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Evaluation of the efficacy, toxicity and safety of vinorelbine incorporated in a lipid emulsion

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ABSTRACT

To reduce the severe adverse effects of vinorelbine (VRB) with the aim of improving patient compliance, a parenteral vinorelbine-loaded lipid emulsion (VLE) has been developed. The objective of the present study was to get insight into the preclinical antitumor efficacy, toxicity and safety of VLE, and compare this with that of the commercial product, Navelbine® i.v. (VS). Comparable antitumor efficacy of VLE and VS was observed in tumor-bearing nude mouse models inoculated with A549 human lung cancer, hepatoma solidity (Heps) G2 cancer and BCAP-37 human breast cancer cells. The median lethal dose (LD_{50}) in mice was 29.3 mg/kg (male) and 32.1 mg/kg (female) for VLE, while the corresponding value was 30.5 mg/kg (male and female) for VS. In the long-term toxicity study, VLE significantly reduced the decreases in RBC, HC, WBC and WBC differential count (DC) levels. Lesions in spleen, thymus, lymph nodes, bone marrow, testis, ovary and injection site induced by VS were much more severe compared with VLE. VLE also exhibited less local venous irritation than VS, as well as no hemolysis or hypersensitivity. Consequently, these observations clearly indicate that the lipid emulsion could be a useful potential parenteral carrier for VRB with equivalent efficacy and lower toxicity.

1. Introduction

Vinorelbine (VRB), a semi-synthetic vinca alkaloid which acts through microtubule disruption (Fig. 1), exhibits less neurotoxicity than the other vinca alkaloids (Gauvin et al., 2000). It has been widely used in the treatment of non-small cell lung cancer (NSCLC) and advanced breast cancer (ABC) (Fumoleau et al., 1993; Mano, 2006). Because VRB is a moderate vesicant which could cause local venous toxicity such as drug-induced phlebitis, the currently clinical formulation, Navelbine® i.v., must be diluted 5- to 20-fold with 0.9% sodium chloride injection or other aqueous i.v. solutions before administration. Before the injection of Navelbine®, the intravenous needle or catheter must be properly positioned. The recommended method of administration of Navelbine® in clinical situations is an intravenous injection over 6–10 min, with a large volume of normal saline or 5% glucose solution flushing the vein after injection. Leakage into surrounding tissue during i.v. injection of Navelbine® may cause considerable irritation, thrombophlebitis and local tissue necrosis. The incidence of phlebitis has been reported to be approximately 16–33% in patients receiving Navelbine® via a 6–10 min drip infusion (Rittenberg et al., 1995; Yoh et al., 2004). Therefore, local venous toxicity should be managed effectively to improve patient confidence.

To prevent VRB-induced injection site irritation, different strategies have been pursued. Nakamaya et al. reported that the administration of a bolus injection of VRB decreased the incidence of drug-induced phlebitis in comparison with common drip infusion (Nakayama et al., 2002). Another experiment performed in rabbits evaluated four different factors (administration rate, dilution, flushing with normal saline and infusion of fat emulsion) for alleviation of VRB-induced phlebitis by infusion (Kohno et al., 2008), and the results revealed that rapid infusion and dilution were effective methods for reducing VRB-induced phlebitis, but the efficacy of flushing with normal saline or infusion of fat emulsion was not confirmed. However, it was also reported that VRB-induced local venous toxicity was observed in 33% of patients with a 6 min infusion and in 24% with a 1 min bolus (Yoh et al., 2007). Moreover, the administration of short bolus of VRB has been reported to be associated with an increased risk of acute lower back pain (Fasce et al., 2000). Unfortunately, all the strategies above could not avoid the occurrence of VRB-induced phlebitis. So, it is of great importance to develop new dosage forms for i.v. VRB with reduced local venous irritation.

One of the efficient ways to reduce the local irritation is to decrease the local concentration of drug. By incorporation of drugs into parenteral fat emulsions, reduced adsorption of drugs on infusion and a reduction in local toxicity have been reported (Xu et al., 2008). Compared with other drug carriers, such as liposomes, nanoparticles and micelles, lipid emulsions are physically stable, biodegradable, biocompatible, and easier to process and manu-

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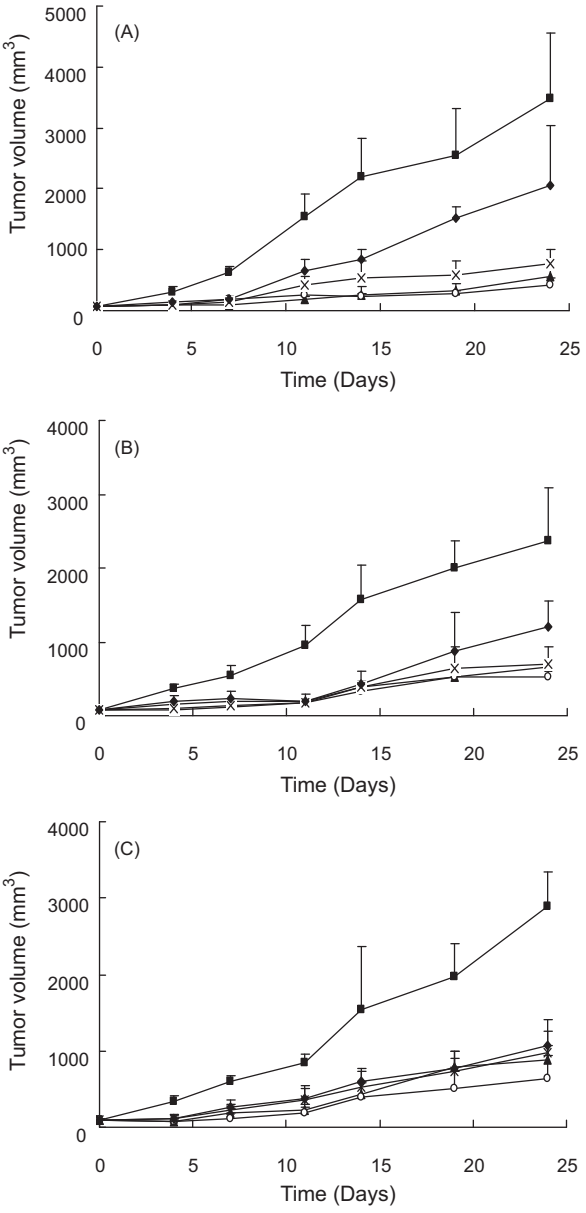

