· Research Papers ·

Alleviation of PEGylated Puerarin on Erythrocyte Hemolysis Induced by Puerarin in Glucose-6-phosphate Dehydrogenase-deficient Rats

LIU Xin-yi[1], LI Jian-rong[2], WANG Nai-jie[3], ZHANG Guang-ping[3], DU Feng[3], YE Zu-guang[2*], XIANG Da-xiong[1*]

1. Key Research Laboratory for Development of New Drug of Traditional Chinese Medicine, The Second Xiangya Hospital of Central South University, Changsha 410011, China
2. China Academy of Chinese Medical Sciences, Beijing 100700, China
3. National Engineering Research Center for R&D of Traditional Chinese Medicine Multi-ingredient Drugs, Beijing 100075, China

Abstract: **Objective** To explore and analyze the reducing hemolytic effects of PEGylated puerarin (PEG-PUE) on erythrocytes induced by PUE in glucose-6-phosphate dehydrogenase (G6PD)-deficient rats. **Methods** The rat model with G6PD-deficiency was established via sc injecting 1% acetylphenyl-hydrazine. Then the G6PD-deficient erythrocyte suspension obtained from this rat model was used to evaluate the hemolytic effects of PUE and the reducing hemolytic effects of PEG-PUE via hemolytic activity and erythrocyte osmotic fragility assay. **Results** It was found that PUE could cause a serious hemolysis to the erythrocyte suspension with the increase of drug concentration and the prolongation of drug incubation time, the hemolytic rate of PUE was up to 40%, while the addition of PEG-PUE to the erythrocyte suspension revealed no significant hemolysis. Additionally, the result of erythrocyte osmotic fragility indicated that PEG-PUE exerted a slight effect on the erythrocyte membranes, and the NaCl concentration that induced 50% hemolysis (32 mmol/L) was about one-third PUE. **Conclusion** These results demonstrate that PEG-PUE could play a significant role in reducing the side effect of hemolysis induced by PUE. The low hemolytic activity of PEG-PUE makes it a favorable candidate for *in vivo* tests and PEG-PUE could also provide the useful insight for the further formulation development as an innovative drug.

Key words: erythrocyte osmotic fragility; glucose-6-phosphate dehydrogenase-deficient rats; hemolytic activity; PEGylated puerarin; puerarin

DOI: 10.7501/j.issn.1674-6384.2013.01.005

Introduction

Puerarin (PUE) is a naturally occurring isoflavone *C*-glycoside isolated from the roots of *Pueraria lobata* (Willd.) Ohwi. The research on the clinical application of PUE injection in recent years has shown that PUE, a vasodilator, was effective to treat a variety of cardio-vascular diseases, such as coronary heart disease, cardiac infarction, arteriosclerosis, and arrhythmia (Wang, 2011; Yeung *et al*, 2006). However, the acute intravascular hemolysis (AIH) of PUE injection is a major drawback for its clinical utilization (Yue *et al*, 2008). The severity and harmfulness of PUE injection on the AIH has aroused a widespread concern and heated discussion. Even worse, it may cause death if the emergency treatment was not managed to save lives of patients in minutes (Zhu, Wang, and Ma, 2003a; Wang

* Corresponding authors: Ye ZG Address: China Academy of Chinese Medical Sciences, Beijing 100700, China
Tel/Fax: +86-10-6401 4411 E-mail: yezuguang88@hotmail.com
Xiang DX Address: Key Research Laboratory for Development of New Drugs of TCM, The Second Xiangya Hospital of Central South University, Changsha 410011, China Tel: +86-731-8529 2129 E-mail: xiangdaxiong@163.com
Received: August 26, 2012; Revised: October 12, 2012; Accepted: November 4, 2012
Fund: Ministry of National Science and Technology Support Program "Twelfth Five-Year Plan" (2011BAI01B08)
Online time: January 12, 2013 Online website: http://www.tiprpress.com/chmen/ch/reader/view_abstract.aspx?file_no=CHM20120508001&flag=1

and Wu, 2009). So it is very necessary to develop an effective strategy to reduce the side effect of hemolysis and exert medicinal actions of PUE injection in clinic.

