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Vesicular phospholipid gels using low concentrations of phospholipids for the sustained release of thymopentin: pharmacokinetics and pharmacodynamics

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Vesicular phospholipid gels (VPGs) with high concentrations of phospholipids are used as implantable depots for sustained release of drugs due to high viscosity. This study aimed to investigate VPGs with low concentrations of phospholipids for subcutaneous injection and sustained release *in vivo*. A small peptide, thymopentin, was selected and incorporated into various VPG formulations. The VPG viscosity was greatly increased with higher concentrations of phospholipids (E80) and thus VPGs based on low lipid contents are more suitable for injection. Additionally, VPGs loaded with 5-hydroxy-fluorescein-thymopetin (5-FAM-TP5-VPGs) were developed and their pharmacokinetic profile was investigated *in vivo*. After subcutaneous injection, the release time of 5-FAM-TP5 was 216 h for 5-FAM-TP5-VPGs (containing 300 mg/g lipid), which was much longer than that of 5-FAM-TP5 solution. The therapeutic efficacy of TP5-VPGs (containing 300 mg/g lipid) after subcutaneous administration once a week was demonstrated to be comparable to that of TP5 solution injected subcutaneously once daily for 7 days. In conclusion, TP5-VPGs with low lipid content (300 mg/g) displayed sustained release properties *in vivo* that may serve as a sustained delivery system for subcutaneous injection.

1. Introduction

More than 20% of new drugs nowadays are proteins or peptides (Pavlou et al. 2004). Most therapeutic proteins or peptides are known to display rapid blood clearance that largely restricts their clinical application. Frequent administration is usually required to maintain stable therapeutic levels in the blood. Thymopentin (TP5, Arg-Lys-Asp-Val-Tyr, CAS: 69558-55-0), a small therapeutic peptide, is the active site of thymopoietin II with 49 amino acids (Tischio et al. 1979). As an immunomodulatory agent, it has been widely used in the treatment of cutaneous T-cell lymphoma (Bernengo et al. 1992), cancer, immunodeficiency (Bodey et al. 2000), rheumatoid arthritis (Ambrogi et al. 1992; Sundal et al. 1994), acquired immunodeficiency syndrome (AIDS) (Coppola et al. 1996; Merigan et al. 1996), and severe acute respiratory syndrome (SARS) (Zhang et al. 2003). Similar to other peptides and protein drugs, only the lyophilized powder of TP5 is available for intramuscular administration or *i.v.* infusions due to its short half-life (~30 s) in plasma and rapid metabolism in the gastrointestinal tract (Tischio et al. 1979). Moreover, the therapeutic effect can last for several months. Therefore, developing a sustained release formulation for TP5 is advantageous as compared to current formulations.

Vesicular phospholipid gels (VPGs) are highly concentrated lipid dispersions where the liposomes form a three-dimensional network (Zhang et al. 2008), normally consisting of 250 mg/g (325 mM) to 600 mg/g (780 mM) lipids (Brandl et al. 2003). The high lipid content leads to a significantly higher ratio of aqueous compartments both within and in-between the liposomal vesicles, rendering the VPGs more suitable for entrapping hydrophilic compounds such as peptides and proteins (Brandl et al. 1998; Kaiser et al. 2003). Due to high lipid content, VPGs display semi-solid properties and a high inner viscosity, making it difficult for drugs to escape from VPGs and thus slowing down the release process (Brandl et al. 1995). Compared with other sustained delivery systems such as microspheres or nanoparticles, VPGs show following advantages: i) simple manufacturing process, *i.e.,* no organic solvent or heating is required which may contribute to the instability of peptide drugs; ii) material biocompatibility, phospholipids are used as the major component. Therefore, VPGs appear to be a good alternative to the current delivery strategies for TP5 with a sustained release profiles.