Fig. 1. Changes in tumor volume as a function of time in tumor-bearing mice after VRB therapy: ■ for blank emulsions (negative control), ▲ for VS (5 mg/kg), ○ for VLE-H (10 mg/kg), * for VLE-M (5 mg/kg), ♦ for VLE-L (2.5 mg/kg). (A) A549 cell line; (B) HeP G2 cell line, and (C) BCAP-37 cell line. Data shown as mean ± SD, $n = 7$.

facture (Lundberg et al., 1996; Fukui et al., 2003). Intravenously administered fat emulsions containing triglycerides as the dispersed phase and phospholipids as the preferred emulsifier have been used in clinical situations to supply calories for decades. These parenteral emulsions can also be used as potential carriers or controlled delivery systems for poorly water-soluble drugs (Xu et al., 2008). They have been used as parenteral delivery carriers for lipophilic drugs, such as prostaglandin E1, diazepam, and non-steroidal anti-inflammatory drugs (Constantinides, 1995; Singh and Ravin, 1986; Yamaguchi and Mizushima, 1994). Therefore, lipid emulsions might be suitable carriers for i.v. administration of VRB, and they are expected to reduce the severe local venous toxicity and improve patient tolerance.

The objective of the present study was to evaluate the preclinical efficacy, toxicity and safety of a VRB-loaded lipid emulsion (VLE), and compare with the commercial product, Navelbine® i.v. (VS). The antitumor efficacy was investigated in tumor-bearing nude mice (A549 human lung cancer, hepatoma solidity (Heps) G2 cancer and BCAP-37 human breast cancer cells). In vivo toxicity and safety evaluation included an acute toxicity study, a long-term toxicity study, a venous irritation study, as well as hemolysis and anaphylaxis testing.

2. Materials and methods

2.1. Drugs and reagents

Vinorelbine ditartrate and Navelbine® i.v. were both purchased from Jiangsu Hengrui Pharmaceutical Co. (Lianyungang, China), Egg yolk lecithin (Lipoid E80®) and medium-chain triglyceride (MCT) were obtained from Lipoid KG (Ludwigshafen, Germany), soybean oil (Tieling Beiya Pharmaceutical Co., Tieling, China), Poloxamer-188 (Pluronic F-68®) (BASF AG, Ludwigshafen, Germany), glycerol (Zhejiang Suichang Glycerol Plant, Lishui, China), sodium oleate (National Pharmaceutical Group Chemical Reagent Co. Ltd., Beijing, China), anhydrous sodium sulfite (Tianjin Boya Chemical Industry Ltd. Co., Tianjin, China), and egg albumin (Sigma–Aldrich Co., St. Louis, MO, USA) were obtained from the sources indicated.

2.2. Preparation and characterization of VLE

The preparation of emulsions with the required characteristics and composition was performed by high-pressure homogenization (Zhang et al., 2008). The formulation contained (%, w/v): 2% soybean oil, 8% medium-chain triglyceride (MCT), 3.0% egg yolk lecithin, 0.5% F-68, 0.1% sodium oleate, 2.5% glycerol, 0.14% total antioxidants (0.1% anhydrous sodium sulfite and 0.04% L-cysteine), and water for injection to 100%. Briefly, the aqueous phase, which consisted of egg yolk lecithin, F-68, oleate sodium, glycerol, and the antioxidants were dispersed in double distilled water stirred at 70 °C. Soybean oil and MCT (2:8) were mixed under stirring at 75 °C to obtain the oil phase. Then, the oil phase was added to the water phase followed by mixing with a high-shear mixer (ULTRA RURRAX® IKA® T18 basic, Germany) at 8000 rpm for 5 min, and then vinorelbine ditartrate was added with further stirring until the primary emulsion was obtained. After adjusting the pH to about 8.0 with 0.1 mol/L NaOH solution and the volume with double distilled water, the final emulsion was obtained using a high pressure homogenizer (Niro Soavi NS10012k, Niro Soavi S.p.A., Via M. Da Erba, Italy) at 900 bar for 10 cycles. The preparation was gassed with N_2 and then sealed in 50 ml glass bottles followed by sterilizing at 121 °C for 15 min. The emulsion contained 1 mg/ml VRB and the percentage was 0.1%(w/v).

The particle size and zeta potential of the lipid emulsions were determined by a laser dynamic light-scattering particle sizer, NICOMPTM^{380} Zeta Potential/Particle Sizer (Santa Barbara, USA), after diluting the emulsion with distilled water. The entrapment efficiency was determined by ultracentrifugation using a Hitachi ultracentrifuge (CS120GXL), and the sample was centrifuged at 50,000 rpm for 2 h at 10 °C. The entrapment efficiency was calculated using the equation: entrapment efficiency (%) = $[(W_{total} - W_{aqueous})/W_{total}] \times 100$ (W_{total} was the amount of drug present initially in the formulation and $W_{aqueous}$ was the estimated amount from the aqueous phase of the formulation). The concentration of VRB in the lipid emulsion and aqueous phase was estimated by high performance liquid chromatography (HPLC) (Zhang et al., 2008). This HPLC method was precise and accurate with a linear response of 2–80 μg/mL ($r^2 = 0.9999$). The percent relative standard deviation (RSD%) of six injections of the same solution was 0.14%, and the RSD% of six samples from the same batch was 0.37%.

2.3. Experimental animals

The Balb/c nude mice used for the antitumor efficacy investigation, the Sprague–Dawley (SD) rats used in the long-term toxicity and hemolysis studies, and the rabbits used in the vein irritation assessment were purchased from the Experimental Animal Center of Medical Department (Beijing University, Beijing, China). The mice used in acute toxicity study were obtained from the Experimental Animal Center in the B&K Universal Group Limited (Shanghai, China). The Hartley guinea pigs for a study of hypersensitivity were obtained from the Experimental Animal Center of Guangdong province (Guangzhou, China). All animals were housed in compliance with Good Laboratory Practice (GLP) standard laboratory conditions. The temperature of the animal room was 22–25 °C during the study and the humidity ranged from 35 to 60%. The animal room had a 12-h light/dark cycle and air was changed 5–10 times per day. The animals were allowed to acclimatize to the standard environment for at least 1 week. Food and water were provided ad libitum. All the animal experiments were evaluated and approved by the local Ethics Committee for the Use of Laboratory Animals (Protocol number: SCXK (Beijing) 2006-0025 and SYXK (Guangdong) 2008-0003) and in compliance with the Guidelines for the Care and Use of Laboratory Animals.

