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

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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

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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 cardiovascular 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...
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-CH₂COOH (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 °C 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 °C, 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 combination 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, ¹H-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 °C. 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.

\[
\text{Hemolysis (\%) } = \left( \frac{A_A - A_0}{A_{100} - A_0} \right) \times 100\%
\]

Where \( A_A \), \( A_0 \), 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 (\( P < 0.05 \)).

**Comparison on osmotic fragility of 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.

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

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

**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 dendrimer showed hemolytic toxicity of $(16.9 \pm 1.93)\%$. But PEGylation of the dendrimer was found to have decreased the hemolysis of RBCs significantly to $(3.4 \pm 0.33)\%$. 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