Research demonstrated the potential of drug loaded VPGs as sustained delivery vehicles (Massing et al. 2008; Kaiser et al. 2003; Tian et al. 2010; Tardi et al. 1988). These studies prepared VPGs containing high lipid contents, for example, more than 450 mg/g phospholipid was used, which contributed to a longer sustained release period than formulations with a lower lipid content *in vitro* (Tian et al. 2010; Tardi et al. 1988). VPGs with high lipid contents have a high viscosity which is not suitable for parenteral administration. Studies were reported on the dispersed VPGs loaded with small molecular drugs such as

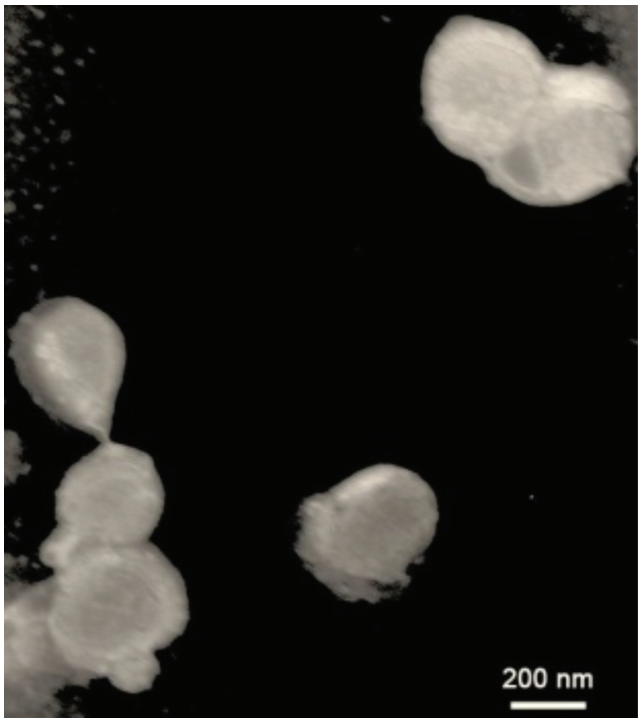

Fig. 1: Microphotograph of the TP5-VPGs dispersions.

vincristine and 5-fluorouracil for *i.v.* injection (Guthlein et al. 2002; Kaiser et al. 2003). VPGs have not been reported as implants directly aiming for subcutaneous injection. Moreover, it remains to be addressed if VPGs with low concentrations of phospholipids could sustain the release of drugs *in vivo* and serve as a long-acting formulation.

In this study, we aimed to develop VPG-based formulations with relatively low lipid contents for injection purposes. TP5 was selected to be loaded in such VPG vehicles. Their *in vitro* release behavior and *in vivo* pharmacodynamic profile were evaluated.

2. Investigations and results

2.1. Morphology

After redispersion in distilled water, TP5-VPGs displayed mainly spherical shapes with co-existent single or congregate lipid vesicles observed by transmission electron microscopy (TEM) (Fig. 1). The congregate particles were mostly likely formed due to the gentle dispersion. Also, the small lipid vesicles might easily resemble after dispersion.

2.2. Determination of particle size of TP5-VPGs

After redispersion in distilled water, multiple peaks in the size distribution were observed for all formulations (Fig. 2). Although there were particles with sizes up to 1000 nm, the VPGs could be used for subcutaneous administrations. When filtered through 1000 nm pores, the mean size of the smaller vesicles was reduced to around 450 nm. After redispersion, the mean size of VPGs displayed an increase in size with increasing amounts of E80 in VPGs (Fig. 3).

