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Panax Notoginsenosides Attenuates Pleural Inflammation in Rabbit Model

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Abstract

Panax Notoginsenosides (PNS) performs the function of enhancing blood circulation. The aim of this study is to investigate the effect of intrapleural PNS on rabbit pleural inflammation reaction, and determine the levels of transforming growth factor β1 (TGF-β1) and Vascular Endothelial Growth Factor (VEGF). Forty New Zealand white rabbits were divided into four groups. The rabbit pleural inflammation reaction model was established by injection of tetracycline hydrochloride solution into pleural cavity. Then PNS, urokinase (UK) and PBS were injected into the pleural cavity as experiment groups. While tetracycline hydrochloride solution was replaced by phosphate buffer solution (PBS) as control group. The pleural effusion was collected at 24 h, 48 h, 72 h and 96 h in all groups, and then biochemical indicators, TGF-β1 and VEGF were detected. On day 14, all animals were killed and pleural tissues were collected to perform hematoxylin eosin (HE) and Masson trichrome staining. The results indicated that levels of TGF-β1 and VEGF were significantly lower in PNS group than that in UK group and PBS group ($P<0.05$); and the levels of VEGF were maximum at 48 h in three experimental groups. The thickness of pleural was thinner in PNS group, and the number of inflammatory cells and fibroblasts was also decreased in PNS group. In conclusion, PNS could reduce production of TGF-β1 and VEGF, reduce number of inflammatory cells and fibroblast, and inhibit collagen production, which had better effects compared with UK. Our findings provide a new treatment strategy for inflammation reaction.

Keywords: Panax notoginsenosides; Pleural inflammation; TGF-β1;VEGF

Introduction

Tuberculous pleurisy can cause exudative pleural effusion disease, thereby resulting in pleural adhesions and fibrosis and reducing lung compliance. Infection, trauma and drugs can lead to pleural reaction, including exudation, inflammation, adhesions, et al; the main mechanism is that after pleura is stimulated, mesothelial cells and subcutaneous cells will release cytokines, leading to vascular wall permeability increase, fibrinogen extravasation, fibrin deposition on the pleura, fibroblast proliferation, collagen production, and ultimately pleural thickening. Now it usually uses hardening agents, such as tetracycline to induce pleural injury model, then applies active drugs, hormones and cytokines to observe the changes of pleural, such as urokinase (UK) [7], transforming growth factor β1 (TGF-β1) [4] and vascular endothelial growth factor (VEGF) [9].

Panax Notoginsenosides (PNS) is the active ingredient extracted from the traditional Chinese medicine Notoginseng, which has function of enhancing blood circulation [3,8,16]. In this study, we observed the levels of TGF-β1 and VEGF in pleural effusion and the changes of pleural pathology in rabbit pleural inflammation after treatment with PNS and UK, and compared the effects of the two medicines.

Materials and Methods

Experimental reagents

PNS were purchased from China Pharmaceutical Biological Products Analysis Institute (Batch number: 120941-200807); UK was purchased from Lizhu Corporation (China); tetracycline hydrochloride was purchased from Sigma (USA); rabbit TGF-β1 and rabbit VEGF immunohistochemistry kit were purchased from Santa Cruz (USA).

Solution preparation

The 19.2 g PNS was dissolved in 240 ml physiological saline to prepare a concentration of 8% PNS; 100,000 IU UK dry powder was dissolved in 200 ml physiological saline to make 500 IU/ml UK; 5.25 g tetracycline hydrochloride powder was dissolved in 150 ml saline to make 3.5% solution. The above reagents were filtered with millipore filter sterilization, and then stored at 4 °C refrigerator.

Animal model

Forty male New Zealand white rabbits (2.5-3.0 kg) were divided into four groups (n=10): PNS group, UK group and control group. The ear vein of rabbits was injected 2 ml/kg 3% pentobarbital solution, after anesthesia, the chest was routine disinfected and skin was cut about 1 cm, next the chest wall muscularis and intercostal muscles were blunt separated, then pleura was broken to insert the rubber drainage tube; at last, the incision was sutured and the drainage tube was fixed. Ten animals were given 20 ml PBS (control group) and thirty animals were given 35 mg/kg tetracycline solution through drainage tube. In PNS group, 1 ml/kg PNS were injected into pleural cavity every 12 h for 8 times; in UK group, 1500 IU/kg UK were injected into pleural cavity every 12 h for 8 times; and 20 ml PBS was injected every 12 h for 8 times in PBS group and control group.

