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Pharmacokinetics and Pharmacodynamics of Recombinant Human EPO-Fc Fusion Protein *In Vivo*

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Abstract

In this study, the *in vivo* pharmacokinetics and pharmacodynamics of a novel recombinant human erythropoietin (rhEPO) Fc fusion protein, rhEPO-Fc, were studied in both rodents and rhesus monkeys. Animal models of anemia induced by irradiation, cyclophosphamide and partial renal ablation were used to evaluate therapeutic effects of rhEPO-Fc. We have demonstrated that serum half-life of rhEPO-Fc was 29.5 to 38.9 h at doses of 8, 25, 80 μg/kg in rhesus monkeys and 35.5 to 43.5 h at doses of 16, 50, 160 μg/kg in rats. In anemia animal models, rhEPO-Fc dose-dependently (7.5–30.0 μg/kg in mice, 5.4–21.4 μg/kg in rats and 5.0–10.0 μg/kg in rhesus monkeys) increased reticulocyte level, followed by an increase of RBC count, hemoglobin and hematocrit levels. At reduced intervention frequency of weekly treatments, rhEPO-Fc showed similar hematopoietic effects as compared with rhEPO given three times a week. These results indicated that rhEPO-Fc could potentially be used in treatment of anemia and warrants future clinical trials.

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Competing Interests: QT, DY, DJ are founders and JY is an employee of Meiyer Biotech Institute, and LHKS, BNCS, CRYS are founders of PharMab, Inc. This affiliation, however, does not in anyway alter the authors' adherence to all the PLOS ONE policies on sharing data and materials. The other authors report no conflict of interest.

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Introduction

Erythropoietin (EPO) is a glycoprotein that stimulates the production of erythrocytes [1,2]. It promotes proliferation, differentiation and maturation of erythroid progenitor cells, and inhibits their apoptosis [3]. In clinical practice, recombinant human EPO (rhEPO) has been used in treatment for anemia associated with chronic renal failure [4], cancer chemotherapy [5], HIV infection [6], and a number of other pathological conditions [7,8].

Since initial clinical usage of rhEPO-α in the 1980s, clinicians quickly recognized the need of frequent administration as one of major drawbacks of the drug. This imposes a burden on both patients and health care providers, as *in vivo* half-lives of rhEPO-α and rhEPO-β administered intravenously or subcutaneously in humans are only about 8.5 and 17 hours respectively [9,10]. Thus, there has been a longstanding need to develop recombinant EPO analogs with longer *in vivo* half-lives.

Attempts have been made to genetically or chemically modify the structure of native EPO protein to either slow down its *in vivo* metabolism or improve its therapeutic properties [11,12]. To extend the half-life, EPO has been chemically conjugated with other moieties to increase its molecular weight. For instance, polyethylene glycol conjugated (PEGylated) EPO has a much higher molecular weight and is protected from being cleared from circulation and therefore has a longer plasma half-life. However, PEGylation may alter the protein structure resulting in unanticipated changes of function and specificity of EPO moiety [13,14]. Other strategies have also been reported to increase the molecular weight of EPO, such as linking EPO molecule to a carrier protein (e.g. human albumin), or forming a homo-dimer of two EPO molecules using linking peptides (3- to 17-amino acids) or chemical cross-linkers [15–18].

To develop a "controlled release" of EPO, we have previously developed a novel recombinant human EPO fused with an Fc domain from a modified human IgG2 without CDC and ADCC function [19,20]. In the present report, its pharmacokinetics and pharmacodynamics were studied. The erythropoietic effects were investigated in various rodent as well as nonhuman primate anemia models.

Materials and Methods

Materials

Recombinant human EPO (rhEPO, 30.4 kD, 98% purity) was purchased from 3SBio Inc. China. The novel EPO fusion protein, rhEPO-Fc (118 kD, ~60,000 IU/mg) was supplied by Shanghai

Meiye Biotech Institute. Briefly, the plasmid encoding rhEPO-Fc fusion protein, containing human EPO, Fc domain of human IgG2 and 16-amino acid linker, was transfected and expressed in Chinese Hamster Ovary (CHO) cells with a yield of 2.5 g/L. The fusion protein was purified using a Protein-A column and the final purity is more than 95% by SDS-PAGE analysis [19,20].