2.3. Rheology

All VPGs prepared in this study appeared to be in the form of semifluid. As shown in Fig. 4, the viscosity of the TP5-VPGs varied with varying concentrations of E80 in the VPGs in a non-linear manner. The VPG viscosity increased dramatically with

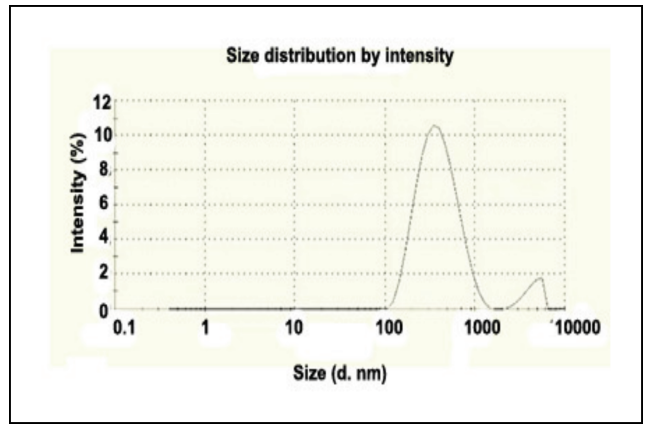

Fig. 2: Particle size distribution of TP5-VPGs dispersions containing 300 mg/g phospholipid by the laser particle size analyzer.

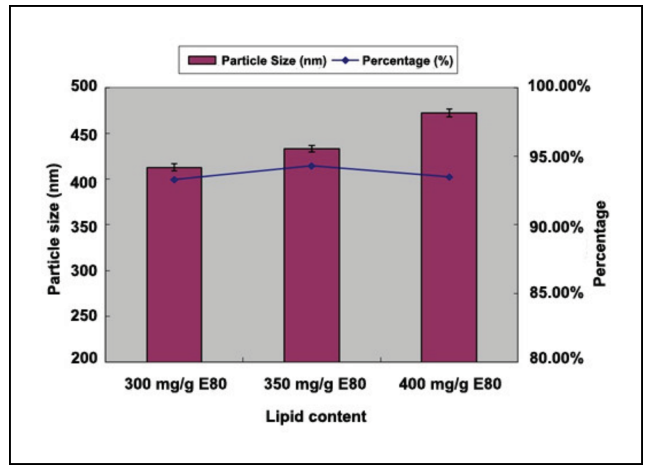

Fig. 3: Particle size of TP5-VPGs dispersions (mean ± SD, n = 3) and percentage of particles below 1000 nm with varying concentrations of E80.

an increase in lipid content to 40%. The VPG with 300 mg/g lipid passed through the syringe more easily than the VPGs with 400 mg/g lipid. Lower viscosity of the VPGs indicated less resistance through the syringe. The VPG with higher lipid content displayed a relatively high viscosity making them less suitable for subcutaneous injection. Therefore, an appropriate lipid con-

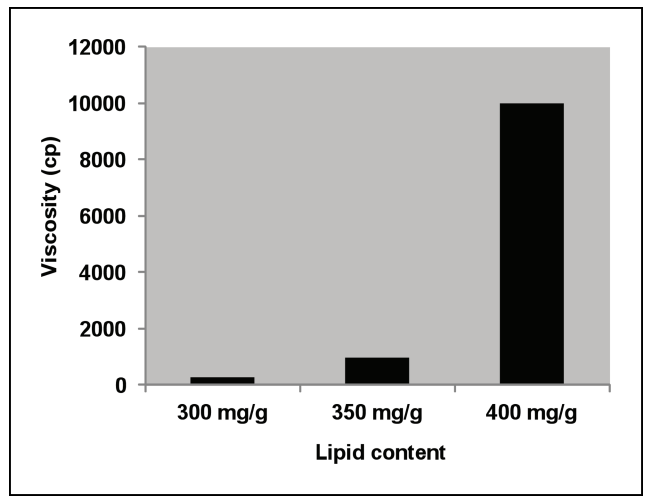

Fig. 4: Viscosity (mean ± S.D., n = 3) of TP5-VPGs containing different concentrations of phospholipid. All gels were based on egg PC (E80) and loaded with 20 mg/g TP5.