Sample collection and analysis

The pleural effusion was extracted through drainage tube before administration at 24 h, 48 h, 72 h and 96 h in three experimental

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groups, and the biochemical analysis was completed within 1 h. The levels of TGF-β1 and VEGF were determined by enzyme-linked immunosorbent assay (ELISA) following the protocol provided by the manufacturer. At day 14, 3% pentobarbital was rapid injected by vein to kill animals, the parietal pleura and inferior lobe of right lung (with visceral pleura) were collected and fixed in 4% formalin for 24 h. The samples were dehydrated and embedded in paraffin, and the embedded samples were cut into 5 μm thick histological sections. Then the sections were stained with hematoxylin and eosin (HE) and Masson-trichrome.

Histomorphometry

Through observation of HE staining under optical microscopy, we measured the thickness of visceral pleura and parietal pleura, and the thickness between mesothelial cells and basal layer. Six image fields (100) were captured on each slide to calculate the average thickness. The fibroblasts have characteristics as followings: a diameter of 10-12 μm, with round or oval nucleus and small nucleoli. The number of pleural fibroblasts was calculated under high power lens (400). In Masson staining, collagen fibers were stained bluish green and muscle fibers and cellulose were stained pink; three image fields (100) were captured on each slide to calculate the average thickness of collagen fibers.

Statistical analysis

Data are expressed as means ± standard deviation and were analyzed by SPSS 13.0 software, data between different groups were analyzed by ANOVA and data between the same group were analyzed by q test. A $P<0.05$ was considered statistically significant.

Results

General observation

Two animals died of hemothorax in PBS group, and one died of pneumothorax after drainage tube shedding in UK group. All animals finished the experiment in PNS group and control group. From the specimens we could see that the pleural adhesions appear in PBS group compared to the control group (Figure 1). The thickness of pleura and collagen fibers was increased in PBS group under microscope. The leukocyte and protein were increased significantly through biochemical detection in PBS group, while these indicators were normal in control group. These results showed that the inflammatory model of the rabbit pleural cavity was established successfully.

The biochemical characteristics of pleural effusion

The protein content of pleural effusion was increased in three experimental groups within 24 h, but there was no significant difference; the protein content was declined from maximum at 48 h in UK group and PBS group, while it began to decline at 24 h in PNS group; the protein content was significantly lower in PNS group than that in UK group and PBS group at 48 h, 72 h and 96 h ($P<0.05$), while the protein content was significantly lower in UK group than that in PBS group ($P<0.05$). There was significant difference in the number of leukocyte between PNS group and PBS group at 72 h and 96 h, but there was no significant difference between PNS group and UK group. The glucose content was significantly more in PNS group than the other two groups

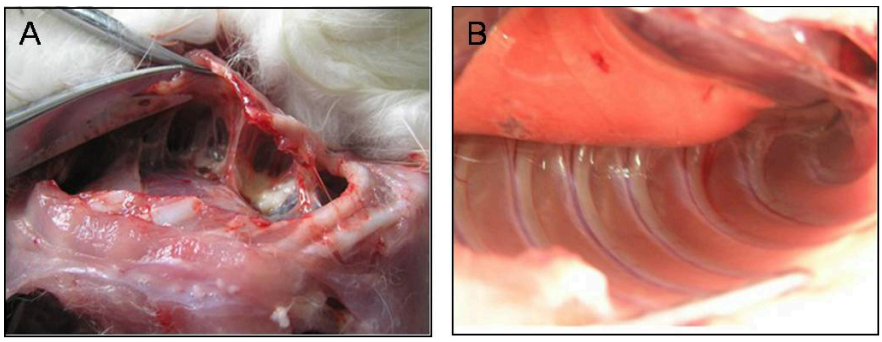

Figure 1: The gross specimen of rabbit pleural cavity in PBS group (A) and control group (B).

