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Title page

Title of manuscript

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Pharmacokinetics and Hemorheology of Phosphocreatine and Creatine in Rabbits: A Directly Comparative Study between Parent Drug and Active Metabolite

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Abstract  
This study is to investigate pharmacokinetics (PK) and hemorheology (HR) of exogenous phosphocreatine (PCr), a cardio-protective agent, and its active metabolite creatine (Cr), with particular focus on the PK and PD comparison between PCr and Cr. A specific ion-pair reversed-phase HPLC-UV assay was used to simultaneously measure PCr, Cr and ATP concentrations in plasma and red blood cells (RBC) samples of rabbits. PK and HR parameters were calculated based on concentration-time (C-T) curves and effect-time (E-T) curves, respectively, obtained after i.v. dosing. Meanwhile the apparent pharmacological activity ratio (R_{app}) and real pharmacological activity ratio (R_{real}) of Cr to PCr were calculated. The PCr disappeared from plasma rapidly and in a biphasic manner; plasma PCr was converted to Cr fast and largely with the elimination rate limited metabolite disposition in vivo (K_m<K). The i.v. administration of PCr led to a markedly elevated and long-lasting ATP level in RBC. After i.v. administration of preformed Cr, plasma Cr displayed similar elimination kinetics behaviors to that of Cr generated metabolically after i.v. PCr. The Cr could also raise ATP level in RBC, but to less extent than PCr. Approximately 43 % of PCr-derived ATP came from Cr-derived ATP in RBC. PCr could significantly reduce whole blood viscosity and RBC osmotic fragility and Cr could do so, but weakly with estimated R_{app} of 0.53~0.68 and R_{real} of 0.38~0.48. PCr also inhibited platelet aggregation significantly, as opposed to Cr. The PCr-caused
improvement of HR is related to the rise in ATP level in RBC. Cr is likely to partially mediate HR effect of PCr.

Keywords: Phosphocreatine; creatine; pharmacokinetics; hemorheology; HPLC; rabbit

1. Introduction

Phosphocreatine (PCr), also known as creatine phosphate, is a phosphorylated creatine molecule naturally occurring in the body as one of high energy phosphates. It serves as an energy shuttle from subcellular site of energy production to sites of energy consumption (Gabr RE, et al., 2011) and as an energy buffer used for resynthesis of ATP via creatine kinase-mediated Lohmann reaction (Guzun R, et al., 2011), and therefore plays vital role in maintaining cellular energy homeostasis. Exogenous PCr has recently gained considerable attention as an efficacious and safe cardioprotectant, which is widely used in cardiac surgery as one of the components of cardioplegic solutions for protection of the heart against intraoperational injury and in acute myocardial ischemic conditions for improving the hemodynamic response and clinical conditions of patients with heart failure (Korge P, et al., 1998; Mastroroberto P, 1992; Ruda MY, 1988; Saks, VA, et al., 1993; Sharov VG, et al., 1986; Strumia E, 2012), as stated in Martindale: The Extra Pharmacopoeia (The Royal Pharmaceutical Society. 1996; Sweetman SC. 2009), and has been extensively reviewed by several scholars of note (Saks VA, et al., 1993; Strumia E, et al., 2012; Belstrino M, et al., 2016; Sun Z, 2016; Han GZ, et al., 2018). More recently, PCr has also been given a great deal of attention for its possible neuroprotective functions (Belstrino M, et al., 2002), and thus shows a very attractive broad R&D prospect.

Besides, most interestingly, creatine as metabolic product of PCr has increasingly been shown also to have extensive pharmacological activities and is under development as one of ergogens that may provide a useful therapeutic strategy to patients with neurological diseases (Beal MF, 2011; Guzun R, et al., 2011; Perasso L, et al., 2011). Although pharmacological effects of PCr and Cr have been extensively investigated, there are few studies on their pharmacokinetics (PK) (Sharov VG, et al., 1986; Korge P, et al., 1998; Lorenzi E, et al., 1987). A detailed studies of their PK are needed for their further development and rational application as well as clarification
of action mechanisms. On the other hand, the fact that PCr is a high-energy phosphate as above stated prompted us to think that PCr is most likely to elevate ATP level in red blood cell (RBC), and thus to improve hemorheology (HR). However, nothing is known about HR of PCr and Cr. The investigation of PCr effects on HR is of importance for elucidation of PCr cardioprotection. In recent years, a growing body of attention has been paid to the metabolic products of drugs in both PK and PD studies. Unfortunately, to date, no information is available regarding the direct comparison in PK and PD of PCr and Cr; so, a comparative study between parent drug and metabolites of PCr becomes desired.

Respecting the above facts, this study is designed to comparatively study: 1) PK of parent drug and main metabolite after intravenous (i.v.) administration of PCr to rabbits with particular focus on the metabolite disposition in plasma and RBC; 2) PK of Cr after i.v. administration of preformed Cr to rabbits; 3) influence of i.v. PCr and Cr on the ATP level; 4) HR effects of PCr and preformed Cr at i.v. equimolar doses to rabbits in order to provide supporting evidence for PCr cardioprotection and to clarify its possible action mechanisms.

2. Materials and methods
2.1. Animal Experiments
2.1.1. Animals
Healthy New Zealand white male rabbits weighing 2.0–2.5 kg, supplied by the Animal Center of Dalian Medical University, were used. All animals were individually housed in a single rabbit cage each and in a temperature- and humidity-controlled animal room with a 12 h:12 h light/dark cycle, and received standard chow and tap water ad libitum prior to experiments. The animal welfare and experimental procedures were strictly in accordance with the institutional ethic guidelines for the care and use of laboratory animals and related regulations.

2.1.2 PK experiments
Twenty rabbits were randomly divided into two groups with 10 rabbits each for PCr PK experiment and Cr PK experiment. Each group was sub-divided into high dose sub-group and low dose sub-group (n=5, each). The PCr group was given i.v. PCr at 500 mg/kg and 250 mg/kg; the Cr group was given i.v. PCr-equimolar chemically synthetic Cr at 228 mg/kg and 114 mg/kg, respectively, via ear marginal vein.
0.4 mL aliquots of rabbit blood were collected from the ear marginal vein into heparinized centrifuge tubes pre-dose and at 2, 6, 10, 20, 30, 40, 60, 90, 120, 150, 180, 210, 240 and 360 min post dose. Then, plasma and RBC samples were prepared by previously described procedures (Lv L, et al., 2013; Muravyov AV, et al., 2009) for HPLC analysis.