Animals

C57BL/6J mice (20.4 ± 0.3 g, 4 weeks old) and Sprague-Dawley rats (232.8 ± 2.9 g, 8 weeks old) were purchased from Sino-British Sippr/BK Lab. Animal Co. Ltd. Rhesus monkeys (3.9 ± 0.3 kg), for *in vivo* bioactivity study, were from Shanghai Public Health Clinical Center, Shanghai, China, and SPF rhesus monkeys (3.5–5.0 kg) for pharmacokinetics study were from Chengdu Greenhouse Biotech Co. Ltd, Chengdu, China.

Animals were housed under specific pathogen free conditions. All experimental protocols were approved by the Animal Experiment Committee of Fudan University, Shanghai, China.

Pharmacokinetic study

For single dose pharmacokinetics study, rats (n = 12 per group) were injected subcutaneously with 16, 50, or 160 μg/kg of rhEPO-Fc, and for rhesus monkeys (n = 6 per group), the doses were 8, 25, or 80 μg/kg. Blood samples (0.3 ml) were collected via retro-orbital venous plexus for rats and femoral vein for rhesus monkeys during 0–168 h after rhEPO-Fc administration, and serum levels of rhEPO-Fc were determined.

For repeated dose pharmacokinetics study, rhesus monkeys (n = 6) were injected subcutaneously with rhEPO-Fc at 25 μg/kg weekly for four consecutive weeks. After the 1st and 4th injection, blood samples were collected in various time points till 168 h. The serum concentration of rhEPO-Fc was determined using an ELISA kit from R&D Systems (Minneapolis, MN, USA).

Pharmacokinetics parameters were obtained by fitting the data to an extravascular administration model with a first order adsorption phase and a first order elimination phase.

Irradiation induced anemia in mice

C57BL/6J mice received a fractionated total body irradiation (TBI) at a dose of 2×4 Gy (5 MeV photons generated by a linear accelerator at a rate of 2.5 Gy/min), on two consecutive days [21]. Two days after irradiation, mice (n = 14 per group) were subcutaneously injected with rhEPO-Fc (7.5, 15.0, 30.0 μg/kg, once a week), control rhEPO (7.5 μg/kg, 3 times per week), or PBS (sterile, endotoxin-free Phosphate Buffer Solution) for a total period of 4 weeks. At indicated time points (0–34 day), red blood cell (RBC), hemoglobin, hematocrit, reticulocyte were determined with an automatic blood analyzer (MEK-8222k, Optoelectronic Industry Co. Ltd, Tokyo, Japan).

Partial renal ablation induced anemia in rats

Seven days after the right kidney was surgically removed, the cortex of the left kidney was partially resected (A quantity corresponding to 2/3 of the weight of the previously resected right kidney). Sham control rats received identical surgical procedures, but no kidney or kidney tissues were removed [22]. Five days after surgery, rats (n = 16 per group) were subcutaneously injected with rhEPO-Fc (5.4, 10.7, 21.4 μg/kg, given weekly), control rhEPO (5.4 μg/kg, given 3 times a week), or PBS for four weeks.

Blood samples were collected weekly during treatment period, and two weeks after treatment ended. RBC, hemoglobin, hematocrit, reticulocyte and blood urea nitrogen (BUN) were evaluated.

Cyclophosphamide induced anemia in rhesus monkeys

Cyclophospamide is an alkylating nitrogen mustard which is clinically used to treat numerous types of cancer and certain autoimmune disorders. Bone marrow suppression, including anemia, is one of the major adverse effects [23,24]. To induce anemia, rhesus monkeys were given intravenously twice a day at a dose of 50 mg/kg cyclophosphamide on two consecutive days. Two days after the second injection, monkeys (n = 4 per group) were subcutaneously injected with rhEPO (2.5 μg/kg, 3 times per week), rhEPO-Fc (5.0, 10.0 μg/kg weekly) or PBS for seven weeks. Blood samples were collected at 0–49 days during treatment period and additional 4 times weekly after treatment ended. RBC, hemoglobin, hematocrit, reticulocyte, blood platelet and leukocyte were determined as described above.

Statistical Analysis

Pharmacokinetics and pharmacodynamics data were analyzed using one-way ANOVA, and a p value ≤ 0.05 was considered statistically significant.