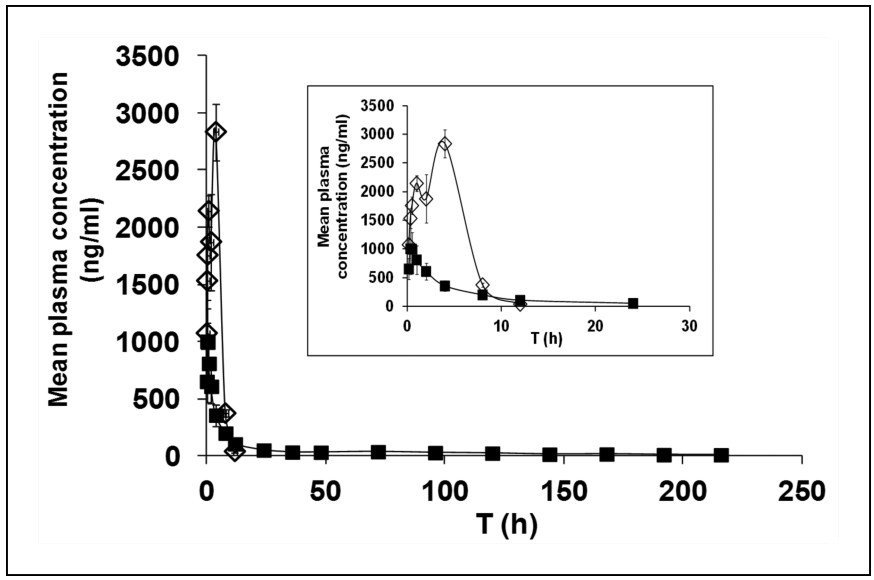

Fig. 5: Mean plasma 5-FAM-TP5 concentration-time curve of 5-FAM-TP5 solution (◇), 5-FAM-TP5-VPGs with 300 mg/g E80 (■) via subcutaneous administration at a dose of 8 mg/kg (n = 5).

tent is critical to the injectable application of VPG formulations *in vivo*.

2.4. In vivo studies

2.4.1. Pharmacokinetics of 5-FAM-TP5-VPGs

To evaluate the release profile of VPGs *in vivo*, pharmacokinetic studies were carried out in rats and the results were compared to *in vitro* release studies previously reported (Chen et al. 2013). The fluidity of VPGs with high lipid contents did not suit the clinical use. Therefore, only TP5-VPGs containing 300 mg/g E80 were evaluated here. Previous studies showed the TP5 was not stable in human plasma with a half-life of ~30 s (Tischio et al. 1979). It is difficult to determine the plasma concentration of TP5 by conventional methods such as HPLC and HPLC-MS. Therefore, TP5 was fluorescently labeled by 5-FAM. Moreover, no abnormal behavior of the animals and no histopathological changes at the injection sites were observed.

As is shown previously (Chen et al. 2013), VPGs containing 300 mg/g phospholipid showed a sustained release of TP5 for about 48 h with a burst phase lasting for about 1 h *in vitro*. In the 5-FAM-TP5-VPGs treatment group, the plasma 5-FAM-TP5 concentration increased rapidly, reaching the first peak within 20 min, followed by a rapid decline from 20 min to 12 h, and followed by a sustained phase of about 204 h (Fig. 5). Important pharmacokinetic parameters are provided in Table 1. The fluorescence signal remained in plasma at 216 h after administering 5-FAM-TP5-VPGs with 300 mg/g E80. However, the fluorescence signal was hardly detected after 12 h in the 5-FAM-TP5 solution treated group. The mean retention time (MRT) value of VPGs group containing 300 mg/g E80 was 46.67 h, which was much longer than 3.52 h of the 5-FAM-TP5 solution group (Table 1). Therefore, VPGs with low concentrations of phospholipids can be potential delivery vehicles with sustained release profile *in vivo*.