Polyethylene glycol (PEG) and its derivatives are compounds with high molecular weight, and they possess excellent properties, such as biocompatibility, non-toxicity, and non-immunogenicity (Khandare and Minko, 2006; Pasut and Veronese, 2007; Silva Freitas and Abrahão-Neto, 2010). The covalent attachment of PEG to prototype drugs (PEGylation) could evade recognition by T cells and macrophages *in vivo* and thus avoid immune response (Nacharaju, Manjula, and Acharya, 2007; Lazarjani *et al*, 2010). Therefore, PEGylation could be used to reduce the immunogenicity and antigenicity of original drugs via a reactive derivative of PEG with the target molecule (Li and Wallace, 2008; Novikov *et al*, 2010). Based on the above theoretical analysis, we aimed to reduce the erythrocyte hemolysis of PUE injection by PEGylation. Although the exact mechanisms of erythrocyte hemolysis induced by PUE injection have not yet been clearly demonstrated, they may be related with the instability of erythrocyte membranes induced by PUE (Hou *et al*, 2008). Finally, PUE injection-induced hemolysis occurs via an osmotic swelling, while PEGylated puerarin (PEG-PUE) might inhibit the interaction of red blood cells (RBCs) with PUE by the effect of the steric shielding or coating of PEG chain (Li and Wallace, 2008; Wang *et al*, 2010). Therefore, we could anticipate that a reduction in the side effect of hemolysis induced by PUE would be achieved.

Clinical experiences have revealed that the incidence of AIH induced by PUE injection among patients was not very high (Zhu, Wang, and Ma, 2003b). Therefore, it requires a larger number of laboratory samples to reflect the significant side effects of hemolysis induced by PUE in normal RBCs. Recent researches have shown that glucose-6-phosphate dehydrogenase (G6PD)-deficient animals were at high risk of developing hemolytic anaemia perhaps due to the fragile erythrocyte membranes (Benatti *et al*, 1978; Abboud and Al-Awaida, 2010). Thus, the RBCs obtained from the rats with G6PD-deficiency were used to increase the sensitivity of RBCs, and which also saved a lot of laboratory samples in our study. And we conducted a comparative study on the hemolytic activities of PUE and PEG-PUE to illustrate the reducing hemolytic effect of PEG-PUE on the erythrocytes of G6PD-deficient rat *in vitro*.

Materials and methods

Materials and reagents

PUE (purity 99.2%, Fig. 1) was purchased from Qingdao Jinfeng Pharmaceutical Co., Ltd. PEG-PUE with a drug load of 4.1% was synthesized in our laboratory. All other reagents were of analytical grade and obtained from commercial sources.

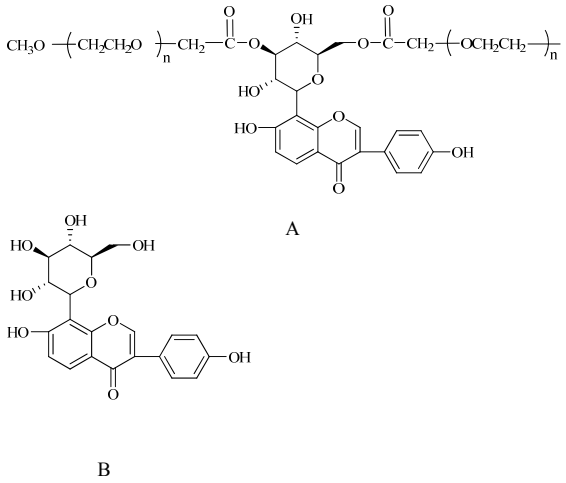

Fig. 1 Chemical structures of PEG-PUE (A) and PUE (B)