Results

Pharmacokinetics

To evaluate pharmacokinetic behavior of the rhEPO-Fc fusion protein *in vivo*, both single and repeated rhEPO-Fc injections were conducted in both rats and rhesus monkeys. Circulating levels of rhEPO-Fc following single injections were measured by ELISA. The mean serum concentration-time curves were shown in Fig. 1A and Fig. 1B for rhesus monkeys and rats, respectively. The pharmacokinetics parameters were summarized in Table 1.

In rhesus monkeys (Fig. 1A and Table 1, the C_{max} and $AUC_{(0-168\ h)}$ positively correlated with dosage. With increasing doses of rhEPO-Fc, C_{max} and $AUC_{(0-168\ h)}$ correspondingly increased, but T_{max}, CL and half-life did not show obvious dose-dependent effects. The serum half-life of rhEPO-Fc was 29.5 to 38.9 h.

In rats (Fig. 1B and Table 1), the similar dose-dependent correlations of C_{max} and $AUC_{(0-168\ h)}$ were also observed. The half-life of rhEPO-Fc was 35.5 to 43.5 h.

In rhesus monkeys receiving repeated injections, there was no rhEPO-Fc detected before the 2nd, 3rd and 4th injection (Fig. 1C). After the 4th injection, the serum concentration-time curve of rhEPO-Fc did not change significantly, similar to that of the 1st injection. This indicated that the repeated injections did not change the pharmacokinetic behavior of rhEPO-Fc.

rhEPO-Fc alleviates anemia induced by irradiation

Next, we tested whether rhEPO-Fc have hematopoietic effects in animal anemia models. We first evaluated the effects of rhEPO-Fc on anemic mice induced by total body irradiation (TBI).

As shown in Fig. 2A, TBI induced severe anemia in C57BL/6 mice. The mean RBC count of PBS control mice decreased to the lowest level (4.9×10^{12}/L, 54% of baseline) on day 7 post irradiation and recovered to 7.2×10^{12}/L (80% of baseline) four weeks later. In mice treated with rhEPO-Fc (7.5, 15.0, 30.0 μg/kg, weekly), rhEPO-Fc treatments attenuated reduction of RBC in a dose-dependent manner. The RBC counts on day 7 were 6.2×10^{12}/L, 5.7×10^{12}/L, 6.1×10^{12}/L in mice treated with rhEPO-Fc at doses of 7.5, 15 and 30.0 μg/kg, decreased to the lowest level on day 9 and then recovered. On day 13 and 27, RBC counts of mice treated with 15 and 30.0 μg/kg rhEPO-Fc were significantly higher than those in PBS control group.

Hemoglobin and hematocrit (Fig. 2B and 2C) were also significantly lowered after irradiation similar to RBC (Fig. 2A).