2.4. In vivo antitumor efficacy

Balb/c nude mice weighing 18–22 g (approximately 6 weeks old) were divided into three groups, and each group was used for one tumor model. Two groups of male mice were separately inoculated with A549 human pulmonary adenocarcinoma cell lines and the HeP G2 hepatocellular carcinoma cell lines, which were supplied by the Department of Pharmacology of the Cancer Institute (Chinese Academy of Medical Sciences, Beijing, China). A group of female mice were inoculated with BCAP-37 human breast cancer cell lines obtained from the First Department of Pharmacology in the Institute of Materia Medica (Chinese Academy of Medical Sciences, Beijing, China). A tumor fragment (1.5 mm × 1.5 mm × 1.5 mm) was inoculated into the right axillary region for every mouse. For each tumor model, 35 mice were used. One week after tumor inoculation (when the tumor volume was around 60–80 mm^3), each treatment was started.

The antitumor efficacy was evaluated in the same manner for the three tumor models. In each tumor model, mice were randomly divided into five groups (seven animals/group) receiving the following treatments intravenously via the tail vein: blank lipid emulsion (negative control, NC), 5 mg/kg VS (positive control), and different doses (2.5, 5, and 10 mg/kg) of VLE (test groups). All treatments were administered at a single dose twice a week for 3 weeks. The body weight and tumor volume were recorded for each mouse regularly after the start of treatment. The tumor volume (V) for each mouse was calculated using the formula: $V = AB^2/2$, where A is the largest and B is the smallest diameter. The tumor diameters were measured twice a week with vernier callipers (01010157, Zhongmeideli Co., Zhengjiang, China) in two dimensions. The tumor inhibition rate (T.I.R.%) was calculated from the equation given below: $\text{T.I.R.\%} = (1 - W_t/W_c) \times 100\%$, here W_t refers to the tumor weight of the therapeutic groups, W_c refers to the total tumor weight of the negative control group. Animals were sacrificed by cervical dislocation three days after the last administration and the tumors were excised and weighed.

2.5. Acute toxicity study

The median lethal dose (LD_{50}) was investigated in the acute toxicity study. Mice with a body weight ranging from 19 to 25 g were divided into five groups of 10 males and 10 females at random. Single intravenous doses of 20, 25, 30, 40 and 50 mg/kg of VLE were injected through the tail vein. Animals were observed immediately at 0.5 h, and at 1, 2, 4 h after injection, then, twice a day for subsequent 14 days with regard to their general condition, toxic symptoms and mortality. Body weights were recorded right before injection and 3, 7, 14 days after administration. On day 15, all the surviving animals were sacrificed and a gross pathological examination was carried out. Finally, the LD_{50} and 95% confidence limits were calculated using a through Bliss analysis. The LD_{50} of VS was also obtained as described above.

2.6. Long-term toxicity study

2.6.1. Treatment

SD rats used in the long-term toxicity study were approximately 7–8 weeks old and their body weight was 170 ± 10.25 g for males and 182 ± 9.35 g for females. The rats were divided into five groups with 32 rats in each group (16 males and 16 females): VLE at low (VLE-L), middle (VLE-M) and high (VLE-H) doses, the positive control (VS) group and the negative control (NC) group. The VLE groups were given VLE at 5, 10, and 20 mg/kg/3-week period. The VS group received VS at 10 mg/kg/3-week period, while the NC group was given VRB-free lipid emulsion in a similar volume to that given to the middle dosage level animals.

The animals were given a intravenous injection every Monday and Thursday for 6 weeks, with every 3 weeks representing one regimen. After the first regimen (3 weeks), 8 rats (half male and half female) in each group and the second regimen (6 weeks), 16 rats (half male and half female) in each group were sacrificed 24 h after the last administration. The remaining eight animals in each group were observed for an additional 3 weeks after treatment and a recovery test was carried out. Then the animals were euthanized, and the blood/serum, urine and organ samples of all animals were collected for further assessment.

2.6.2. Clinical observations and body weights

All animals were observed for morbidity and mortality twice daily, and clinical examinations were performed once daily. Detailed physical examinations were conducted on all animals weekly. In addition, body weights were recorded before the start of dosing, weekly thereafter, and final body weights were recorded prior to the scheduled necropsy.

2.6.3. Clinical pathology

Blood and urine samples for clinical pathology evaluations (haematology, clinical biochemistry and urinalysis) were collected from all animals prior to the scheduled necropsy. Blood was collected after the animals had fasted overnight. Blood samples were collected via cardiac puncture under ketamine anaesthesia at the time of necropsy. All blood samples were then subjected to haematology and clinical biochemistry analyses. Urine samples were collected over a 24-h period using metabolism cages and then urinalysis was carried out.

Hematology parameters were determined using an ACL-200 blood autoanalyzer (Beckman Coulter Corp., USA). The parameters included: red blood cell count (RBC), haemoglobin concentration (HB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), reticulocyte count (Ret), white blood cell count (WBC), WBC differential count (DC), platelet count (PLT) and coagulation time (CT).

The clinical biochemistry parameters were determined using an AU640 blood biochemical autoanalyzer (Olympus Corp., JAP). The parameters included: aspartate aminotransferase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), blood urea nitrogen (BUN), total protein (TP), albumin (ALB), globulin (GLB), creatinine (Cr), total bilirubin (TBiL), glucose (GLU), total choles-

terol (TCHO), triglyceride (TG), creatinine kinase (CK), gamma glutamyl transferase (GGT), sodium (Na), potassium (K) and chloride (Cl).

Standard urinalysis parameters including the pH, nitrite, glucose, and protein levels, urine specific gravity, occult blood, bilirubin in urine, urobilinogen in urine, urinary ketone and white blood cell were examined using a Uritest-300 Urine autoanalyzer (Uritest Corp., USA).

2.6.4. Necropsy and histopathology

A complete necropsy was performed on all animals for gross lesions. Tissues and organs of interest were retained. Heart, lung, liver, spleen, kidney, brain, adrenal gland, thoracic gland, testes, epididymus, uterus and ovary of all animals were examined and weighted soon after dissection to calculate the organ coefficients. Organ to final body weight ratios were calculated.