2.4.2. Pharmacodynamics of TP5-VPGs

TP5-VPGs containing 300 mg/g E80 were evaluated in immunosuppressed rats. In immunological suppressed or deficient patients, the values of $CD4^+$ and $CD8^+$ T-lymphocyte subsets in blood are usually abnormal with an altered $CD4^+/CD8^+$ ratio (Bodey et al. 2000; Wang et al. 2006). As an immunomodulator, TP5 was demonstrated to regulate the $CD4^+/CD8^+$ ratio to a normal level (Bodey et al. 2000; Wang et al. 2006). Thus, the ratio of $CD4^+$ and $CD8^+$ T-lymphocyte subsets was used to evaluate the therapeutic efficacy of TP5 in various formulations. The values of $CD4^+/CD8^+$ in blood of the immunosuppressed rats were higher than that of normal rats ($p < 0.05$) (Table 2), indicating the successful establishment of the immunodepression model. Given TP5 solution and TP5-VPGs as described above, the values of $CD4^+/CD8^+$ appeared to be comparable to that of normal rats ($p > 0.05$). These results demonstrated the feasibility of VPGs for a sustained release of TP5 *in vivo*.

3. Discussion

TP5 loaded VPGs were prepared by the magnetic stirring method. The size analysis showed at least two particle size populations of the redispersed VPGs which was also reported by other groups (Kaiser et al. 2003). Despite the multiple size distribution peaks, the VPGs obtained in this study should still be suitable for subcutaneous use.

The viscosity of VPGs increased as their contents of E80 increased (Fig. 2). The resistance of the VPGs through the syringe increased with increased viscosity. When the E80 concentration was high, it was difficult for VPGs to pass through the syringe thus presenting concerns with accurate dosage. However, VPGs based on low lipid contents such as 300 mg/g were demonstrated to have a proper viscosity and pass through the syringe easily. Therefore, VPGs with low lipid content (300 mg/g) are more promising to serve as a sustained delivery system for subcutaneous injection in the long run.

There are studies (Massing et al. 2008; Kaiser et al. 2003; Tian et al. 2010; Tardi et al. 1988) about sustained release VPG-based formulations, but no *in vivo* studies were reported. In our study, however, VPGs with low lipid content were demonstrated to have a sustained release profile *in vivo*. TP5-VPGs containing 300 mg/g E80 sustained the release of TP5 from VPGs with a burst effect, which was similar with the *in vitro* release behavior reported previously (Chen et al. 2013). Two mechanisms may explain the drug release profile from the VPGs: i) erosion of the phospholipid matrix with release of drug-loaded liposomes and drugs that are trapped between the liposomes; ii) diffusion of the drug through the membranes (Tardi et al. 1988). The low lipid content might facilitate the erosion which is the dominant

Table 1: Pharmacokinetic parameters of 5-FAM-TP5 solution and 5-FAM-TP5-VPGs at a dose of 8 mg/kg (n = 5)

Parameters	5-FAM-TP5 solution	5-FAM-TP5-VPGs containing 300 mg/g E80
$C_{max(ng/l)}$	3834.32 ± 246.48	1076.40 ± 351.81*
$T_{max\ (h)}$	4.00	0.50 ± 0.53*
$AUC_{0\rightarrow t(ng/l*h)}$	15505.86 ± 1405.08	9547.78 ± 2256.43*
$MRT0\rightarrow t_{(h)}$	3.52 ± 0.05	46.67 ± 7.86*

*$p < 0.05$ versus 5-FAM-TP5 solution

driving force for drug release. Therefore, TP5 molecules trapped between the liposomes were rapidly released with the erosion of the matrix, which contributed to the burst effect. Additionally, the release of TP5 from the VPGs was sustained for up to 216 h, which was longer than the *in vitro* release (Chen et al. 2013). A dialysis method was previously used to study the release properties *in vitro*, and sink conditions were established by using 250 mL of release medium which was greatly larger than the tissue fluid in rats. The erosion of the VPGs *in vitro* was suggested to be more rapid than the erosion of VPGs *in vivo*, which might contribute to the enhanced higher rate of TP5 release from the VPGs *in vitro*.