Preparation of PEG-PUE

PEG-PUE was synthesized by a common procedure. Briefly, mPEG-CH_2COOH (0.2 mmol) was dissolved in 12 mL anhydrous dichloromethane (DCM) at room temperature. And then EDC·HCl (0.24 mmol) and DMAP (0.066 mmol) at 4 ℃ were added. Then PUE (0.25 mmol) previously dissolved into 4 mL *N*,*N*-dimethylformamide (DMF) was dropped into the solution. The reaction was proceeding overnight under stirring at room temperature in the dark. The solvent was then removed by the concentration under reduced pressure and dropped into 40 mL anhydrous ether. After 5 h at 4 ℃, the product PEG-PUE was washed with 2-propanol (10 × 30 mL) to remove the remaining impurities. Then the precipitate was purified by gel-filtration chromatography using Sephadex LH-20 resin eluted with DMF. The fractions were analyzed by HPLC to determine the presence of PEG-PUE and PUE, respectively, and those containing the conbination were pooled and concentrated under vacuum. The purified

product was finally washed thrice with anhydrous ether and dried under vacuum. The product was characterized by HPLC, ^{1}H-NMR spectroscopy, and MALDI-TOF MS (Liu *et al*, 2010).

Preparation of erythrocytes in G6PD-deficient rats

G6PD-deficient rat model was established via sc injecting 1% acetylphenylhydrazine into the nuchal region (Ukab *et al*, 1981; Caprari *et al*, 1991). Heparinized blood samples were centrifuged under the cooled conditions for 5 min at 1500 r/min and washed for several times with physiological saline (PS) at pH 7.4 until the supernatant was clear and colorless (Abboud and Al-Awaida, 2010).

Comparison on hemolytic activity of PUE and PEG-PUE injection

The hemolytic studies were performed in erythrocyte of G6PD-deficient suspension in PBS with the hematocrit at 10%. Both PUE and PEG-PUE were added from stock solution to the erythrocyte suspension and incubated at 37 ℃. After the incubation periods, the erythrocyte suspension was centrifuged at 1500 r/min for 5 min and the percentage of hemolysis was determined by comparing the absorbance (*A*) of the supernatants with that of the control samples totally hemolysed by distilled water. The *A* of the supernatant in PS was taken as zero hemolysis and the total hemolysis (100%) was assigned when PBS was replaced by distilled water. The degree of hemolysis was determined by the following equation.

$$\text{Hemolysis (\%)} = (A_s - A_0) / (A_{100} - A_0) \times 100\%$$

Where A_s, A_0, and A_{100} are the *A* values of the sample, a solution of 0% hemolysis, and a solution of 100% hemolysis, respectively (Piriou *et al*, 1987; Isomaa, Engblom, and Hägerstrand, 1988; Munday and Munday, 2003; Li and Liu, 2008)

Comparison on osmotic fragility of PUE and PEG-PUE injections

The osmotic fragility experiments were carried out according to Dacie and Lewis (1995). Both PUE and PEG-PUE were incubated with NaCl concentration increasing from 30 to 140 mmol/L, respectively. After 30 min at room temperature, the tubes were centrifuged at 1500 r/min for 5 min and the erythrocytes lysis of G6PD-deficient rats was followed by measuring the *A* of the supernatants at 540 nm. The *A* values were plotted against NaCl concentration and fitted to suitable mathematical models by Sigma Plot 10.0 software. The NaCl concentration that induced 50% hemolysis (osmotic fragility, $H_{50\%}$) during a 30 min incubation was obtained from corresponding mathematical models and used as an important parameter to compare the osmotic fragility of PUE and PEG-PUE (Udden and Patton, 2005).

Statistical analysis

Comparison on the measured properties between the two groups was performed using two-tailed *t*-test. $P < 0.05$ indicated that the difference was statistically significant.

Results

Hemolytic activity

The effects of PUE and PEG-PUE on the isolated erythrocytes of G6PD-deficient rats were assayed as the function of incubation time and drug concentration in rat erythrocyte suspension (Fig. 2). This figure showed the time-dependent hemolytic difference between 2.5 mg/mL PUE and PEG-PUE (PUE equivalent). Statistically significant differences in hemoglobin levels were noticed between the two groups ($P < 0.05$).