Histopathological assessments were performed for organs of interest retained from all animals of the VLE-H and VS groups and, if necessary, from the VLE-M and VLE-L groups. The following tissues and organs were dissected and then preserved in 10% neutral buffered formalin for histopathological examination: heart, lung, liver, spleen, kidney, brain (cerebellum, mesencephalon and brain stem), sciatic nerve, pituitary, spinal cord, adrenal gland, pancreas, stomach, duodenum, jejunum, ileum, colon, trachea, esophagus, thyroid gland, prostate gland, gonad (testes, epididymus, uterus and ovary), bladder, submandibular gland, sternum, thoracic gland, thoracic aorta, mesenteric lymph node and the tissue at the injection site. For pathological studies, all histopathological tests were performed using standard laboratory procedures. The organs were treated with alcohol, embedded in paraffin blocks, 4–6 μm setion were obtained and then stained with hematoxylin and eosin (HE) before microscopic examination. The degree of lesions was scored according to the description rating in Table 1.

2.7. Safety studies

2.7.1. Intravenous irritation assessment

20 male rabbits weighting 2.42 ± 0.11 kg were divided into four groups ($n = 5$). Two groups were given VLE and VS intravenously at a dose of 0.8 mg/kg into the right ear marginal vein, and this was repeated after 3 days. An equivalent volume of VRB-free lipid emulsion and normal saline were given intravenously to the other two groups in the same way. After injection, any paradoxical reaction at the injection site was recorded. Three rabbits from each group were sacrificed 24 h after the last administration, and the remaining two rabbits were sacrificed after a 14-day recovery period. At localization of the injection site to the proximal region, the vascular tissues were removed and fixed in 10% buffer-formalin solution for histopathological examination.

2.7.2. Hemolysis test

2.7.2.1. Hemolysis test in vitro. Erythrocytes isolated from fresh whole rabbit blood were used to test the hemolysis effect of VLE in vitro. Different amounts of VLE and VS, 0.1, 0.2, 0.3, 0.4 and 0.5 ml, were added to five tubes along with 2.5 ml 2% erythrocyte dispersion in each. Then, normal saline was added to every tube to obtain a final volume of 5 ml. As a comparison, the positive and negative controls were prepared by addition of 2.5 ml water and 2.5 ml normal saline to 2.5 ml 2% erythrocyte dispersion, respectively. After vortexing, the tubes were incubated at 37 °C. Then at 20 min, 40 min, 1 h, 2 h and 3 h, the samples were centrifuged at 1000 rpm for 10 min. Finally, the optical density (OD) was measured in a 752-UV-spectrophotometer at a wavelength of 550 nm (Beisida Instrument Factory, Chengdu, China).

2.7.2.2. Hemolysis test in vivo. Wistar rats weighing 160–180 g were used for the in vivo hemolysis test. Rats were randomly assigned to three groups ($n = 8$, equal numbers of males and females): (1) negative control group (0.9% saline injection), (2) positive control group (VS) and (3) test group (VLE). Animals were given an i.v. injection of 3.3 mg/kg/3-week, and this was repeated every 3 or 4 days for a total of six times. The anaphylactic response was recorded 3 h after the challenge injection. Then, 2 days after the last administration, the rats were sacrificed and the blood and urine were collected. The following parameters were determined using an ACL-200 Blood autoanalyzer (BeckmanCoulter Corp., USA) and a Uritest-300 Urine analyzer (Uritest Corp., USA): red blood cell count (RBC), hematocrit (HCT), reticulocyte count (Ret), bilirubin in urine (U-BIL) and urobilinogen in urine (URO)

2.7.3. Hypersensitivity reaction

Hartley guinea pigs weighing 240–280 g were randomly divided into six groups ($n = 6$, 3 males and 3 females): (1) negative control group (0.9% saline injection); (2) positive control group (1.0% egg albumen solution); (3) VS-L (0.19 mg/kg VS); (4) VS-H (0.37 mg/kg VS); (5) VLE-L (0.19 mg/kg VLE); (6) VLE-H (0.37 mg/kg VLE). All the animals were given an intraperitoneal injection of 0.5 ml of the solutions every other day for a total of three times. Twelve days after the last injection, each animal was given a challenge dose of corresponding solution into the vein at the lateral of the crus curvilineum. The challenge dose was 3-time the administration dose. The animals were monitored for 3 h after the challenge injection to record any hypersensitivity reaction, such as nose scratching, sneezing, erect hair, twitching, dyspnea, convulsion and death.

2.8. Statistical analysis

All calculated values were expressed as means ± standard deviation (SD). The values were analyzed statistically by one-way analysis of variance and Student's *t*-test. A significant difference was taken as $p < 0.05$.

3. Results and discussion

3.1. Characterization of VLE

The average diameter of VLE given by the In-Wt Gaussian distribution was 165.3 ± 50.5 nm and the diameter D99% was less than 268.0 nm. Microscopic observation showed that no particles were larger than 5 μm in the emulsions. The zeta potential and pH value were determined as −26.53 mV and 8.02, respectively. The formulation contained 1.012 mg/ml VRB and the entrapment efficiency was 90.46%. The long-term stability investigation was carried out for 18 months. There were no significant changes in the main parameters, the results showed that VLE was stable during a 18-month storage at 6 ± 2 °C, indicating that VLE would be very suitable as a potential parenteral formulation.

3.2. In vivo antitumor efficacy

The in vivo antitumor efficacy of VLE was evaluated in tumor-bearing nude mice after intravenous injection of VRB-free lipid emulsions (negative control, NC group), VS (5 mg/kg) and three different doses of VLE (2.5, 5, 10 mg/kg). The dose was chosen by transferring from the skin surface area conversion of the clinical dose of 25–30 mg/m^2 and the maximum tolerated dose for mice in a pre-test experiment. The tumor volumes in Balb/c-nu mice after administration are shown in Fig. 1. In all three tumor models, the NC group failed to show any effect on tumor weight and progression, but the VS group and all VLE groups effectively inhibited tumor growth, and there were statistically significant differences

Table 1
Scoring value for tissue lesions.

Tissue	Value Classification	0 No lesions	0.5 Weak lesions	1 Mild lesions	2 Moderate lesions	3 Severe lesions	4 Very severe lesions
Bone marrow	Hematopoietic cell decreased by	Hardly noticeable	Less than 5%	Between 5% and 25%	Between 25% and 50%	Between 50% and 75%	More than 75%
Spleen	Lymphocytes decreased by	Hardly noticeable	Less than 5%	Between 5% and 25%	Between 25% and 50%	Between 50% and 75%	More than 75%
Thymus	Lymphocytes decreased by	Hardly noticeable	Less than 5%	Between 5% and 25%	Between 25% and 50%	Between 50% and 75%	More than 75%
Injection site	Grade of Inflammatory cell infiltration	Hardly noticeable	Weak	Mild	Moderate	Severe	Very severe

($p < 0.05$) between the therapeutic groups and the NC group. Table 2 lists the tumor inhibition rates of all therapeutic groups, and all groups showed significant tumor inhibition (inhibition rate more than 30%, $p < 0.05$). It was obvious that the VLE exhibited a dose-dependent antitumor activity in the range of 2.5–10 mg/kg. In addition, there was no significant difference between the tumor volumes following treatment with VS at 5 mg/kg and with VLE at the same dose ($p > 0.05$). Although the antitumor efficacy was not improved, the local venous irritation and systematic toxicity might be reduced significantly leading to a more effective drug treatment.