To gain further insight into the *in vivo* performance of the TP5-VPGs with low lipid content, the therapeutic efficacy of the gels after subcutaneous injection was compared to the TP5 solution given once daily in rats. After giving the TP5-VPGs via subcutaneous injection, the $CD4^+/CD8^+$ ratios of the immunosuppression rats returned to the normal level, which was similar to the TP5 solution treated group (Table 2). The VPGs based on low concentration of phospholipid were demonstrated to have a well sustained release behavior *in vivo*.

4. Experimental

4.1. Materials

Thymopetin (TP5) was purchased from KaiJie Biotechnology Co. Ltd. (Chengdu, China). 5-Hydroxy-fluorescein-thymopetin (5-FAM-TP5) was synthesized by KaiJie Biotechnology Co. Ltd. (Chengdu, China, purity 98.49%). Cyclophosphamide was obtained from Sigma (USA). FITC conjugated mouse IgM, PE Mouse IgG2a, PE anti-rat CD8a, FITC anti-rat CD3, PE/Cy7 anti-rat CD4, PE/CY 7 Mouse IgG1 and hemolysin were supplied by Biolegend (San Diego, CA, USA). Egg lecithin (E80) containing phosphatidylcholine (80–85%), phosphatidylethanolamine (7.0–9.5%), lysophosphatidylcholin (less than 3%), sphingomyelin (2–3%) and triglycerides (less than 3%) were purchased from Lipoid (Germany). Ultrapure water was used for all solutions and dilution. All other reagents were of analytical grade.

4.2. Preparation of TP5-VPGs

The preparation of TP5-VPGs with various contents of E80 was carried out as follows. TP5 was dissolved in the 0.02 M acetic acid buffer (pH 6.78). The drug solution was transferred to a round bottom flask, then mixed with E80 and stirred for 90 min at room temperature by a magnetic agitator (Shanghai Sile instrument Co. Ltd.). The resulting TP5-VPGs were loaded with 20 mg/g of TP5.

4.3. Morphology

To analyze the morphology, TP5-VPGs were diluted with pure water, and redispersed under magnetic stirring for 30 min. The morphology of the vesicles of redispersed TP5-VPG was examined by transmission electron microscopy (TEM). One drop of redispered TP5-VPGs was placed on copper grids without films and then stained with 2% (w/v) phosphotungstic acid. The excess staining solution was removed with filter paper. After being air dried, the films were observed with a transmission electron microscope (H-600, Hitachi, Japan).

4.4. Particle size analysis

After redispersed in pure water, the size distribution of the lipid vesicles was examined by a laser particle size analyzer (Malvern Zetasizer Nano ZS90, Malvern instruments Ltd, UK) at 25 °C.

4.5. Rheology

The rheological behavior of TP5-VPGs with different lipid contents (300 mg/g, 350 mg/g, 400 mg/g) was determined by the rotational viscometer (Brookfield engineering labs. INC. Middleboro Ma 02346 USA). The temperature and rotation speed were set at 25 °C and 100 rpm, respectively. All samples were prepared as described above and loaded with 20 mg/g of TP5.

4.6. In vivo studies

Healthy male Wistar rats were provided by West China Experimental Animal Center of Sichuan University (China). The protocol was approved by the Institutional Animal Care and Use Committee of Sichuan University. The rats were housed in cages under controlled conditions.