2.1.3. HR experiment

Thirty rabbits were randomly divided into PCr-treated group (n=10, sub-grouped and dosed like PK experiment), Cr-treated group (n=10, sub-grouped and dosed like PK experiment), saline-treated group (n=5, dosed at equal volume of physiological saline as negative control) and pentoxifylline(PX)-treated group (n=5, dosed i.v. PX 50 mg/kg as positive control).

Then, an aliquot (4 mL) of blood was taken by cardiac puncture predose and at 2, 10, 30, 60, 120, and 180 min postdose, with 3 mL blood being anticoagulated by heparin (final concentration 25 IU/mL) for viscosity determination, and 1 mL blood anticoagulated by sodium citrate (3.8 % sodium citrate/blood=1:9, v/v) for antiplatelet determination. The 1 mL of the above heparinized blood was subjected to whole blood viscosity (ηb) determination, the remaining 2 mL heparinized blood was centrifuged at 3000 r/min×10 min, Plasma was separated for the determination of plasma viscosity (ηp); the pellet was washed 3 times with saline to yield the RBC suspension for the determination of the RBC osmotic fragility.

2.2. Chemicals and reagents

Test drug PCr, formulated as creatine phosphate disodium salt tetrahydrate (C4H8N3O5PNa2.4H2O, MW 327.15) with purity>98 % (by HPLC) and as sterile powder for injection, was a generous gift from Bolai Pharm (Harbin, China) and the test preformed Cr, formulated as creatine monohydrate (C4H9N3O2.H2O, MW 149.15) with purity 98 % (by HPLC), was from Bailinwei Chemicals (Shanghai, China). PX injection (100 mg/2 mL) was obtained from Shenyang 1st Pharmaceutical Factory (Shenyang, China). Reference standards of PCr, Cr and ATP (>99 % purity, respectively) were purchased from Merck (Germany and USA, respectively), trimethoprim (TMP) as an internal standard (IS) (99 % purity) came from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China); MeOH (Tedia, USA) was of HPLC grade, Tetrabutyl ammonium hydrogen sulphate (TBA) used as ion-pairing reagent (JK Co., Ltd, USA) and other chemicals including...
KH₂PO₄, NaOH, K₂CO₃, heparin, sodium citrate and perchloric acid (PCA) were all of analytical grade; The bidistilled water was used for preparation of mobile phase and solutions.

2.3. Determination of drug concentrations

The PCr, Cr, and ATP in plasma or RBC samples were simultaneously measured by a newly developed and published ion-pair reversed phase HPLC assay (IP-RPHPLC) (Lv L, et al., 2013). Briefly, the samples were deproteinized with ice-cold 6 % PCA, followed immediately by neutralization with pre-cold 2 mol/L K₂CO₃ to pH 7.0. Then, a suitable volume (20 µL) of supernatant following centrifugation at 4 °C was injected into chromatograph. The chromatographical separation of analytes was achieved using an Agilent 1100 Series LC System on a Chromasil C18 column (250 mm×4.6 mm, 5 µm) (Ilite, Dalian, China) with a gradient mobile phase composed of TBA-containing KH₂PO₄ buffer and MeOH, at a flow rate of 0.8~1.3 mL·min⁻¹; the detection wavelength was set at 210 nm for PCr and Cr as well as 260 nm for TMP (IS) and ATP. For quantification, the peak area ratio-based internal standard (IS) method was used with TMP serving as IS. A blank sample pre-dosing was initially run for baseline subtraction. This assay showed high specificity with good resolution between analytes and no interference from matrix. The validation of methodology for PCr, Cr and ATP was carried out by 6 replicate analyses of QC high, medium and low concentrations. The intra- and inter-day precisions were determined to be <9 % and the accuracies between 98 %~105 % in plasma and RBC. The extraction recovery was found to be 92 %~98 % and the lower limit of quantification was 10 µg/mL plasma and 5 µg/mL RBC for PCr, Cr and ATP. The analytes proved to be stable after blood withdrawal and immediate storage at -20 °C, as detailed in section Discussion.

2.4. PK analysis

Plasma and RBC concentrations obtained from conventional full PK study underwent PK analysis using the commercially available software 3P97 version1.1 (China Math Pharmacology Society, Beijing, China). Concentration(C)-time(T) data were fitted on the basis of compartmental technique for parent drug PCr and preformed Cr and non-compartmental technique for PCr-derived metabolite Cr. In this program, the AUC (area under C-T curve) and AUMC (area under the first moment curve) of the compounds of interest were calculated using the trapezoidal method and extrapolated to infinity using terminal rate constant (λ) estimated from the slope of terminal phase
ln-linear regression. The observed PK parameters were the $C_{\text{max}}$ (maximum drug concentration) and $t_{\text{max}}$ (the time to reach $C_{\text{max}}$). The derived PK parameters were $t_{1/2}$ (elimination half life) = $\ln(2)/\lambda$; the CL (total body clearance) = dose$_{iv}$/AUC; the $V_d$ (apparent volume of distribution) = CL/$\lambda$; the MRT (mean residence time) = AUMC/AUC; the $t_{1/2}k_{\text{fam}}$ (formation half-life of metabolite) being calculated in the same way as absorption half-life of oral drug; the $f_m$(%) (formation fraction of metabolite Cr derived from PCr) = AUC$_{(m)}$/AUC’$_{(m)}$$\times$100, where AUC$_{(m)}$ and AUC’$_{(m)}$ were AUC of Cr produced after i.v. equimolar doses of PCr and chemically synthesized Cr, respectively; the $f_{\text{ATP,Cr/PCr}}$ (%)(extent of Cr- made contribution to PCr- caused rise in ATP level in RBC) = (AUC$_{\text{ATP,Cr}}$/AUC$_{\text{ATP,PCr}}$)$\times$ $f_m$$\times$100, where AUC$_{\text{ATP,Cr}}$ and AUC$_{\text{ATP,PCr}}$ were AUC of related ATP in RBC after i.v. equimolar doses of Cr and PCr, respectively.

2.5. Determination of HR parameters

2.5.1. Viscosity

Heparinized blood and plasma (1 mL samples) were subjected to rotational viscometric determination of the $\eta_b$ at low shear rate (20 s$^{-1}$) and high shear rate (150 s$^{-1}$) and $\eta_p$, respectively, by means of CNY-N6 HR Instrument (Pulisheng Instrument Center, Beijing, China).