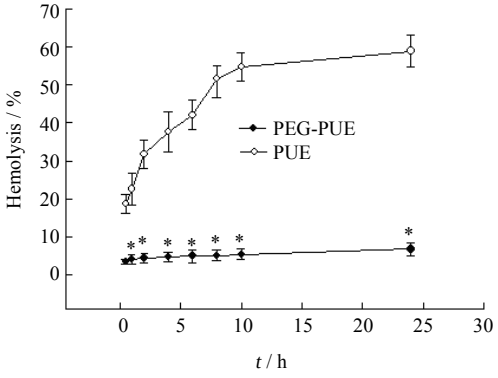

Fig. 2 Hemolytic effects induced by PUE and PEG-PUE in erythrocytes of G6PD-deficient rats

*$P < 0.05$ *vs* PUE group, same as below

Overall, the curve of hemolysis induced by PUE (2.5 mg/mL) showed an upward trend, and it presented an inflexion smooth transition of accelerated speed at 8 h, while PEG-PUE showed no significant hemolysis after incubation for 24 h. The dose-response curve of PUE hemolytic activity was approximate sigmoid shape in erythrocyte suspension of rats, with sharply increased hemolysis between 0.3 and 2.5 mg/mL PUE (Fig. 3),

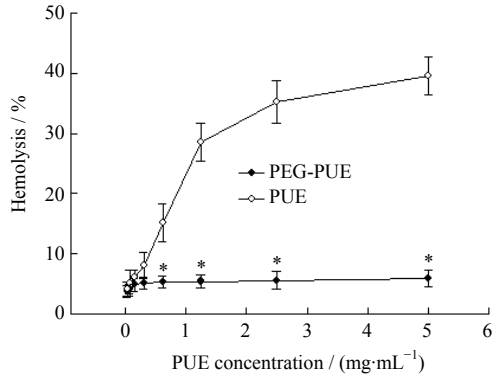

Fig. 3 Comparison on hemolytic effects induced by PUE and PEG-PUE in erythrocytes of G6PD-deficient rats, as a function of drug concentration

whereas the hemolytic activity of the same concentration of PEG-PUE (PUE equivalent) was far lower than that of PUE ($P < 0.05$).

Erythrocyte osmotic fragility

Evaluation of the osmotic fragility of RBCs is commonly used to detect the changes in the form and flexibility of their membranes, which could explain the lysis of these cells. As compared with PUE, this result indicated that there was a significant decrease of erythrocyte osmotic fragility of G6PD-deficient rats for PEG-PUE (Fig. 4). Using Sigma Plot 10.0, optimal mathematical models for erythrocyte osmotic fragility of PUE and PEG-PUE were established. Interestingly, the osmotic fragility of PUE was well fitted with the Boltzmann equation and the $H_{50\%}$ value was 91 mmol/L (Table 1). As for PEG-PUE, the $H_{50\%}$ value was only 32 mmol/L and was much smaller than that of PUE injection ($P < 0.05$), suggesting that PEG-PUE had a much weaker hemolytic action than PUE.

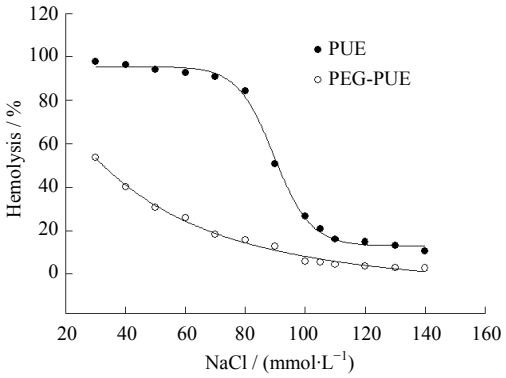

Fig. 4 Osmotic fragility curves in erythrocytes of G6PD-deficient rats in presence of PUE and PEG-PUE

Table 1 Curvilinear equations of osmotic fragility curves for PUE and PEG-PUE in erythrocytes of G6PD-deficient rats

Drugs	Curvilinear equation	r	$H_{50\%}$ / (mmol·L^{-1})
PUE	$y = 12.9880 + \frac{82.5842}{1+e^{(x-89.6312)/6.1271}}$	0.9989	91
PEG-PUE	$y = -10.7708 + \frac{108.7435}{1+\left(\frac{x}{37.6639}\right)^{1.5952}}$	0.9985	32