In our previous study, the in vivo tissue distribution in mice was investigated. The results showed that the $AUC_{0-48\,h}$ was significantly higher in the liver and a little lower in the lung after VLE administration compared with VS. However, unexpectedly at the same dose it exhibited no significant difference between VLE and VS in the tumor inhibition rate of the A549 human pulmonary adenocarcinoma cell lines and the HeP G2 hepatocellular carcinoma cell lines. The following factors might be involved. The previous study showed that VRB is liable to the tissues with abundant blood perfusion (Sun et al., 2008). The concentration of VRB in tissues after 0.5 h IV administration was in the following order: lung > kidney > spleen > intestine > stomach > liver > heart > brain. However, the tumor fragment was inoculated into the right axillary region where the blood is not rich, so it is reasonable that the difference between the two formulations was reduced. For further understanding the antitumor efficacy, more studies should be carried out.

3.3. Acute toxicity

The purpose of the acute toxicity study was to compare the LD_{50} and short-term exposure toxicity of VLE with that of VS. The LD_{50} values of both VLE and VS were calculated and the toxic effects on major organs were examined to determine the acute toxicity of VLE. Determination of LD_{50} is the first step to provide information on health hazards likely to arise from short-term exposure to drugs (Ihsan et al., 2010). The LD_{50} of VLE was 29.3 and 32.1 mg/kg for male and female mice, respectively, and the 95% confidence limits were in the range of 26.4-32.2 and 28.3–36.0 mg/kg, respectively. The corresponding LD_{50} of VS was 30.5 mg/kg for both male and female mice, with similar 95% confidence limits calculated as 27.4–33.7 mg/kg. The clinical signs noted following the administration of both VLE and VS in mice receiving 50, 40, 30 and 25 mg/kg were pronation and convulsion within the first 0.5 h, which disappeared after 4 h. No adverse effect was observed during the subsequent 14-day observation period. All deaths occurred in the first 4 h. There were no marked toxic effects evident in the experimental animals injected with VLE or VS at a dose of 20 mg/kg. In addition, no macroscopic abnormalities were recorded at necropsy in any of the animals. These results suggested that there was no significant difference in the LD_{50} values between VLE and VS, and VLE exhibited the same safety profile for i.v. administration as VS during short-term exposure.

After being incorporated in the lipid emulsion, it takes time for the drug to release from the emulsion, but the nature of the drug is not changed. Our previous pharmacokinetics study in rats showed that the VRB concentration was different between VLE-treated rats and VS-treated rats at the first 15 min, but then these differences disappeared. It might be concluded that the lipid emulsion cannot be a stable drug carrier system for VRB in vivo. After injection, VRB released from the emulsion rapidly. So after a high-dose intravenous injection, the VLE showed no difference at the acute toxicity compared with VS.

3.4. Long-term toxicity

In this paper, the long-term toxicity was performed to assess the systematic toxicity of VLE, and it was evaluated by general clinical observations, body weights, clinical pathology evaluations (haematology, clinical biochemistry and urinalysis analyze), necropsies and pathological observations. Very interesting results were obtained from the long-term toxicity experiment.

All animals survived until the scheduled necropsy in the long-term toxicity study. During the course of the scheduled treatment and recovery periods, the rats in VLE-H and VS groups experienced weight loss, reduced appetite, emaciation and depilation, dry feces and white particles in the feces. No severe adverse effects were observed in the VLE-M and VLE-L groups. Changes in body weight in the long-term toxicity study are indicative of toxic effects in the laboratory animals. Lower mean body weight gains were noted in

Table 2
Tumor weights and tumor inhibition rates of all therapeutic groups on the 24th day. The results are expressed as means ± SD ($n = 7$).

Groups	A549		HeP G2		BCAP-37	
	Tumor weight (g)	T.I.R.%	Tumor weight (g)	T.I.R.%	Tumor weight (g)	T.I.R.%
Negative control	2.30 ± 0.69	–	2.03 ± 0.48	–	3.08 ± 0.66	–
VS	0.31 ± 0.12[a]	86.33	0.73 ± 0.18[a]	64.09	1.37 ± 0.32[a]	55.47
VLE-H	0.33 ± 0.08[a]	85.71	0.61 ± 0.26[a]	69.72	0.99 ± 0.54[a]	67.9
VLE-M	0.33 ± 0.08[a]	62.73	1.03 ± 0.29[a]	49.3	1.36 ± 0.33[a]	65.75
VLE-L	1.57 ± 0.38[b]	31.68	1.23 ± 0.69[b]	39.44	1.18 ± 0.32[a]	61.55

[a] $p < 0.05$ when compared with the NC group.
[b] $p < 0.05$ when compared with the VS group.

Table 3
Body weight (g) of the rats in all groups at different time points in long-term toxicity study. The results are expressed as means ± SD.

	Weeks	NC	VS	VLE-H	VLE-M	VLE-L
Male	3	364.31 ± 20.81	274.31 ± 24.17[a]	300.50 ± 26.30[a]	332.50 ± 17.72[a,b]	340.69 ± 19.27[a]
	6	416.75 ± 31.97	350.58 ± 21.12[a]	369.58 ± 32.40[a]	409.00 ± 21.09[b]	398.08 ± 16.31[a]
	9	484.5 ± 39.78	410.75 ± 16.50[a]	460.50 ± 9.29	462.50 ± 37.49[b]	474.25 ± 28.57
Female	3	251.63 ± 12.60	218.31 ± 15.04[a]	229.31 ± 20.02[a]	229.88 ± 16.56[a,b]	231.00 ± 18.02[a]
	6	283.08 ± 35.73	270.00 ± 14.85	250.00 ± 15.48[a]	272.08 ± 17.86	268.25 ± 17.04
	9	296.50 ± 15.55	267.25 ± 13.94[a]	278.78 ± 27.68	285.75 ± 26.24	291.50 ± 11.00

[a] $p < 0.05$ when compared with the NC group.
[b] $p < 0.05$ for the VLE-M group when compared with the VS group.