2.5.2. Platelet aggregation rate

The 1 mL of citrated blood was centrifuged at differentiated velocity to isolate platelet–rich plasma (PRP) and platelet–poor plasma (PPP) by routine procedures (Han GZ, et al., 1997). The PRP was adjusted with PPP to give platelet count of $3\times10^8$/mL, and then was measured by turbidimetric method of Dr. Born (Born GVR, 1962) for its maximal platelet aggregation rate (PAR) at 5 min induced by ADP (final concentration 3 μmol/L) by means of LNY-N12 Four Channel Blood Aggregameter (Pulisheng Instrument Center, Beijing, China).

2.5.3. RBC osmotic fragility

The RBC suspension was prepared from heparinized blood by procedures of Dr Muravyov, et al. (Muravyov A, et al., 2009). Subsequently, the method reported by Dr. Luo, et al was followed (Luo C, et al., 2009) with minor modification. Briefly, an aliquot of 30 μL of the RBC suspension was added to 8 mL of 0.90 % or 0.45 % NaCl aqueous solution, or distilled water. Following gentle mixing and centrifugation at 3000 RPM for 10 minutes, the supernatant was subjected to absorbance measurement.
with UV2100 model spectrophotometer (Younike Instrument Co., Ltd, Shanghai, China) at 545 nm. The absorbance of the 0.9 % NaCl-treated RBC sample was taken as zero absorbance. RBC osmotic fragility was measured by assessing the Hemolysis rate (HLR) as follows:

$$\text{HLR} \, (\%) = \frac{A_{0.45 \% \text{NaCl}}}{A_{\text{water}}} \times 100 \quad \text{………………………… (1)}$$

where $A_{0.45 \% \text{NaCl}}$ is absorbance measured for 0.45 % NaCl solution-treated RBC, and $A_{\text{water}}$ for water-treated RBC.

2.6. HR data processing

The percent inhibitory rate (I %) of $\eta_b$, $\eta_p$, PAR and HLR served to express HR effect intensity (E) and was estimated from:

$$I \, (\%) = \frac{(V_{\text{pre}} - V_{\text{post}})}{V_{\text{pre}}} \times 100 \quad \text{………………………… (2)}$$

where $V_{\text{pre}}$ and $V_{\text{post}}$ were the measured value predose and postdose, respectively. The maximum effect intensity ($E_{\text{max}}$) and corresponding time ($t_{\text{max,E}}$) were determined visually from E-T profile as actual value measured. The action duration is the lasting time of the effect with statistical significance. From $E_{\text{max}}$ and area under E-T curve (AUC$_E$) measured by trapezoidal rule, the $E_{\text{max}}$– and AUC$_E$– based apparent activity ratio ($R_{\text{app}, \text{Emax}}$ and $R_{\text{app}, \text{AUC}_E}$) and real activity ratio ($R_{\text{real}, \text{Emax}}$ and $R_{\text{real}, \text{AUC}_E}$) of Cr to PCr could be found, respectively, according to:

$$R_{\text{app}, \text{Emax}} = \frac{E_{\text{max,Cr}}}{E_{\text{max,PCr}}} \quad \text{………………………… (3)}$$

$$R_{\text{real}, \text{Emax}} = R_{\text{app}, \text{Emax}} \times f_m \quad \text{………………………… (4)}$$

$$R_{\text{app}, \text{AUC}_E} = \frac{\text{AUC}_{E,\text{Cr}}}{\text{AUC}_{E,\text{PCr}}} \quad \text{………………………… (5)}$$

$$R_{\text{real}, \text{AUC}_E} = R_{\text{app}, \text{AUC}_E} \times f_m \quad \text{………………………… (6)}$$

Where $E_{\text{max,Cr}}$ and $E_{\text{max,PCr}}$ are the $E_{\text{max}}$ obtained after i.v. equimolar doses of Cr and PCr, respectively; $\text{AUC}_{E,\text{Cr}}$ and $\text{AUC}_{E,\text{PCr}}$ are the $\text{AUC}_E$ obtained after i.v. equimolar doses of Cr and PCr, respectively; $R_{\text{real}}$ was the $R_{\text{app}}$ corrected by $f_m$.

2.7. Statistical analysis

Data were presented as arithmetic mean ± standard deviation (SD). The mean values of data between groups were compared using one-way ANOVA. All statistical analysis were performed with SPSS software package for windows (version 11.5, SPSS Japan Inc., Tokyo, Japan). The statistical significance was defined as $p<0.05$.

3. Results

3.1. Plasma PK following i.v. administration of PCr
As seen from Fig.1-A, after i.v. administration of PCr at high and low doses, PCr level in plasma were declined in a bi-exponential fashion with a time inflexion point at 40 min, thus, the plasma C-T curve could be best fitted to the two-compartment model. This modeling was further confirmed using the statistic fitting algorithm such as $S_w$ (sum of weighted residual squared, $W_i=1/C^2$), AIC (Akaike’s information criterion) and $R^2$ (goodness of fit) (Gibaldi M, et al., 1982). The $S_w$ and AIC calculated based on the two-compartment model were all smaller than those based on the one- or three-compartment model, but $R^2$ was larger; so, the two-compartment model was chosen.

As summarized in table1, the $t_{1/2\beta}$ was as short as 27 min, indicating that PCr eliminated from body very rapidly. The $V_d$ was 697~737 mL/kg, which is much larger than the rabbit plasma volume of 38.8 mL/kg (Liu CX, et al., 1999), indicating a widespread distribution of PCr in rabbits. The measured plasma levels and AUCs showed clear dose-proportionality and with increasing dose the $t_{1/2}$, CL and $V_d$ did not change significantly ($p>0.05$), implying that PCr followed linear kinetics at doses studied.

It was apparent from Fig.1-A and table1 that the PCr-derived metabolite Cr appeared in plasma as quickly as 2 min after i.v. administration of parent drug PCr, and had $t_{1/2ka(m)}$ of only 3~4 min. The metabolite showed an initial progressive ascending and subsequent slow descending C-T curve with the terminal phase being not parallel to that of PCr. The generated Cr peaked soon ($t_{max}$ 30 min) and had peak concentration ($C_{max}$) of 354 μg·mL$^{-1}$ and 162 μg·mL$^{-1}$ at high and low dose of PCr, respectively. The clear dose-proportionality of plasma level and AUC was also observed. The metabolite half life ($t_{1/2(m)}$) was 45.36~48.54 min vs PCr $t_{1/2\beta}$ of 26.7~27.5 min ($p<0.05$). This larger $t_{1/2(m)}$ gained support from i.v preformed Cr experiment ($t_{1/2(Cr)}$ 57~58 min) (table2). Besides, no ATP could be detected in plasma.