Discussion

The AIH induced by PUE injection has attracted a great attention of society, and it has to be noticed in the "Warnings" section in the drug label, which was officially required by SFDA. Experimental studies on the mechanisms of hemolysis induced by PUE injection were reported in a large number of literature reports, but until now, there were still no consensus. Maybe they resulted from many factors, such as PUE itself, the solubilizer contained in PUE injection, and its preparation technology. From the analysis on clinical medicines, it was reported that some patients suffering from the adverse effects of iv hemolysis were due to long-term injection of PUE (Zhu, Wang, and Ma, 2003a; Wang and Wu, 2009). Some experts suggested one possible reason for this iv hemolysis: PUE itself might have a stimulating effect on blood-vessel of patients. This stimulating effect might be very slight and it was insufficient to induce the hemolytic effect at once after administration. But if the patients were treated with long-term and frequent administration of PUE injection, in addition to poor physique or physically handicapped, it was very possible that AIH induced by PUE injection may occurred in these special crowds (Yue *et al*, 2008). The accumulated damage effect of PUE injection might be an ideal substantial reason for erythrocyte hemolysis. It was just consistent with the side effect reports of PUE injection in clinical applications.

Based on the analysis of our research, PUE caused hemolytic activity in a time- and dose-dependent manner (Murugesh *et al*, 1981). Nonetheless, previous research had suggested that the hemolytic activity induced by PUE was not very serious in intact rat RBCs, implying that the probability of hemolytic effect of PUE on normal erythrocytes was not very high. Whereas, the present data obtained from *in vitro* experiments of hemolytic activity and erythrocyte osmotic fragility could provide credible evidence that erythrocytes of G6PD-deficient rats were extremely sensitive to PUE. A plausible explanation for the susceptibility of erythrocytes in G6PD-deficient rats to PUE was that their cell membranes were more fragile and less flexible, leading to inability to respond effectively when attacked, as compared with that of intact erythrocytes. Our experiments could also verify that PUE tended to destroy those vulnerable erythrocytes, which was just in line with the actual clinical situations. Based on these observations, the erythrocytes of G6PD-deficient model was applied to evaluate the hemolytic activity of PUE and PEG-PUE.

PEGylation technology could be applied to develop a potentially valuable and interesting drug delivery system. In view of the potent blood compatibility and highly water-soluble PEG, the attachment of PUE to PEG could avoid at least the potential hemolytic activity of solubilizing agent. Moreover, PEG itself is a total bond of long-chain structure of macromolecules. Once PUE was connected to PEG polymer, PUE could be almost entirely surrounded by PEG chain. Thus it could luckily inhibit the interaction of RBCs with PUE by the effect of steric shielding or coating of PEG chain. In addition, PEG could also confer its excellent hemocompatibility upon PUE and its special characteristic of long-term sustained release could also decrease the accumulation of the highest concentration of PUE. Therefore, the risk of hemolytic activity of PUE may be reduced dramatically. As shown in Figs. 2 and 3, the hemolytic activity of PEG-PUE was far less than that of PUE. In general, PEG-PUE-induced hemolysis of rat RBCs was less than 6%, which could be regarded as negligible factor. But PUE had a certain destructive effect on the erythrocytes of G6PD-deficient rats, and the hemolytic rate increased to 40%. Additionally, the experimental result of erythrocyte osmotic fragility could also verify that PEG-PUE exerted a slight effect on the erythrocyte membranes, its $H_{50\%}$ value was obviously smaller than that of PUE. This could also provide the further explanation for the fact that the side effect of hemolysis induced by PUE could be reduced via the attachment of PUE to water-soluble polymers, namely PEG-PUE. Some relevant researches have also come up with some similar conclusions in the scientific literatures (Neu *et al*, 2007; Noh *et al*, 2010; Zhu *et al*, 2010). For example, Gajbhiye *et al* (2009) reported that the amine-terminated charged PPI 5.0G dentrimer showed hemolytic toxicity of (16.9 ± 1.93)%. But PEGylation of the dendrimer was found to have decreased the hemolysis of RBCs significantly to (3.4 ± 0.33)%. This was due to the inhibition of the interaction of RBCs with the charged quaternary ammonium ion by the effect of the steric shielding or coating of PEG chain (Gajbhiye *et al*, 2009). Besides, according to our previous researches on the pharmacodynamics and toxicology of PEG-PUE, we have not observed obvious toxic reactions, such as hemolysis in rats after iv administration of PEG-PUE combination.