4.6.1. Pharmacokinetic studies of 5-FAM-TP5-VPGs

In this study, 5-FAM-TP5 was used and the 5-FAM-TP5-VPGs were prepared in advance. Ten rats (220 ± 20 g) were divided into two groups at random. The 5-FAM-TP5 solution, 5-FAM-TP5-VPGs with 300 mg/g phospholipid were administrated Subcutaneously on the back of the rats at a TP5 dose of 8 mg/kg (Li et al. 2010). After injection, blood samples (more than 0.3 ml for each sample) were immediately collected from the caudal veins at predetermined intervals, and placed in heparinized tubes. Plasma was separated from blood samples by centrifugation at 2304 × g for 5 min twice. The obtained plasma was stored at −80 °C until analysis. All plasma samples were deproteinized by a five-fold dilution in dehydrated alcohol and centrifuged at 4096 × g for 10 min. The supernatant was analyzed by fluorescent spectrophotometer (Shimadzu RF-5301PC, Japan) to determine the concentration of 5-FAM-TP5 (excitation wavelength, 485 nm; emission wavelength, 526 nm; slit for excitation, 5 nm; slit for emission, 5 nm). The calibration graph was linear in the range of 3.2∼32 ng/ml ($R^2 = 0.996$) with a lowest limit of detection (LOD) of 0.8 ng/ml.

Table 2: Values of $CD4^+/CD8^+$ T-lymphocyte subsets in blood of rats. (n = 5)

Group	Treatment	$CD4^+$%	$CD8^+$%	$CD4^+/CD8^+$
1	Normal control	71.07 ± 0.25	33.33 ± 0.57	2.14 ± 0.05^a
2	Immunosurppression control	72.93 ± 3.61	28.17 ± 6.03	2.59 ± 0.04^b
3	TP5 solution	66.17 ± 3.61	31.93 ± 1.31	2.07 ± 0.03^c
4	TP5-VPGs	65.33 ± 4.37	37.37 ± 11.33	2.19 ± 0.08^d

[c] $p > 0.05$ versus group 4.
[d.c] $p > 0.05$ versus group 1.
[d.c.a] $p < 0.05$ versus group 2.

4.6.2. Pharmacodynamic studies of TP5- VPGs

Twenty healthy male rats were randomly divided into four groups. The rats of group 1 received normal saline solution (N.S.) once a day for 10 days as a normal control. The rats of the other three groups (group 2, group 3 and group 4) received cyclophosphamide solution intraperitoneally at a dose of 35 mg/kg once a day for three consecutive days to construct immunodepression models. Then, the rats of group 2 and group 3 were given N.S. and TP5 solution (0.09 mg/kg) subcutaneously once daily for 7 days. The rats of group 4 were subcutaneously injected with TP5-VPGs containing 300 mg/g E80 (0.9 mg/kg). Lymphocyte subsets in blood were determined by multiparameter flow cytometry with three-color analysis (Wang et al. 2006). The blood specimens were treated as follows: First, as an isotype control, 100 μl of anticoagulated blood was mixed with 5 μl IgM-FITC, 8 μl IgG1-PE/Cy7 and 20 μl IgG2a-PE. Then, another 100 μl blood was mixed with 5 μl CD3-FITC, 8 μl CD4-PE/Cy7 and 8 μl CD8-PE as a sample. The red blood cells were vortexed for 30 s and incubated for 20 min at 25 °C away from light. Red blood cells in control and sample tubes were treated by hemolysin in accordance with the manufacturer's instruction. Subsequently, the specimens were centrifuged at 350 × g for 5 min at 4 °C to collect the cells in sediment. Then the cells were washed with PBS twice and finally suspended in 0.5 ml of PBS. The samples were kept in the dark at 4 °C and the T-lymphocyte subsets analysis was performed within 4 h using flow cytometer (Cytomices FC 500, Beckman coulter, USA). The $CD4^+/CD8^+$ ratios were calculated by the amounts of labeled $CD4^+$ and $CD8^+$ in the blood samples, and the mean value ± standard deviations (SD) of the different groups of rats were recorded. In order to evaluate the release of TP5 from the VPGs, the two-tailed t-test was performed for the significance and a value of $p < 0.05$ was regarded statistically significant.

4.7. Statistical analysis

Results were analyzed by Drug and Statistics (DAS2.0, Anhui, China) software. Significance was tested by a two-tailed *t*-test. A value of $p < 0.05$ was considered statistically significant and the data were expressed as mean ± S.D.

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