3.2. Plasma PK following i.v. administration of preformed Cr

Fig.1-B depicted time course of Cr in plasma of rabbits receiving i.v. administration of chemically synthetic Cr. As done for PCr, the C-T data of Cr was also submitted to a two-compartment model analysis assuming linear kinetics. Interestingly, the PK parameters of Cr obtained by i.v. preformed Cr was shown to be consistent with those of Cr generated metabolically by i.v. PCr, as presented in table1 and table2. It is worthy of note that the Cr had $t_{1/2\beta}$ of 56~58 min, which was unequivocally larger than that of PCr. On the other hand, similar to PCr, Cr had $V_d$ much larger than
plasma volume of the rabbit, indicating, also for Cr, a widespread distribution in rabbits. From ratio of AUC to AUC', f_m was calculated as 70 %, meaning that much Cr was formed via bioconversion from PCr entering systemic circulation.

Figure 1. Plasma C-T curves after i.v. administration of PCr (A) and preformed Cr (B) at high and low doses to rabbits (n=5)

Animals were grouped and administered as described in Materials and Methods. The doses of 228 mg/kg and 114 mg/kg for Cr were equi-molar to the doses of 500 mg/kg and 250 mg/kg for PCr, respectively. Plasma drug concentration was measured by ion-pair HPLC-UV assay.

Table 1. Main PK parameters of PCr and Cr in plasma of rabbits receiving i.v. PCr (n=5, values are means and SD)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Analyte PCr</th>
<th>Parameters</th>
<th>Analyte Cr</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>i.v. PCr 500 mg/kg</td>
<td>i.v. PCr 250 mg/kg</td>
<td>i.v. Cr 228 mg/kg</td>
</tr>
<tr>
<td>AUC/ (µg/mL)·min</td>
<td>27806.0±1602.9</td>
<td>12992.3±361.1</td>
<td>43630.7±2159.9</td>
</tr>
<tr>
<td>CL/ L/kg/min</td>
<td>0.0181±0.0009</td>
<td>0.0207±0.0016</td>
<td>3.67±0.54</td>
</tr>
<tr>
<td>t_{1/2 α}/ min</td>
<td>7.08±0.72</td>
<td>7.05±0.75</td>
<td>48.54±3.95</td>
</tr>
<tr>
<td>t_{1/2 β}/ min</td>
<td>26.70±1.96</td>
<td>27.47±2.21</td>
<td>354.18±42.58</td>
</tr>
<tr>
<td>t_{max}/min</td>
<td>0.697±0.084</td>
<td>0.737±0.092</td>
<td>30</td>
</tr>
<tr>
<td>t_{1/2 kα(m)} /min</td>
<td>7.08±0.72</td>
<td>7.05±0.75</td>
<td>48.54±3.95</td>
</tr>
<tr>
<td>t_{1/2 kβ(m)} /min</td>
<td>26.70±1.96</td>
<td>27.47±2.21</td>
<td>354.18±42.58</td>
</tr>
<tr>
<td>V_d / L/kg</td>
<td>66.67±1.52</td>
<td>62.36±3.69</td>
<td>71.1</td>
</tr>
</tbody>
</table>

Note: AUC: area under C-T curve extrapolated to infinity; CL: total body clearance; t_{1/2 α}: distribution half life; t_{1/2 β}: elimination half life; V_d: apparent volume of distribution; C_{max}: maximal plasma drug concentration; t_{max}: time corresponding to C_{max}; t_{1/2 kα(m)}: elimination half life of metabolite; t_{1/2 kβ(m)}: formation half life of metabolite; MRT: mean residence time.

Table 2. Main PK parameters of Cr in plasma of rabbits receiving i.v. preformed Cr (n=5, values are means and SD)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Cr dose (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>228</td>
</tr>
<tr>
<td>AUC/ (µg/mL)·min</td>
<td>61326.8±1749.3</td>
</tr>
<tr>
<td>CL/ L/kg/min</td>
<td>0.0085±0.0004</td>
</tr>
</tbody>
</table>
### 3.3. RBC PK following i.v. administration of PCr

As shown in Fig.2-A, following i.v. dosing PCr to rabbits, Cr as a metabolite and related ATP were detectable in RBC samples very soon (2 min postdose), and showed bi-exponential C-T curves reflecting Cr and ATP formation and elimination in RBC; however, PCr was undetectable in RBC. As presented in table3, the Cr and ATP levels peaked at 120 min and 60 min, respectively, the t\(\text{max}\) and t\(\frac{1}{2}\text{ka(m)}\) being much longer than that of Cr as metabolite in plasma PK study, and were maintained at relatively high levels during a long period of time (240 min~300 min). The t\(\frac{1}{2}\text{km}\) of about 70 min for Cr indicated that Cr entering RBC eliminated slowly as compared with the Cr in plasma. Also, the \(C_{\text{max}}\) and AUC showed dose-proportionality.

### 3.4. RBC PK following i.v. administration of preformed Cr

As shown from Fig.2-B and table3, the Cr and related ATP levels in RBC after i.v. dosing synthetic Cr had \(t_{\text{max}}, t_{\frac{1}{2}\text{km}}, t_{\frac{1}{2}\text{ka(m)}}\) and MRT consistent with those in RBC after i.v. dosing PCr. When dose was doubled, the \(C_{\text{max}}\) and AUC were also doubled, while \(t_{\frac{1}{2}}\) was kept unchanged basically. By comparison between Fig.2-A and Fig.2-B, it was found that PCr-derived ATP level was larger than Cr-derived ATP. Based on \(\text{AUC}_{\text{ATP(Cr)}}\) and \(\text{AUC}_{\text{ATP(PCr)}}\) (table3) and \(f_{\text{m}}\) (table1), \(f_{\text{ATP, Cr/PCr}}\) was calculated as 42.9 % (high dose) and 43.6 % (low dose), meaning that about 43 % of the PCr-derived ATP came from Cr-derived ATP. Contrary with Cr and ATP, PCr was undetectable in RBC samples after i.v. dosing both PCr and Cr.