	Time (h)	PNS group	UK group	PBS group
Protein content (g/L)	24	4.00 ± 0.60	4.20 ± 0.55	4.60 ± 0.50
	48	3.04 ± 0.33*#	4.69 ± 0.74	5.20 ± 0.80
	72	3.20 ± 0.11*#	4.10 ± 0.31	5.00 ± 0.28
	96	2.40 ± 0.25*#	3.72 ± 0.32	4.80 ± 0.62
Leukocytes (×109/L)	24	6.00 ± 1.33	6.12 ± 1.21	6.68 ± 1.34
	48	5.08 ± 0.31	5.24 ± 0.45	5.34 ± 0.78
	72	2.90 ± 0.42*	2.99 ± 0.38	3.21 ± 0.22
	96	0.97 ± 0.13*	1.22 ± 0.36	2.10 ± 0.11
LDH(IU/L)	24	3242 ± 635	3546 ± 721	3678 ± 810
	48	2621 ± 392	2729 ± 362	2750 ± 380
	72	2326 ± 189	2338 ± 292	2230 ± 420
	96	1538 ± 126	1653 ± 177	1820 ± 232
Glucose content (mmol/L	24	2.74 ± 0.57	2.62 ± 0.48	2.13 ± 0.72
	48	3.49 ± 0.29*#	3.11 ± 0.39	2.24 ± 0.78
	72	4.58 ± 0.77*#	3.53 ± 0.68	2.57 ± 0.96
	96	5.23 ± 0.26*#	4.67 ± 0.35	3.35 ± 0.23

Table 1: The biochemical characteristics of pleural effusion.

at 48 h, 72 h and 96 h (P<0.05). There was no significantly difference in lactate dehydrogenase (LDH) among the three groups. Data was shown in Table 1.

The levels of TGF-β1 and VEGF

The level of TGF-β1 was significantly lower in PNS group than that in UK group and PBS group at 24 h and 48 h (P<0.05). The level of TGF-β1 was significantly lower in PNS group and UK group than that in PBS group at 72 h and 96 h (P<0.05); however, there was no significant difference between PNS group and UK group. In PBS group, the level of TGF-β1 in pleural effusion was increased rapidly from 48 h, and the levels of TGF-β1 were also increased, but with a gentle curve (Table 2). The level of VEGF was significantly lower in PNS group than that in UK group and PBS group at 24 h, 48 h, 72 h and 96 h (P<0.05). The level of VEGF reached maximum at 48 h in three groups (Table 3).

The changes of pleural pathology

HE staining showed that the thickness of visceral pleura and parietal pleura was 20 μm, which was composed of a layer of mesothelium and basement membrane, and there were no inflammatory cells and fibroblasts (Figure 2A). In PBS group, the thickness of visceral pleura and parietal pleura became thicker, and there were lots of inflammatory cells and fibroblasts in subcutaneous connective tissue (Figure 2B). The thickness of visceral pleura and parietal pleura was thinner in PNS group (Figure 2C) and UK group (Figure 2D) than that in PBS group, and the number of inflammatory cells and fibroblasts was also decreased in PNS group and UK group. Masson staining showed that only a small amount of collagen was observed in mesothelial cells (Figures 3A, 3B); the blue connective tissues of visceral pleura and parietal pleura were significantly increased in PBS group (Figures 3C, 3D); the collagen fibers were decreased in PNS group compared to PBS group (Figures 3E, 3F). The results suggested that collagen fibers and fibroblasts were decreased in PNS group than that in PBS group. The pleural thickness, fibroblast number and collagen fiber thickness were shown in Table 4.

Discussion

Pleural adhesions and fibrosis are the clinical outcome of many pleural diseases, the serious one would develop into pleural thickening, which further appear calcification and shrink, resulting in intercostals space narrowing to limit ventilator function. Fibrinolytic balance destruction of the pleural cavity is an important reason for the occurrence of pleural adhesions and fibrosis, this imbalance would

Group	24 h	48 h	72 h	96 h
PBS group	35.80 ± 2.31	36.47 ± 1.25	58.96 ± 3.21	72.92 ± 4.23
PNS group	26.15 ± 0.95*	31.01 ± 2.02*	36.67 ± 1.20*	37.31 ± 0.77*
UK group	39.29 ± 4.28	39.30 ± 1.23	42.97 ± 2.35**	44.31 ± 5.28**

*P<0.05 compared to PBS group and UK group; ** P<0.05 compared to PBS group.

Table 2: The level of TGF-β1 in three groups (ng/L) ($\bar{x}$ ±s).