Conclusion

In summary, the erythrocytes of G6PD-deficient rats show to be more susceptible to hemolysis than intact erythrocytes and provoke the high incidence of false negative in RBCs assay with blood of rats. Thereby, the difference in hemolytic activity between PUE and PEG-PUE is efficiently demonstrated via this disease experimental model. PEG-PUE induces hemolysis, but to a much smaller extent compared with PUE, perhaps due to the effect of the steric shielding or coating of PEG chain. Moreover, PEG-PUE could exert less influence on the G6PD-deficient erythrocyte membranes, and its $H_{50\%}$ value is much smaller than that of PUE. These studies indicated that the feasibility of PEG-PUE was a promising way for reducing adverse effects of PUE.

References

Abboud MM, Al-Awaida W, 2010. Synchrony of G6PD activity and RBC fragility under oxidative stress exerted at normal and G6PD deficiency. *Clin Biochem* 43: 455-460.

Benatti U, Morelli A, Frascio M, Melloni E, Salamino F, Sparatore B, Pontremoli S, De Flora A, 1978. Glucose 6-phosphate dehydrogenase activity in membranes of erythrocytes from

normal individuals and subjects with Mediterranean G6PD deficiency. *Biochem Biophys Res Commun* 85: 1318-1324.

Caprari P, Bozzi A, Ferroni L, Giuliani A, La Chiusa BF, Strom R, Salvati AM, 1991. Membrane alterations in G6PD- and PK-deficient erythrocytes exposed to oxidizing agents. *Biochem Med Metab Biol* 45: 16-27.

Dacie SJ, Lewis SM, 1995. *"Practical Haematology" Churchill Living Stone*. Elsevier Science Health Science div: New York.

Gajbhiye VG, Kumar PV, Tekade RK, Jain NK, 2009. PEGylated PPI dendritic architectures for sustained delivery of H2 receptor antagonist. *Eur J Med Chem* 44: 1155-1166.

Hou SZ, Li G, Lai XP, Ye MR, Chen JN, Su ZR, 2008. Experimental studies on mechanism of hemolysis induced by puerarin injection. *ADRJ* 10: 1-6.

Isomaa B, Engblom AC, Hägerstrand H, 1988. On the time-dependence of amphiphile-induced haemolysis. *Toxicology* 48: 285-291.

Khandare J, Minko T, 2006. Polymer-drug conjugates: Progress in polymeric prodrugs. *Prog Polym Sci* 31: 359-397.

Lazarjani HA, Vasheghani-Farahani E, Barani L, Hashemi-Najafabadi S, Shojaosadati SA, Zahediasl S, Tairahi T, Atyabi F, 2010. Effect of polymer concentration on camouflaging of pancreatic islets with mPEG-succinimidyl carbonate. *Artif Cells Blood Substit Immobil Biotechnol* 38: 250-258.

Li C, Wallace S, 2008. Polymer-drug conjugates: Recent development in clinical oncology. *Adv Drug Deliv Rev* 60: 886-898.

Li GX, Liu ZQ, 2008. The protective effects of ginsenosides on human erythrocytes against hemin-induced hemolysis. *Food Chem Toxicol* 46: 886-892.

Liu XY, Yu BY, Wang NJ, Zhang B, Du F, He C, Ye ZG, 2010. A validated stability-indicating HPLC method for the determination of PEGylated puerarin in aqueous solutions. *J Chromatogr B* 878: 2061-2066.

Munday R, Munday JS, 2003. Comparative haemolytic activity of bis(phenylmethyl) disulphide, bis(phenylethyl) disulphide and bis(phenylpropyl) disulphide in rats. *Food Chem Toxicol* 41: 1609-1615.