<table>
<thead>
<tr>
<th></th>
<th>(t_{\frac{1}{2}a} / \text{min})</th>
<th>(t_{\frac{1}{2}b} / \text{min})</th>
<th>(V_d / \text{L/kg})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>18.36±1.98</td>
<td>17.88±2.36</td>
<td>0.717±0.101</td>
</tr>
<tr>
<td></td>
<td>58.45±5.73</td>
<td>56.70±4.36</td>
<td>0.683±0.079</td>
</tr>
</tbody>
</table>

Note: AUC, CL, \(t_{\frac{1}{2}a}, t_{\frac{1}{2}b}\) and \(V_d\): same as in the table1.
Figure 2. RBC C-T curves of Cr and ATP after i.v. administration of PCr (A) and preformed Cr (B) to rabbits (n = 5)

Table 3. Main PK parameters of Cr and ATP in RBC of rabbits receiving i.v. PCr and Cr (n=5, values are means and SD)

<table>
<thead>
<tr>
<th>Drug</th>
<th>Dose /mg/kg</th>
<th>Analyte</th>
<th>AUC(µg/mL)-min</th>
<th>t½ Ka (m) /min</th>
<th>t½ k (m) /min</th>
<th>Cmax /µg/mL</th>
<th>tmax /min</th>
<th>MRT /min</th>
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<tbody>
<tr>
<td>PCr</td>
<td>500</td>
<td>ATP</td>
<td>25285.7±467.9</td>
<td>49.4±2.8</td>
<td>52.4±4.8</td>
<td>112.65±9.74</td>
<td>60</td>
<td>108</td>
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<td></td>
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<td>Cr</td>
<td>8519.6±337.9</td>
<td>68.4±3.9</td>
<td>71.5±6.8</td>
<td>36.51±1.62</td>
<td>120</td>
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<td></td>
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<td>ATP</td>
<td>11798.7±1432.7</td>
<td>47.9±3.6</td>
<td>50.7±4.9</td>
<td>63.21±7.68</td>
<td>60</td>
<td>112</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cr</td>
<td>3932.9±119.4</td>
<td>69.3±4.2</td>
<td>70.0±1.2</td>
<td>17.65±1.18</td>
<td>120</td>
<td>148</td>
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<tr>
<td>Cr</td>
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<td>ATP</td>
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<td>47.4±6.8</td>
<td>55.2±2.9</td>
<td>69.87±6.25</td>
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<td>118</td>
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<tr>
<td>Cr</td>
<td>114</td>
<td>ATP</td>
<td>7178.8±1136.8</td>
<td>49.9±3.4</td>
<td>53.3±4.2</td>
<td>34.96±2.46</td>
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<td>120</td>
</tr>
<tr>
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<td></td>
<td>ATP</td>
<td>5960.9±486.9</td>
<td>65.9±3.7</td>
<td>69.6±5.7</td>
<td>25.12±1.36</td>
<td>120</td>
<td>132</td>
</tr>
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Note: AUC, t½ Ka(m), t½ k(m), Cmax and MRT: same as in table 1.

3.5 Effects of i.v. PCr and i.v. preformed Cr on whole blood viscosity and plasma viscosity

From Fig. 3, it was shown that i.v. administration of PCr resulted in a significant reduction in $\eta_{b,150s-1}$ and $\eta_{b,20s-1}$, with the $t_{max,E}$ of 30 min and $E_{max}$ being reduced by 39.0 % and 27.5 % for $\eta_{b,150s-1}$ ($p<0.01$) and by 39.1 % and 27.9 % ($p<0.01$) for $\eta_{b,20s-1}$ at high and low doses, respectively, as compared with pre-dosing corresponding values measured. PX as a positive control at 50 mg/kg also markedly reduced $\eta_{b}$, but with $t_{max,E}$ of 10 min, and $E_{max}$ being reduced by 26 % for $\eta_{b,150s-1}$ and 20 % for $\eta_{b,20s-1}$ only. The reduction in $\eta_{b}$ started at 2 min postdose and lasted for as long as 120 min.

However, after Cr was i.v. administered at doses equimolar to PCr, the reduction in $\eta_{b}$, although it was statistically significant ($p<0.01$ vs predose), was less than that induced by PCr ($p<0.01$), reflected by $E_{max,cr}$ of 25.3 % and 23.1 % for $\eta_{b,150s-1}$ and 18.3 % and 18.0 % for $\eta_{b,20s-1}$ ($p<0.01$) at high and low doses, respectively, and of shorter duration (60 min).

As opposed to $\eta_{b}$, no significant reduction in $\eta_{p}$ was evident following i.v. administration of both PCr and Cr. The $\eta_{b}$ and $\eta_{p}$ in saline-treated group as negative control did not change significantly with multiple collection of blood samples by cardiac puncture.
Figure 3. The whole blood viscosity and plasma viscosity after i.v. administration of PCr and Cr to rabbits (n=5, the bars of plots A, A', B, B', C, C' represent means and SD)

A, A': $\eta_{b\cdot20s\cdot1}$ after i.v. PCr, Cr, respectively. B, B': $\eta_{b\cdot150s\cdot1}$ after i.v. PCr, Cr, respectively. C, C': $\eta_{p}$ after i.v. PCr, Cr, respectively. A'', B'' and C'': % inhibition-time curves corresponding to A, A'; B, B' and C, C' respectively. $\eta_{b}$ and $\eta_{p}$: whole blood viscosity and plasma viscosity, respectively.

*p<0.05, **p<0.01 and *p>0.05 vs pre-dose.
For clarity, the data in saline treated group was not presented; the data in PX treated group was presented only at pre-dose and $t_{\text{max,E}}$ (10 min postdose).

3.6 Effects of i.v. PCr and i.v. preformed Cr on platelet aggregation
As shown in Fig. 4, i.v. administration of PCr led to a significantly reduced ADP-induced platelet aggregation rate vs pedose. The $t_{\text{max}}$ was 30 min and $E_{\text{max}}$ reached 43% and 31% ($p<0.01$) at high and low doses, respectively, compared with $E_{\text{max}}$ of only 16.9% ($p<0.05$) for PX 50 mg/kg. However, after i.v. equimolar doses of Cr, no significantly reduced platelet aggregation was observed. Besides, a long-lasting (120 min) antiplatelet effect of PCr was also evident.
Figure 4. The platelet aggregation after i.v. administration of PCr (A) and Cr (A') to rabbits (n=5, the bars of plots A and A' represent means and SD) and corresponding % inhibition-time curves (A''). Platelet aggregation rate (PAR), which was measured by turbidimetric method of Dr. Born, with ADP (3 µmol/L) as inducer of aggregation. *p<0.05, **p<0.01 and ▲p>0.05 vs pre-dose.