Group	24 h	48 h	72 h	96 h
PBS group	13.52 ±1.39	22.19 ± 3.42	21.83 ± 2.45	19.86 ± 2.18
PNS group	8.31 ± 0.90*	11.17 ± 1.30*	8.00 ± 1.18*	6.42 ± 0.73*
UK group	14.58 ± 2.13	17.12 ± 2.09	14.91 ± 1.36	15.92 ± 1.89

*P<0.05 compared to PBS group and UK group.

Table 3: The level of VEGF in three groups (ng/L) ($\bar{x}$ ±s).

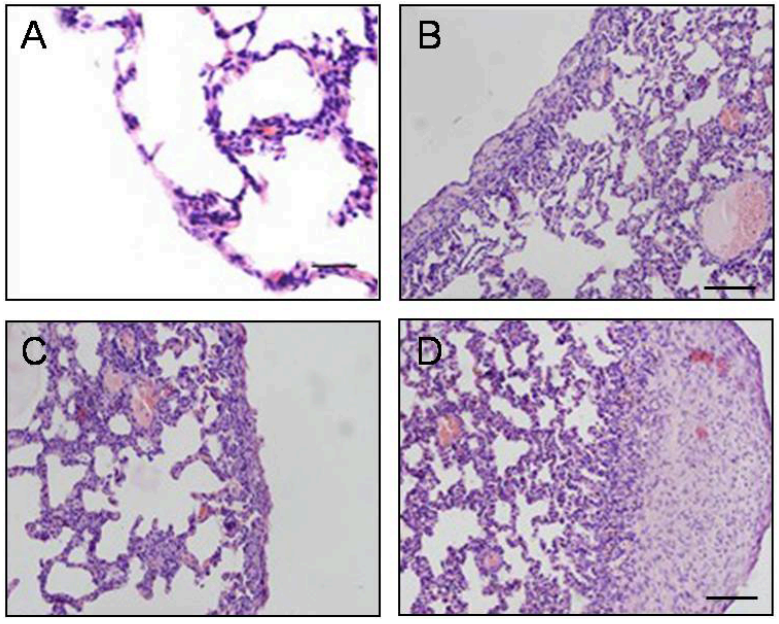

Figure 2: HE staining of visceral pleura in control group (A, ×400), PBS group (B, ×100), PNS group (C, ×100) and UK group (D, ×100).

cause pleural thickening [3]. Fibrinolytic system suppression would promote fibrin accumulation to form a fibrin mesh, which provide the attachment site for fibroblast and prevent the spread of inflammation, eventually resulting in fibrosis development. At last, mesothelial cells were damaged and decreased, and replaced by collagen fiber.

Using hardening agent in pleural cavity is a common method of studying pleural inflammation reaction and pleural adhesions, such as tetracycline and talc; these drugs would damage pleural mesothelial cells to release a variety of enzymes, complement and biologically active substances, thereby causing increased capillary permeability and exudative pleural effusion [5]. In the pleural effusion, the protein content and LDH were increased, and glucose was decreased. Injection of tetracycline into pleural cavity stimulated pleural mesothelial cells to produce VEGF, which would promote angiogenesis and increase capillary permeability [10]. The leukocytes, fibrinogen and coagulation factors also went to pleural cavity from blood vessels, all these reacted with plasminogen activator inhibitor (PAI) to activate thrombin, which was conducive to form fibrin and accelerate the process of fibrosis [12,2]. Notoginseng is the main ingredients of Yunnan White, which could promote blood circulation. PNS is the active ingredients extracted from Notoginseng, mainly containing ginsenoside and notoginsenoside. It has been found that PNS could reduce blood viscosity and platelet aggregation, and enhance fibrinolytic activity; it also could significantly increase serum superoxide dismutase (SOD) and tPA levels, reduce lipid peroxidation (LPO) and PAI-1 inhibitor in patients with coronary heart disease; in addition, PNS could reduce the production and release of IL-8, inhibit the activation of neutrophil, and reduce the inflammatory response around ischemic brain tissue during cerebral ischemia-reperfusion injury. In this study, the contents of leukocytes and protein were significantly lower in PNS group than UK group and PBS group, however, the glucose content was higher in PNS group, suggesting that PNS could reduce the inflammation of exudates. A large number of cytokines also appeared in pleural effusion, including TGF-β1 and VEGF. VEGF could stimulate endothelial cell proliferation and promote angiogenesis, but also improve the permeability of the blood vessels to cause the extravasation of plasma proteins. Malignant effusion promoted pleural cavity to release VEGF [6], on the contrary, anti-VEGF antibody (bevacizumab) prevented acute pleurisy reaction caused by talc [11]. TGF-β1 was the chemotactic agent of fibroblasts, which could reduce the production of tPA and stimulate mesothelial cells to synthesize collagen matrix protein. TGF-β1 was significantly increased in malignant effusion, it seems to promote angiogenesis and hinder anti-tumor response mediated by immune [1]. In our study, the levels of TGF-β1 and VEGF were consistent with previously research after application of tetracycline, which lays a foundation for further study.