Table 4
The hematology parameters in all the groups after the second regimen in the long-term toxicity study. The results are expressed as means ± SD ($n = 16$).

	NC	VS	VLE-H	VLE-M	VLE-L
RBC ($\times 10^{12}$/L)	8.47 ± 0.39	4.83 ± 0.73[a]	5.38 ± 0.89[a]	6.53 ± 0.40[a,b]	7.04 ± 0.80
HB (g/L)	159.38 ± 7.23	99.75 ± 15.26[a]	108.00 ± 16.13[a]	129.88 ± 5.00[a,b]	136.63 ± 12.48[a]
HCT (%)	48.69 ± 2.36	31.14 ± 4.72[a]	33.20 ± 4.66[a]	39.89 ± 1.66[a,b]	41.84 ± 3.62[a]
MCV (fL)	57..49 ± 1.17	64.56 ± 3.26[a]	61.96 ± 2.42[a]	61.76 ± 3.62[a]	59.61 ± 2.18
MCHC (g/L)	327.38 ± 4.31	320.25 ± 3.45[a]	324.75 ± 4.89[a]	325.63 ± 3.96	326.50 ± 6.00
Ret (%)	2.86 ± 0.36	0.99 ± 0.52[a]	1.19 ± 1.23[a]	1.33 ± 1.15[a]	1.75 ± 0.61
PLT ($\times 10^9$/L)	713.60 ± 73.99	605.89 ± 194.84	577.63 ± 212.95	620.14 ± 124.25	765.85 ± 150.72
CT (s)	193.25 ± 29.36	180.88 ± 37.64	182.88 ± 44.43	180.38 ± 29.31	160.00 ± 26.72
WBC ($\times 10^9$/L)	10.19 ± 4.24	2.68 ± 1.30[a]	3.14 ± 1.29[a]	4.75 ± 1.96[a,b]	4.28 ± 1.09[a]
Neu	15.88 ± 6.70	3.66 ± 2.06[a]	3.91 ± 3.79[a]	4.40 ± 5.31[a,b]	11.41 ± 5.68
Lymp	80.04 ± 6.39	95.32 ± 2.43[a]	92.90 ± 10.24[a]	93.37 ± 7.56[a]	87.00 ± 7.57
Eosinophils	1.15 ± 0.61	0.24 ± 0.30	0.09 ± 0.18[a]	0.18 ± 0.15[a]	0.44 ± 0.33[a]
Monocytes	2.42 ± 1.14	0.92 ± 0.64[a]	0.74 ± 0.48[a]	2.09 ± 2.53	1.63 ± 2.04

[a] $p < 0.05$ when compared with the NC group.
[b] $p < 0.05$ for the VLE-M group when compared with the VS group.

all the therapeutic groups compared with the NC group during the entire study. Adverse effects and loss in body weight were noted in a dose-related manner. Although the clinical observations and body weights in the VLE-H and VS returned to normal during the recovery period, adverse effects were still present, and the body weights failed to recover to the level of the NC group. Some statistically significant differences in the body weight of male rats were noted when the VLE-M and VS groups were compared, as shown in Table 3. In the VLE-M group, reductions in body weight by 1.0%, 2.8%, and 11.8% were observed after the first regime, second regime and recovery period, respectively while, in the VS group, decreases in body weight by 5.3%, 3.7%, 15.8% were observed for the same time period.

In this work, a number of clinical pathology parameters were measured to evaluate the long-term toxicity of VLE in rats. The hematology measurements are summarized in Table 4. There were statistically significant differences between the therapeutic groups and the NC group. Lower mean RBC, HC, WBC and WBC differential counts (DC) were noted in the VLE-H and VS groups during the treatment period, and all the changes were related to the dose and time. After the second regimen, decreases in RBC, HC, WBC and WBC differential counts (DC) showed a statistically significant difference between the VLE-M and VS groups. The RBC, HCT and WBC decreased by 22.90%, 18.07% and 53.79% in the VLE-M group compared with 42.78%, 36.04% and 73.70% in the VS group. In addition, the neutrophil count decreased by 5% and 87.08% in the VLE-M group after the first regimen and the second regimen, respectively, compared with 50.79% and 93.94% in the VS group. After the 3-week recovery period, the RBC and HCT of the VLE-H and VS groups were still a little lower than in the NC group. The marked reduction in mean RBC, HB, WBC and WBC differential counts (DC) in the therapeutic groups reflected the damage to the hematopoietic system and immune system induced by VRB.

The clinical biochemistry values are presented in Table 5. Among the biochemistry parameters measured in this study, only a reduction in the TP and ALB levels was recorded after the second regimen in the VLE-M, VLE-H and VS groups relative to the NC group. The TP was 66.36 g/L and 73.37 g/L for the VLE-M and VS groups, respectively, compared with 77.46 g/L for the NC group. ALB was 38.78 g/L and 38.98 g/L for the VLE-M and VS groups, respectively, while it was 46.78 g/L for the NC group. Moreover, a reduction in the AST levels was recorded after the second regimen in the VLE-H, VLE-M

Table 5
Main serum biochemistry parameters in all the groups after the second regimen in the long-term toxicity study. The results are expressed as means ± SD ($n = 16$).

	NC	VS	VLE-H	VLE-M	VLE-L
ALT (U/L)	63.00 ± 11.80	55.06 ± 8.63	53.81 ± 8.35	56.23 ± 12.48	62.43 ± 8.82
AST (U/L)	267.63 ± 30.91	266.94 ± 47.21	219.55 ± 29.30[a]	214.70 ± 37.69[a,b]	203.59 ± 39.11[a]
TP (g/L)	72.10 ± 3.20	67.61 ± 2.74[a]	63.59 ± 3.42[a]	65.56 ± 2.94[a]	63.11 ± 1.30[a]
ALB (g/L)	41.19 ± 2.18	37.20 ± 1.65[a]	33.54 ± 4.17[a]	36.29 ± 1.42[a]	37.45 ± 2.02[a]
TBIL (Umol/L)	1.53 ± 0.54	0.83 ± 0.29[a]	1.36 ± 0.54	1.28 ± 0.59	1.12 ± 0.39
ALP (U/L)	216.64 ± 45.51	187.01 ± 49.53	165.35 ± 37.91[a]	168.48 ± 34.45[a]	242.18 ± 42.50
GLB (g/L)	32.16 ± 3.54	30.44 ± 2.02	30.05 ± 2.54	29.28 ± 3.34	28.66 ± 1.24
TG (mmol/L)	0.83 ± 0.31	0.66 ± 0.12	0.63 ± 0.13	0.71 ± 0.23	0.87 ± 0.42
BUN (mmol/L)	5.42 ± 0.73	5.78 ± 0.96	7.69 ± 4.33	5.68 ± 2.99	6.10 ± 1.16
GLU (mmol/L)	6.91 ± 1.21	7.21 ± 0.53	7.62 ± 0.63	7.85 ± 0.36[b]	7.62 ± 0.63

[a] $p < 0.05$ when compared with the NC group.
[b] $p < 0.05$ for the VLE-M group when compared with the VS group.