3.7 Effects of i.v. PCr and i.v. preformed Cr on RBC fragility
The RBC hemolysis rate, as a measure of RBC fragility, and its inhibition by i.v. PCr and Cr were presented in Fig.5. The $E_{\text{max, HLR}}$, expressed as the % maximal inhibition against low osmotic saline–induced RBC hemolysis, was found 32.5 % and 23.4 % (p<0.05) with action duration of 120 min and 60 min for PCr at high and low doses, respectively, compared with the $E_{\text{max, HLR}}$ of only 22.7 % and 13.5 % (p<0.05) for Cr at corresponding equimolar doses. In contrast, no significant inhibitory effect was found for i.v. PX ($E_{\text{max, HLR}}$ of 7.1 %, p>0.05). PCr and Cr showed same $t_{\text{max, E}}$ of 30 min but different action duration (60~120 min for PCr vs 30~60 min for Cr).

Figure 5. The RBC hemolysis after i.v. administration of PCr (A) and Cr (A') to rabbits (n=5, the bars of plots A and A' represent means and SD) and corresponding % inhibition-time curves (A''). RBC hemolysis rate (HLR) was calculated using equation 1. *p<0.05, **p<0.01 and ▲p>0.05 vs pre-dose.

3.8 The HR activity ratio of Cr to PCr
In accordance with equation 2~5, the $R_{\text{app}}$ and $R_{\text{real}}$ of HR were found (table4). The Cr- and PCr- induced reduction in $\eta_b$ and RBC, HLR showed $R_{\text{app}}$ of 0.59~0.68 and $R_{\text{real}}$ of 0.38~0.48 at high dose, and of 0.53~0.65 and 0.38~0.46 at low dose. This
indicated that HR effect of metabolite Cr could account for 38 %~48 % of that of parent drug PCr, namely, about 40 % or more of HR effects caused by PCr came from those caused by Cr. It was also found that the $R_{\text{app}}$ and $R_{\text{real}}$ calculated based on $E_{\text{max}}$ gave almost the same results as those based on $\text{AUC}(E)$. The Cr had no effects on $\eta_p$ and PAR.

### Table 4. The HR activity ratio of Cr to PCr

<table>
<thead>
<tr>
<th>Parameter</th>
<th>$R_{\text{app}}$</th>
<th>$R_{\text{real}}$</th>
<th>$R_{\text{app,AUC}(E)}$</th>
<th>$R_{\text{real,AUC}(E)}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\eta_{b,150s-1}$</td>
<td>0.59:1 0.64:1 0.68:1 0.45:1 0.68:1 0.61:1 0.48:1 0.44:1</td>
<td>0.63:1 0.65:1 0.46:1 0.53:1 0.57:1 0.38:1 0.40:1</td>
<td>0.63:1 0.65:1 0.46:1 0.53:1 0.57:1 0.38:1 0.40:1</td>
<td>0.63:1 0.65:1 0.46:1 0.53:1 0.57:1 0.38:1 0.40:1</td>
</tr>
<tr>
<td>$\eta_{b,20s-1}$</td>
<td>0:0</td>
<td>0:0</td>
<td>0:0</td>
<td>0:0</td>
</tr>
<tr>
<td>PAR</td>
<td>0:1</td>
<td>0:1</td>
<td>0:1</td>
<td>0:1</td>
</tr>
<tr>
<td>HLR</td>
<td>0.65:1 0.65:1 0.46:1 0.54:1 0.53:1 0.38:1 0.38:1</td>
<td>0.65:1 0.65:1 0.46:1 0.54:1 0.53:1 0.38:1 0.38:1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a high dose, *ΔΔ* low dose. $\eta_{b,150s-1}$ and $\eta_{b,20s-1}$: whole blood viscosity at high and low shear rate. PAR: platelet aggregation rate. HLR: hemolysis rate of RBC. $R_{\text{app},E_{\text{max}}}$ and $R_{\text{real},E_{\text{max}}}$: apparent activity ratio and real activity ratio calculated based on $E_{\text{max}}$ using equation 3 and 4, respectively. $R_{\text{app,AUC}(E)}$ and $R_{\text{real,AUC}(E)}$: apparent activity ratio and real activity ratio calculated based on $\text{AUC}(E)$ using equation 5 and 6, respectively.

### 4. Discussion

The present study investigated PK properties and HR effects of PCr and Cr, on a comparative basis, which provide a powerful backing for cardioprotective effects of PCr. Furthermore, to the best of our knowledge, this investigation is the first published study to offer direct comparative data on PK and PD between parent drug PCr and metabolite Cr. For the direct comparison, the preformed Cr is used.

PCr is a highly polar molecule which is not easily extractable from biological samples. Organic solvents are apparently not suited to extract it from plasma and RBC matrix. Thus, acid precipitation is the method chosen. In order to avoid the instability of PCr in strong acid condition, all extraction procedures were conducted under low temperature including an ice-cold 6 % PCA as an acidic protein-precipitant and pre-cold 2 mol/L $\text{K}_2\text{CO}_3$ solution as an alkaliifying reagent as well as centrifugation at 4 °C. Besides, experiment processing was quickly carried out, and deproteinization was followed immediately by neutralization to pH 7.0 with $\text{K}_2\text{CO}_3$. In this way PCr becomes acid – endurable and stable, as evidenced by extraction recovery of >93 % in both plasma and RBC. Also, after above handling of PCr-containing samples no Cr was found based on the chromatogram, indicating the absence of chemical
degradation. In fact, this kind of acid-precipitation method has long been used by many researchers to extract high-energy phosphate compounds from biological samples (Ally A, et al., 1992; Lorenzi E, et al., 1987; Teerlink T, et al., 1993; Han GZ, et al., 2011; Xu L, et al., 2014). Long term stability test also demonstrated that the QC samples stored at -20 °C was stable for at least 1 month, reflected by a recovery of >97 % in plasma and RBC. In contrast, those at room temperature for 2-h was instable with a recovery of <87 %, implying the presence of degradation of PCr in samples during room temperature storage; thus, in present study the anticoagulant samples collected from vein were prescribed to be immediately frozen and used within 2 weeks for further experiment.