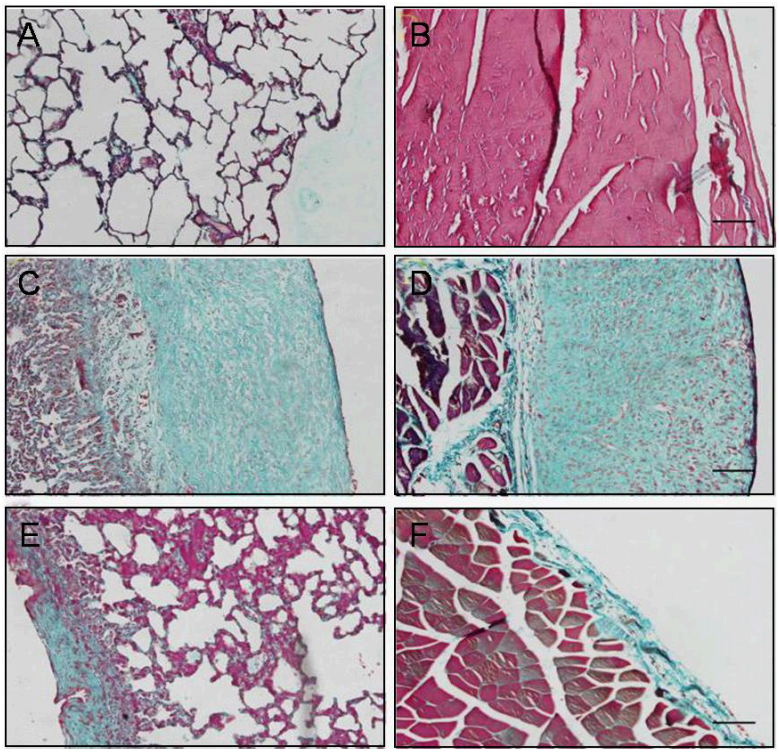

Figure 3: Masson staining of visceral pleura (A) and parietal pleura (B) in control group, visceral pleura (C) and parietal pleura (D) in PBS group, and visceral pleura (E) and parietal pleura (F) in PNS group (×100).

Group	PBS group	PNS group	UK group	F	P
Pleural thickness (μm)	335.37 ± 72.23	69.01 ± 32.02*	100.00 ± 57.01*	43.41	0.000*
Fibroblast number (n/mm^2)	198.25 ± 75.90	90.21 ± 24.31*	113.55 ± 35.87*	12.57	0.000*
Collagen fiber thickness (μm)	328 ± 74.34	121 ± 36.11*	125 ± 41.10*	45.11	0.000*

*P<0.001 compared to PBS group.

Table 4: The pleural thickness, fibroblast number and collagen fiber thickness in four groups ($\bar{x}$ ±s).

Peritoneal adhesions are common complication after abdominal surgery, it has been reported that PNS could inhibit peritoneal adhesions of animals (14,13,8). In our study, the collagen deposition was alleviated by Masson staining in PNS group, suggesting PNS could prevent pleural collagen proliferation. The number of fibroblast was significantly less in PNS group than that in UK group and PBS group; the thickness of collagen also significantly thinner in PNS group, suggesting PNS could reduce pleural effusion and inhibit pleural thickening caused by tetracycline. Our findings provide a new treatment strategy for inflammation reaction.