Table 6
Organ coefficients of rats after the second regimen in the long-term toxicity study of VLE.(g/100 g body weight). The results are expressed as means ± SD (n = 16).

	NC	VS	VLE-H	VLE-M	VLE-L
Heart	0.36 ± 0.05	0.38 ± 0.04	0.41 ± 0.05	0.38 ± 0.02	0.35 ± 0.0
Liver	3.03 ± 0.38	3.84 ± 0.22[a]	3.45 ± 0.47	3.61 ± 0.32	3.40 ± 0.33
Spleen	0.18 ± 0.02	0.29 ± 0.15[a]	0.25 ± 0.06[a]	0.20 ± 0.02	0.18 ± 0.03
Lung	0.38 ± 0.03	0.43 ± 0.04	0.47 ± 0.07	0.44 ± 0.04	0.34 ± 0.15
Kidneys	0.78 ± 0.07	0.91 ± 0.15	0.85 ± 0.07	0.79 ± 0.03	0.78 ± 0.08
Brain	0.50 ± 0.06	0.59 ± 0.04	0.54 ± 0.10	0.53 ± 0.03	0.40 ± 0.08
Thymus	0.14 ± 0.02	0.09 ± 0.02[a]	0.08 ± 0.01[a]	0.08 ± 0.02[a]	0.10 ± 0.03
Adrenals	0.024 ± 0.006	0.030 ± 0.006	0.024 ± 0.005	0.024 ± 0.003	0.023 ± 0.005
Testes	1.50 ± 0.23	1.52 ± 0.13	1.64 ± 0.36	1.61 ± 0.34	1.40 ± 0.16
Uterus	0.31 ± 0.07	0.33 ± 0.16	0.34 ± 0.07	0.46 ± 0.12	0.33 ± 0.03
Ovaries	0.08 ± 0.01	0.07 ± 0.02	0.07 ± 0.02	0.07 ± 0.02	0.08 ± 0.01

[a] $p < 0.05$ when compared with the NC group.

and VLE-L groups, it was 219.55, 214.70 and 203.59 U/L, respectively, while 267.63 U/L for the NC group and 266.94 U/L for the VS group.

There was no treatment-related adverse effect on the urinalysis parameters for male and female rats during the entire study (data not shown). All the animals showed red blood cells in urine, but this effect was not considered to be drug-related.

Table 6 shows the coefficients of the examined organs in terms of body weight (mg/g, wet weight of tissues/body weight). After the second regimen, the organ coefficient of the spleen was significantly higher in male rats in the VLE-H and VS groups, and the organ coefficient of the thymus was higher in male and female rats in the VLE-M, VLE-H and VS groups, in comparison with the NC group. There was no statistically significant difference in the other organ coefficients.

The NC treatment did not cause any histopathology abnormalities in any of the organs, while histopathology changes in the spleen, thymus, lymph nodes, bone marrow, testis, ovary and injection site were observed in the therapeutic groups, and all the injuries were time- and dose-related. The scores for organ lesions in the long-term toxicity study are listed in Table 7. Only the histopathological evaluations of the injured organs have been described. During the treatment period, lesions to bone marrow, testis and lymph nodes were observed in the VLE-M, VLE-H and VS groups. Damage to bone marrow was associated with a decrease in hematopoietic cells and an increase in adipocytes. This, along with the results obtained from the biochemical studies, suggests that the bone marrow is severely damaged. Degenerative changes in the testis were reflected by mild atrophy, degeneration of the seminiferous tubules, along with a decrease in the spermatid and spermatozoa counts in male rats. Injury to lymph nodes was associated with attenuation on the cortex, and a decrease in lymphocytes. Lesions observed in the lymph nodes consisted of cell swelling, granular and ballooning degeneration, subsequent progressive vacuolation, and necrosis of the glomerulosa cells. Atrophy of the thymus and spleen was apparent in all the therapeutic groups. Injury to the ovary was observed only in the VS group, and this was associated with mild denaturation, swelling and pycnosis, together with injury to follicle cells in female rats. In addition, obvious macroscopic changes such as excoriation and swelling, and microscopic changes such as degeneration and mortification were observed at the injection site of all rats in the VS group during the treatment period. The VLE-H group also exhibited slight midrange irritation, and microscopic changes such as degeneration were observed but there were no macroscopic changes. Very mild changes at the injection site were observed for the VLE-M and VLE-L groups. The lesions of the spleen, testis, bone marrow and at the injection site were still present in the VS group after the 3-week recovery period, while no obvious injury was noted in the VLE groups.

The bone marrow has been documented to be the most common toxicological target of VRB. In order to further explore the lower toxicity of VLE on bone marrow, the VRB concentrations in rat bone marrow after i.v. injection of VLE and VS were examined using the UPLC/MC/MC method described elsewhere. The results showed that after a single i.v. administration, VLE and VS reached almost equal concentrations in bone marrow at the first 24 h. Then, VLE exhibited a significantly lower VRB concentration compared with VS at 36 h, 48 h and 72 h. The lower drug concentration of VLE in the bone marrow at the posterior time might help in reducing the bone marrow toxicity, because granulocyte nadirs occurs between 8 and 10 days after dosing (Khayat et al., 1995).

The main adverse effects of VRB in clinical practice are neutropenia, leucopenia, and thrombocytopenia, with neutropenia being the primary dose-limiting toxicity (Carrato et al., 1997). Chemotherapy related neutropenia can lead to life-threatening events. As neu-

Table 7
Scores for organs lesions in the long-term toxicity study. The results are expressed as means ± SD (n = 8 for 3 and 9 weeks, n = 16 for 6 weeks).