Following *i.v.* PCr, significantly elevated Cr level was detected in RBC. The possible explanation is that plasma Cr derived from *i.v.* PCr entered RBC mainly via a specific Cr transporter (CrT)-mediated active transport mechanism or even via a passive diffusion mechanism, if any. The hypothesis that the elevated Cr in RBC may at least in part be released from PCr within RBC could be probably excluded by the following reasons: 1) PCr is very difficult to enter RBC via passive diffusion due to its high polarity and also unable to enter RBC via a carrier-mediated transport mechanism because of absence of specific PCr transporter on membrane of RBC; 2) If the PCr could enter RBC, it still cannot release Cr via a creatine kinase (CK) - catalyzed Lohmann reaction because RBC is devoid of mitochondria and CK activity (Wyss M, et al., 2000) and 3) If the PCr could enter RBC and release Cr, this implies that the Cr in RBC comes from two different routes and mechanisms, i.e., release by PCr within RBC and transport of plasma Cr. In this case, the C-T curve of Cr in RBC would have probably displayed a double-peak. The single peak C-T curve observed supports, differently, CrT-mediated active transport or Cr passive diffusion only.

After *i.v.* administration, PCr and Cr showed similar PK characteristics. Bi-exponential models well fitted their C-T profiles, and the compounds showed fast elimination and linear kinetics. But, as compared with PCr, Cr has longer elimination half life, i.e., the $K_m<K$. This indicates that the metabolite kinetics is characterized by elimination rate limitation (ERL) instead of formation rate limitation (FRL), meaning that Cr transformed from PCr eliminates depending on its own elimination rate and independently of degradation of parent drug PCr. This ERL metabolite disposition is
directly validated by assessing the disposition of both parent drug and preformed metabolite after administration of not only PCr but also its metabolite Cr. Our study has also demonstrated that after *i.v.* administration to rabbits PCr-derived ATP level is larger than Cr-derived ATP level, which are in line with recent studies of Zou LL and Xu L, who found similar results in mice and rats (Xu L, et al., 2014; Zou LL, et al., 2011). The difference between PCr and Cr is probably explained by the dual action mechanisms exerted on ATP by PCr (unchanged PCr itself plus Cr as active metabolite), as opposed to single action mechanism only for Cr. Radically, this difference comes from difference in molecular structure: PCr, but not Cr, is an energy-riched compound. Specifically, the former as a high energy phosphoryl group donor can phosphorylate ADP to ATP, but Cr, a compound free from high energy phosphoryl group, is unable to do so. It seems reasonable to suppose that the unchanged PCr itself combined with metabolite Cr may constitute a so-called PCr/Cr system (Balestrino M, et al., 2016), and thus exert synergistic action on RBC energy metabolism and beneficial HR-improving effects.

The influence of PCr on cellular energy metabolism is considerably complicated, especially on mitochondria- and CK-free RBC. Despite to date many studies and reviews dealing with action mechanisms of PCr on energy metabolism of cardiomyocytes, little is known about those of RBC. The present study has only experimentally demonstrated rise of RBC ATP after *i.v.* PCr, but we still cannot exactly answer the reasons why PCr can do so. Much more work needs to be done.

On the other hand, the stability test during sample handling showed that PCr is stable in RBC matrix, as evidenced by extraction recovery of 95 %~99 % (Lv L, et al., 2013). It is believed that the Cr appearing after *i.v.* PCr cannot be derived from degradation of PCr during handling. As far as the HR characteristics is concerned, both PCr and Cr reduce \( \eta_b \) and RBC fragility and exhibit no effect on \( \eta_p \). After *i.v.* Cr, the reduction in \( \eta_b \) was less than that induced by PCr, evidenced by Cr to PCr \( R_{app} \) of 0.5~0.7:1 and \( R_{real} \) of 0.4~0.5:1. This is because \( \eta_b \) relies on the RBC aggregability (RBCA), which is strongly influenced by negative charge on RBC membrane surface (Fernandes HP, et al., 2011; Muravyov A, et al., 2009), and RBC deformability (RBCD), which is closely related to energy status of RBC (Saks VA, 1993). Since PCr, a zwitterionic molecule (Saks VA, 1993), may, we suppose, significantly increase negative charge on the RBC membrane surface via PCr/phospholipid interaction,
whereby increase repulsion between cells and, consequently, reduce the RBCA; on the other hand, PCr elevates ATP level of RBC and thus improves its energy status including the improved ATPase (e.g. Ca\(^{2+}\) pump) activity, whereby avoiding intracellular Ca\(^{2+}\) overload and the resulting membrane stiffening and also Gardos effect (i.e., the increased intracellular viscosity induced by Ca\(^{2+}\)-activated K\(^+\) efflux) and, hence enhancing topology and plasticity of RBC (Saks VA, 1993; Tokarska-Schlattner M, et al., 2012; Kaiserova K, et al., 2002). In this way, PCr heightens RBC deformability. However, Cr, relative to PCr, is a less negatively charged molecule and induces a less significantly elevated ATP level, and, therefore, reduces RBCA and increases RBCD to a smaller extent than PCr. So, this is not surprising that i.v. Cr vs i.v. equimolar PCr leads to a less significant reduction in \(\eta_b\).

It has been also noted that in relation to Cr, PCr has more marked RBC fragility-lowering effect, manifested by significantly reduced hemolysis due to low osmotic saline (0.42 % NaCl solution). This significant resistance to hypoosmotic stress can be explained by the membrane stabilizing effect of PCr, which, as above-mentioned, is a zwitterionic molecule able to interact with opposite charges of phospholipid polar heads in the membrane (Strumia E, et al., 2012; Tokarska-Schlattner M, et al., 2012), whereas Cr is a non-zwitterionic molecule unable to produce the above interaction and causes less membrane-stabilizing effect and, consequently, produces the less HR-improving effect than its parent drug PCr. Besides, our experiment results are supported by studies of Dr. Tokarska-Schlattner M, et al., who found using in vitro RBC and large unilamellar vesicle models that PCr, but much less so Cr, could protect RBC from permeabilization induced by melittin, doxorubicin, hypoosmotic stress or saponin (Tokarska-Schlattner M, et al., 2012).