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References

1. Atanackovic D., Cao Y., Kim J.W., Brandl S., Thom I., Faltz C., Hildebrandt Y., Bartels K., de Weerth A., Hegewisch-Becker S., Hossfeld D.K. & Bokemeyer C. The local cytokine and chemokine milieu within malignant effusions. Tumour. Biol., 2008, **29**:93-104.
2. Baldwin J.F., Sood V., Elfline M.A., Luke C.E., Dewyer N.A., Diaz J.A., Myers D.D., Wakefield T. & Henke P.K. The role of urokinase plasminogen activator and plasmin activator inhibitor-1 on vein wall remodeling in experimental deep vein thrombosis. J. Vasc. Surg., 2012, **56**:1089-1097.
3. Benjamin I.J. Matrix metalloproteinases: from biology to therapeutic strategies in cardiovascular disease. J. Investig. Med., 2001, **49**:381-397.
4. Border W.A. & Noble N.A. Transforming growth factor-β in tissue fibrosis. N. Engl. J. Med., 1994, **331**:1286-1292.
5. Carvalho P., Knight L.L., Olson R.D., Crowley J.J., Hawk P.A. & Charan N.B. Effects of erythromycin on the rabbit pleura: its potential role as a pleural sclerosant. Am. J. Respir. Crit. Care. Med., 1995, **151**:1228-1232.
6. Duysinx B.C., Corhay J.L., Hubin L., Nguyen D., Henket M. & Louis R. Diagnostic value of interleukine-6, transforming growth factor-beta 1 and vascular endothelial growth factor in malignant pleural effusions. Respir. Med., 2008, **102**:1708-1714.
7. Komissarov A.A., Mazar A.P., Koenig K., Kurdowska A.K. & Idell S. Regulation of intrapleural fibrinolysis by urokinase-alpha-macroglobulin complexes in tetracycline-induced pleural injury in rabbits. Am. J. Physiol. Lung. Cell. Mol. Physiol., 2009, **297**:L568-L577.
8. Liu G., Wang B., Zhang J., Jiang H. & Liu F. Total panax notoginsenosides prevent atherosclerosis in apolipoprotein E-knockout mice: Role of downregulation of CD40 and MMP-9 expression. J. Ethnopharmacol., 2009, **126**:350-354.
9. Marchi E., Vargas F.S., Takemura R.L., Acencio M.M., Antonangelo L., Teixeira L.R. & Light R.W. Monoclonal antibodies anti-TGFβ1 and anti-VEGF inhibit the experimental pleurodesis induced by silver nitrate. Growth Factors, 2012, **30**:304-309.
10. Oltean S., Neal C.R., Mavrou A., Patel P., Ahad T., Alsop C., Lee T., Sison K., Qiu Y., Harper S.J., Bates D.O. & Salmon A.H. VEGF165b overexpression restores normal glomerular water permeability in VEGF164-overexpressing adult mice. Am. J. Physiol. Renal. Physiol., 2012, **303**:F1026-F1036.
11. Ribeiro S.C., Vargas F.S., Antonangelo L., Marchi E., Genofre E.H., Acencio M.M. & Teixeira L.R. Monoclonal anti-vascular endothelial growth factor antibody reduces fluid volume in an experimental model of inflammatory pleural effusion. Respirology, 2009, **14**:1188-1193.
12. Strange C., Baumann M.H., Sahn S.A., Idell S. Effects of intrapleural heparin or urokinase on the extent of tetracycline-induced pleural disease. Am. J. Respir. Crit. Care. Med., 1995, **151**:508-515.
13. Su B.H., Li Z., Fan J.M., Wang M. & Tang R. Effects of panax notoginseng saponins on the process of renal interstitial fibrosis after unilateral ureteral obstruction in rats. Sichuan. Da. Xue. Xue. Bao. Yi. Xue. Ban., 2005, **36**: 368-371.
14. Sun H.X., Qin F. & Ye Y.P. Relationship between haemolytic and adjuvant activity and structure of protopanaxadiol-type saponins from the roots of Panax notoginseng. Vaccine, 2005, **23**:5533-5542.
15. Xu Q.F., Fang X.L., Chen D.F. & Li J.C. Studies on formulations of Panax notoginsenosides for intranasal administration. Yao. Xue. Xue. Bao., 2003, **38**:859-862.
16. Zhang X.P., Wang C., Wu D.J., Ma M.L. & Ou J.M. Protective effects of ligustrazine, kakonein and Panax notoginsenosides on multiple organs in rats with severe acute pancreatitis. Methods. Find. Exp. Clin. Pharmacol., 2010, **32**:631-644.