	Weeks	NC	VS	VLE-H	VLE-M	VLE-L
Bone marrow	3	0.00 ± 0.00	0.69 ± 0.53[a]	0.75 ± 0.66[a]	0.00 ± 0.00[b]	0.00 ± 0.00
	6	0.00 ± 0.00	3.97 ± 0.13[a]	3.25 ± 1.34[a]	2.19 ± 0.91[a,b]	1.38 ± 0.62[a]
	9	0.00 ± 0.00	1.13 ± 0.64[a]	0.38 ± 0.23[a]	0.00 ± 0.00[b]	0.00 ± 0.00
Spleen	3	0.00 ± 0.00	0.38 ± 0.23[a]	0.25 ± 0.38[a]	0.00 ± 0.00[b]	0.00 ± 0.00
	6	0.00 ± 0.00	1.28 ± 0.78[a]	0.81 ± 0.66[a]	1.03 ± 0.56[a]	0.19 ± 0.31[a]
	9	0.00 ± 0.00	0.44 ± 0.50[a]	0.13 ± 0.23[a]	0.00 ± 0.00[b]	0.00 ± 0.00
Thymus	3	0.00 ± 0.00	1.19 ± 0.75[a]	1.31 ± 0.59[a]	0.00 ± 0.00[b]	0.00 ± 0.00
	6	0.00 ± 0.00	2.75 ± 0.45[a]	2.34 ± 0.98[a]	1.75 ± 0.45[a,b]	0.16 ± 0.30[a]
	9	0.00 ± 0.00	0.31 ± 0.37[a]	0.00 ± 0.00	0.00 ± 0.00[b]	0.00 ± 0.00
Injection site	3	0.00 ± 0.00	2.19 ± 0.75[a]	0.13 ± 0.23	0.13 ± 0.23[b]	0.19 ± 0.26
	6	0.00 ± 0.00	2.75 ± 0.44[a]	0.72 ± 0.77[a]	0.44 ± 0.51[a,b]	0.69 ± 0.48[a]
	9	0.00 ± 0.00	1.88 ± 0.83[a]	0.00 ± 0.00	0.00 ± 0.00[b]	0.00 ± 0.00

[a] $p < 0.05$ when compared with the NC group.
[b] $p < 0.05$ for the VLE-M group when compared with the VS group.

tropenia and lymphopenia have been reported to be the major toxicities associated with a decreased number of white blood cells (Schaeppi et al., 1974), the statistical significance on the WBC change between VLE-M and VS groups suggested that VLE could markedly reduce the incidence of neutropenia and lymphopenia induced by VRB In addition, it was worth noting that the lesions in the bone marrow, spleen, thymus as well as the injection site were much more severe in the VS group than VLE-M group throughout the entire treatment and recovery period. All these results strongly suggested that the systematic toxicity and local injection site toxicity induced by VLE were much less serious compared with VS.

3.5. Intravenous injection safety test

3.5.1. Rabbit ear vein irritation assessment

There was no obvious visible damage at the injection site and surrounding tissues of the VRB-free lipid emulsion and normal saline groups. Additionally, there was no thrombus or vascular congestion in blood vessels at or distant from the site of injection and degeneration of endothelial cells in any groups. 1 day after administration, erythema and edema in the surrounding tissues in one rabbit, and phlebectasia and congestion in all three rabbits were observed in the VS group, while phlebectasia and congestion in two rabbits were observed in the VLE group. 14 days after administration, phlebectasia and congestion in two rabbits were observed for the VS group, while there was no obvious visible damage at the injection site for the VLE group.

Local venous toxicity, such as drug induced-phlebitis, is one of the unpleasant toxicities for patients undergoing cancer (Yoh et al., 2007). By loading into lipid emulsions, part of the drug is incorporated in the oil core or in the oil–water interfacial film, and direct contact of the drug with the body fluids can be avoided to minimize any possible adverse effects. As a result, the entrapment efficiency is an important parameter characterizing the emulsion. The VRB entrapment efficiency in the present study was found to be about 90%. The high drug entrapment efficiency might be due to the high lipophicity of VRB, as the octanol-buffer (pH 7.2) partition coefficient was 16 (Levˆeque and Jehl, 1996), and it exhibits good solubility in lipophilic solvents and poor solubility in water. As most of the drug was incorporated, the local concentration of VRB will be much lower than that of VS when injected into the vein. As expected, the results of the local vein irritation assessment and histopathology of the injection site of rats in the long-term toxicity study both showed that VLE exhibited much lower the local venous toxicity than VS.

3.5.2. Hemolysis test

The hemolytic potential of VLE was evaluated to ensure its hemocompatibility. Complete hemolysis was observed in tubes of the positive control within 20 min. The tubes of the VLE, VS and negative control groups appeared to be clear, with erythrocyte precipitation at the bottom during the 3 h observation period. No significant difference was noted in the OD values between the VLE and VS tubes. These experimental results demonstrated that both VLE and VS at different concentrations up to 0.040 mg/ml would not induce hemolysis or erythrocyte agglutination at 37 °C.

No statistically significant difference in the parameters was observed between the VLE and NC groups while the values of RBC, HCT, and Ret obtained in the VS group were significantly different from those of the NC group. In brief, the values of RBC, HCT, and Ret were $6.79 \pm 0.41 \times 10^{12}$/L, $43.03 \pm 2.36\%$, $3.49 \pm 1.56\%$ and $6.14 \pm 1.42 \times 10^{12}$/L, $38.55 \pm 6.63\%$, $1.81 \pm 0.61\%$ for VLE and VS groups, respectively, compared with $7.48 \pm 0.40 \times 10^{12}$/L, $45.08 \pm 2.31\%$, $4.00 \pm 0.40\%$ for the NC group. The decrease in the values of RBC, HCT, Ret in the VS group might be the result of the bone marrow depression caused by VS.

3.5.3. Hypersensitivity reaction

Following the challenge, all the guinea pigs in the positive control group exhibited obvious hypersensitivity symptom including dyspnea, shock and convulsions, and then died within 2 min. However, no anaphylaxis symptoms were observed in the NC group, VS and VLE groups. These results suggested that the intravenous administration of VLE did not induce hypersensitivity reactions.

4. Conclusion

In the present paper, we have evaluated the antitumor efficacy, toxicity and injectable safety of VLE in comparison with VS. The results showed that VLE maintained the antitumor activity against A549 human lung cancer, hepatoma solidity (Heps) G2 cancer and BCAP-37 human breast cancer cells. It also significantly reduced the local venous irritation and systemic toxicity. In addition, VLE was safe for intravenous injection. The comparable antitumor efficacy, lower local venous irritation and bone marrow toxicity shown by VLE make it a useful potential parenteral carrier for VRB in cancer treatment.

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