 and TNF-α in tumour-bearing mice. In addition, the appropriate dose of PGS could also increase the IgG concentration in immunosuppressed mice, as well as markedly enhance the IgM content in tumour-bearing mice. Therefore, one of the most important mechanisms of the immunostimulatory and antitumour effects of PGS was to overcome the suppressed immune function by promoting both cellular and humoral immune activation.

Cancer remains one of the most common causes of death and is a disease with an infiltrative and destructive nature that has the potential to spread to various organs from its site of origin. Therefore, it is important that anticancer compounds can selectively inhibit the proliferation of tumour cells and reduce the growth of solid tumours in the body. To investigate whether PGS can directly impact tumour growth, the solid tumour inhibition rate *in vivo* and the tumour cell inhibition rate *in vitro*, respectively, were calculated in this study. Compared with the tumour control model, a moderate to high dose of PGS was found to significantly inhibit the growth of solid tumours in S180 transplanted mice, with the highest tumour inhibition rate of 48.5%. In addition, PGS was also associated with a dose-dependent inhibition of the proliferation of S180 cells *in vitro* and it's IC_{50} was 15.63±2.18 μg ml^{-1}, less than 30 μg ml^{-1}. Therefore, one of the another important mechanisms of the anti-tumour effects of PGS was the direct inhibitory effects on the growth of solid tumours in mice and the proliferation of tumour cells *in vitro*.

The experimental results of both the *in vitro* and *in vivo* experiments in the present study indicate that PGS has anticancer effects and immune activating properties. Therefore, PGS could potentially restore the suppressive immune function of animals and possess therapeutic activities against certain types of tumours.

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