Murugesh N, Kumar VR, Vembar S, Damodaran C, 1981. Studies on erythrocyte membrane. V. Haemolytic effect of methylsalicylate and its possible mechanism. *Toxicol Lett* 9: 225-229.

Nacharaju P, Manjula BN, Acharya SA, 2007. Thiolation mediated pegylation platform to generate functional universal red blood cells. *Artif Cells Blood Substit Immobil Biotechnol* 35: 107-118.

Neu M, Germershaus O, Behe M, Kissel T, 2007. Bioreversibly crosslinked polyplexes of PEI and high molecular weight PEG show extended circulation times *in vivo*. *J Controlled Release* 124: 69-80.

Noh SM, Park MO, Shim G, Han SE, Lee HY, Huh JH, Kim MS, Choi JJ, Kim K, Kwon IC, Kim JS, Baek KH, Oh YK, 2010. Pegylated poly-l-arginine derivatives of chitosan for effective delivery of siRNA. *J Controlled Release* 145: 159-164.

Novikov BN, Grimsley JK, Kern RJ, Wild JR, Wales ME, 2010. Improved pharmacokinetics and immunogenicity profile of organophosphorus hydrolase by chemical modification with polyethylene glycol. *J Controlled Release* 146: 318-325.

Pasut G, Veronese FM, 2007. Polymer-drug conjugation, recent achievements and general strategies. *Prog Polym Sci* 32: 933-961.

Piriou A, Tallineau C, Chahboun S, Pontcharraud R, Guillard O, 1987. Copper-induced lipid peroxidation and hemolysis in whole blood: Evidence for a lack of correlation. *Toxicology* 47: 351-361.

Silva Freitas D, Abrahão-Neto J, 2010. Batch purification of high-purity lysozyme from egg white and characterization of the enzyme modified by PEGylation. *Pharm Biol* 48: 554-562.

Udden MM, Patton CS, 2005. Butoxyacetic acid-induced hemolysis of rat red blood cells: Effect of external osmolarity and cations. *Toxicol Lett* 156: 81-93.

Ukab WA, Sato J, Wang YM, Eys JV, 1981. Xylitol mediated amelioration of acetylphenylhydrazine-induced hemolysis in rabbits. *Metabolism* 30: 1053-1059.

Wang CS, 2011. Effect of puerarin Injection on hemorheology of acute coronary syndrome patients with no reperfusion treatment. *Chin Tradit Herb Drugs* 42: 563-565.

Wang DC, Wu CP, 2009. Puerarin injection-induced hemolytic reactions: Literature analysis of 44 cases. *China Pharmacy* 20: 943-945.

Wang W, Xiong W, Zhu Y, Xu H, Yang X, 2010. Protective effect of PEGylation against poly(amidoamine) dendrimer-induced hemolysis of human red blood cells. *J Biomed Mater Res B Appl Biomater* 93: 59-64.

Yeung DKY, Leung SWS, Xu YC, Vanhoutte PM, Man RYK, 2006. Puerarin, an isoflavonoid derived from *Radix Puerariae*, potentiates endothelium-independent relaxation via the cyclic AMP pathway in porcine coronary artery. *Eur J Pharmacol* 552: 105-111.

Yue PF, Yuan HL, Zhu WF, Cong LB, Xie H, Liu ZG, Wang LJ, Xiao XH, 2008. The study to reduce the hemolysis side effect of puerarin by a submicron emulsion delivery system. *Biol Pharm Bull* 31: 45-50.

Zhu SJ, Hong MH, Tang GT, Qian LL, Lin JY, Jiang YY, Pei YY, 2010. Partly PEGylated polyamidoamine dendrimer for tumor-selective targeting of doxorubicin: The effects of PEGylation degree and drug conjugation style. *Biomaterials* 31: 1360-1371.

Zhu YZ, Wang B, Ma MX, 2003a. Exploration of pathogenic mechanism of acute hemolysis and renal failure induced by puerarin. *Chin J New Drugs Clin Rem* 22: 699-702.

Zhu YZ, Wang B, Ma MX, 2003b. Laboratory diagnosis and the possible mechanism of puerarin poisoning. *Beijing Med J* 25: 390-392.