The \(f_m\) approaching approximately 70 % indicates that most of PCr in plasma is converted to Cr, the latter has been shown by numerous previous studies and the present study to be pharmacologically active (Beal MF, 2011; Belstrino M, et al., 2002; Guzun R, et al., 2011; Perasso L, et al., 2011). It is reasonable to believe that PCr exerts its pharmacological effects following 3 ways: 1) via high energy-riched PCr intact molecule to re-synthesize ATP; 2) via active metabolite Cr; 3) via both PCr and Cr, which should occur in most of conditions. In the case of 2), PCr actually becomes a pro-drug or carrier for Cr; in the case of 3), PCr-induced pharmacological effects actually are synergistic effect of the parent drug PCr plus metabolite Cr.
In this study not only PCr and Cr PK in plasma but also Cr and ATP PK profiles in RBC have been studied. After *i.v.* dosing of PCr and Cr, the related ATP level in RBC has been significantly elevated. A possible explanation is that Cr entering RBC after *i.v.* dosing PCr and Cr may stimulate glycolysis in RBC, a cell free from oxidative phosphorylation energy-producing mechanism, which is supported by the fact that Cr has been demonstrated to promote glycolysis in the skeletal muscle, myocardium and brain (Beauloye C, et al., 2002; Gallo M, et al., 2008; Guzun R, et al., 2011; Oláh J, et al., 2008). However, a complicated situation is that the ATP level derived from *i.v.* PCr is much higher than that from *i.v.* equimolar dose of Cr, just as opposed to the Cr level derived from PCr being much lower than that from *i.v.* equimolar dose of Cr, while PCr is undetectable in RBC after *i.v.* dosing PCr (Fig.2 and table 3). A possible more complex mechanism might exist for PCr. Further exploration is warranted.

By calculation, \( f_{ATP, Cr/PCr} \) is around 43.25 % (42.9 %~43.6 %), meaning that about 43 % of PCr-derived ATP comes from Cr-derived ATP. Very interestingly, by calculation based on equation 4 and 6, Cr to PCr \( R_{real} \) is around 43.0 % (38 %~48 %), which is in good agreement with the above \( f_{ATP, Cr/PCr} \). This excellent coincidence undoubtedly indicates that the improved HR caused by PCr and Cr is related to the rise in ATP level in RBC.

In the present study, a significant platelet aggregation inhibitory effect of PCr, as opposed to Cr, has been noted. This should be attributed to double effects of PCr on platelet aggregation: 1) removal of ADP, a powerful platelet agonist, by PCr via Lohmann reaction (PCr+ADP → Cr+ATP) under catalysis of CK and 2) antagonism of aggregation by resultant ATP, a well-documented antagonist at the P2Y(12) receptor on membrane of platelet (Kauffenstein G, et al., 2004). However, Cr cannot do so due to the absence of high energy phosphoryl group in its molecule.

In this study a new approach on pharmacological activity ratio of metabolite to its parent drug using \( R_{app} \) and \( R_{real} \) has been put forth for the first time. Here \( R_{app} \) is defined as the pharmacological activity ratio of metabolite to its parent drug measured after respective administration of parent drug and preformed metabolite at equimolar doses, whereas \( R_{real} \) is defined as the pharmacological activity ratio of parent drug-derived metabolite to its parent drug measured after administration of parent drug or simply defined as \( R_{app} \) corrected by \( f_m \). Because parent drug converts to metabolite generally in a certain fraction but not wholly, thus, \( R_{app} \) must be multiplied by \( f_m \), the
metabolite formation fraction, to provide real activity ratio in the body, namely \( R_{\text{real}} \). The same reason is suitable for \( f_{\text{ATP}}, C_{\text{ATP}} \). It is noteworthy that the above new approach that we put forth is defined as the ratio of equimolar dosed effects and is different from the conventional approach for comparison of drugs i.e., potency ratio or relative potency, which is defined as the ratio of equieffective doses.

Again, in this study a negative control group dosed with saline and a positive control group dosed with PX, a frequently used HR-improving agent (Muravyov A, et al., 2009), were designed. The former shows that blood collection via cardiac puncture for 6 times did not substantially change HR parameters; the latter shows that the HR-improving effect of PCr is better than that of PX at doses studied, whereas Cr is not.

It is well known that PX, a non-selective phosphodiesterase (PDE) inhibitor able to elevate intracellular cAMP level, possesses multiple potent pharmacological activities, including reduced RBC and platelet aggregability, enhanced RBC deformability, etc., (Muravyov A, et al., 2009). As a result, PX is often used clinically to treat microcirculation dysfunction and abnormal HR-related disorders. Despite the fact that PX is a non-high energy phosphate compound and thus has different action mode from PCr and Cr, owing to its clear HR-improving effect, it has widely be used to serve a positive control drug in HR study. So, it was selected here as a positive control drug.

5. Conclusions

By direct comparison between PCr and Cr dosed on a equimolar basis, the present study has demonstrated that 1) plasma PK profiles of both \( i.v. \) PCr and \( i.v. \) preformed Cr can be described by two-compartment models and are characterized by fast elimination; 2) PCr entering systemic circulation is mostly converted to Cr with \( K_m < K \) and ERL metabolite disposition in vivo; 3) \( i.v. \) PCr induces significant rise in ATP level in RBC and improvement of HR to a greater extent as compared to \( i.v. \) preformed Cr; 4) this improvement in HR seems to be related to the elevated ATP level in RBC; 5) It is reasonable to believe that Cr as metabolite is likely to partially mediate PCr-caused HR improvement; 6) the improved HR is in favor of cardioprotection of PCr.

Acknowledgements
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**Conflict of interest**

The authors declare that they have no conflict of interest related to this review.

**References**


Graphical abstract

RBC: red blood cell
PLT: platelet
RBC−: negative change RBC
RBCA: RBC aggregability
RBCD: RBC deformability

PK analysis

C-T profile

PCr/Cr

PCR /cr
plasma

PCr/Cr

PK analysis

C-T profile

membrane stabilization

hyposmotic stress resistance

RBC fragility

RBC O

RBCD

RBCA

R_{app}: apparent pharmacological activity ratio
R_{real}: real pharmacological activity ratio
C-T: concentration-time
Cr_m*: metabolite Cr derived from PCr