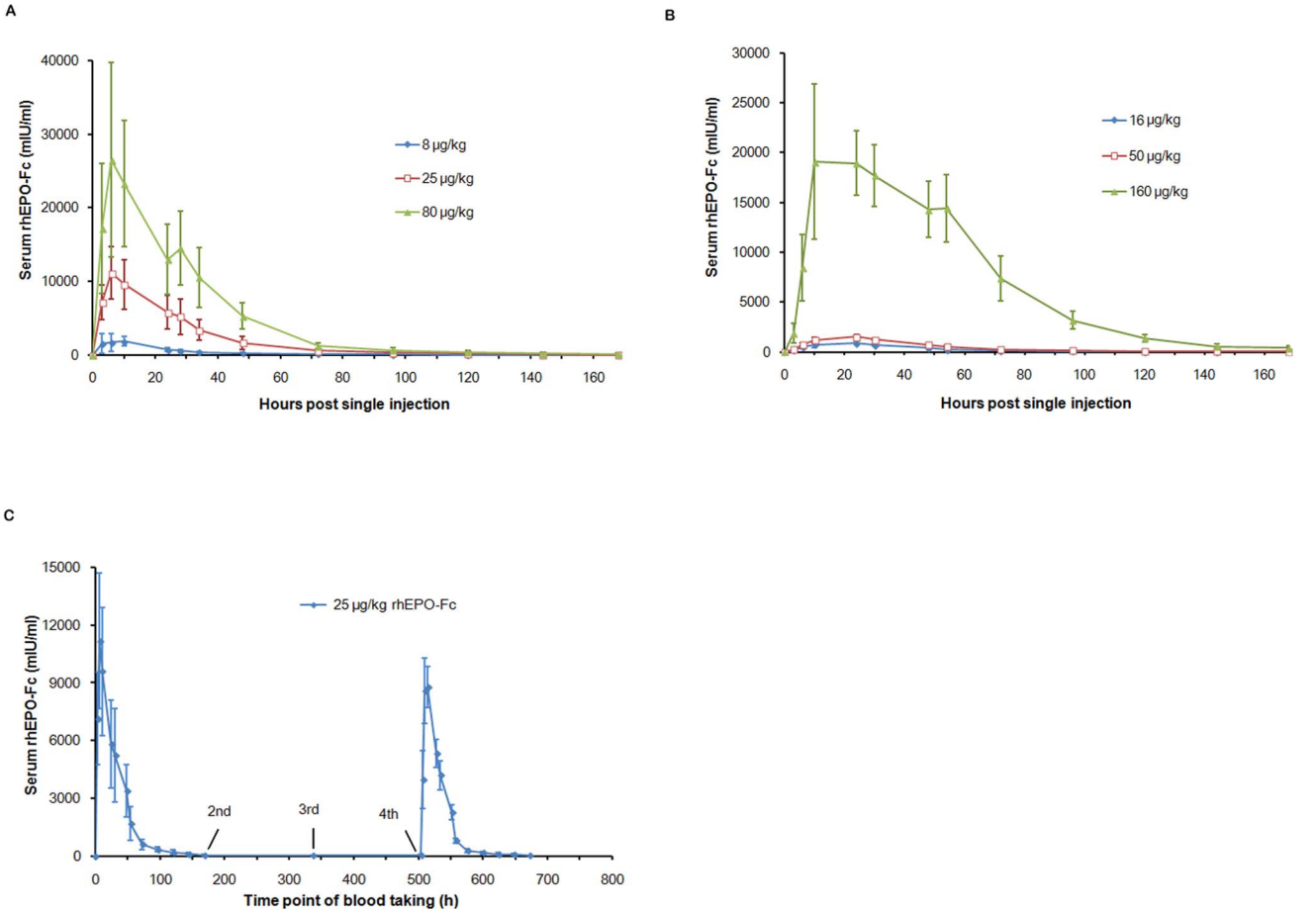

Figure 1. Pharmacokinetics of rhEPO-Fc with single and repeated injections. Mean serum rhEPO-Fc concentration versus time after single subcutaneous injection in rhesus monkeys (Fig. 1A), rats (Fig. 1B) and repeated subcutaneous injections in rhesus monkeys (Fig. 1C) were plotted. Data were mean±SD for six rhesus monkeys or twelve rats per group.
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Treatment with rhEPO-Fc also dose-dependently alleviated the reduction of hemoglobin and hematocrit ($p \leq 0.05$, compared with PBS control). At the weekly dosing of 30.0 μg/kg, rhEPO-Fc exerted similar effects on RBC, hemoglobin and hematocrit, compared with rhEPO (7.5 μg/kg, 3 times a week).

After irradiation, reticulocyte counts of PBS control mice reduced to the lowest level on day 3, then rapidly reached the highest level on day 13 and then decreased gradually. The rhEPO-Fc treatment showed dose-dependent stimulating effects on reticulocytes, which significantly elevated at doses of 15.0,

Table 1. Pharmacokinetic parameters of rhEPO-Fc following single subcutaneous injection in rhesus monkeys and rats.

Species	Dose	N	Cmax[a]	Tmax[b]	$AUC_{(0-168)}$[c]	CL[d]	Half-life
	(μg/kg)		(IU/L)	(h)	(IU/ h·ml)	(ml/h/kg)	(h)
Rhesus monkey	8	6	2193.1±902.3	7.5±3.0	56.3±19.0	0.4±0.2	38.9±16.3
	25	6	12186.1±2695.5	8.0±2.2	335.0±81.1	0.2±0.1	29.5±4.2
	80	6	26772.4±13066.6	6.7±1.6	837.0±295.2	0.3±0.1	33.1±14.1
Rat	16	12	899.6±141.9	21.7±5.7	39.0±5.9	0.5±0.1	38.6±22.9
	50	12	1522.5±209.1	24.0±0.0	69.2±6.7	0.5±0.3	35.5±8.9
	160	12	22191.4±4422.3	23.0±17.4	1238.0±116.5	0.3±0.3	43.5±32.0

[a]Maximal drug concentration.
[b]Time of maximal drug concentration.
[c]Area under the curve (0–168 h).
[d]Clearance.
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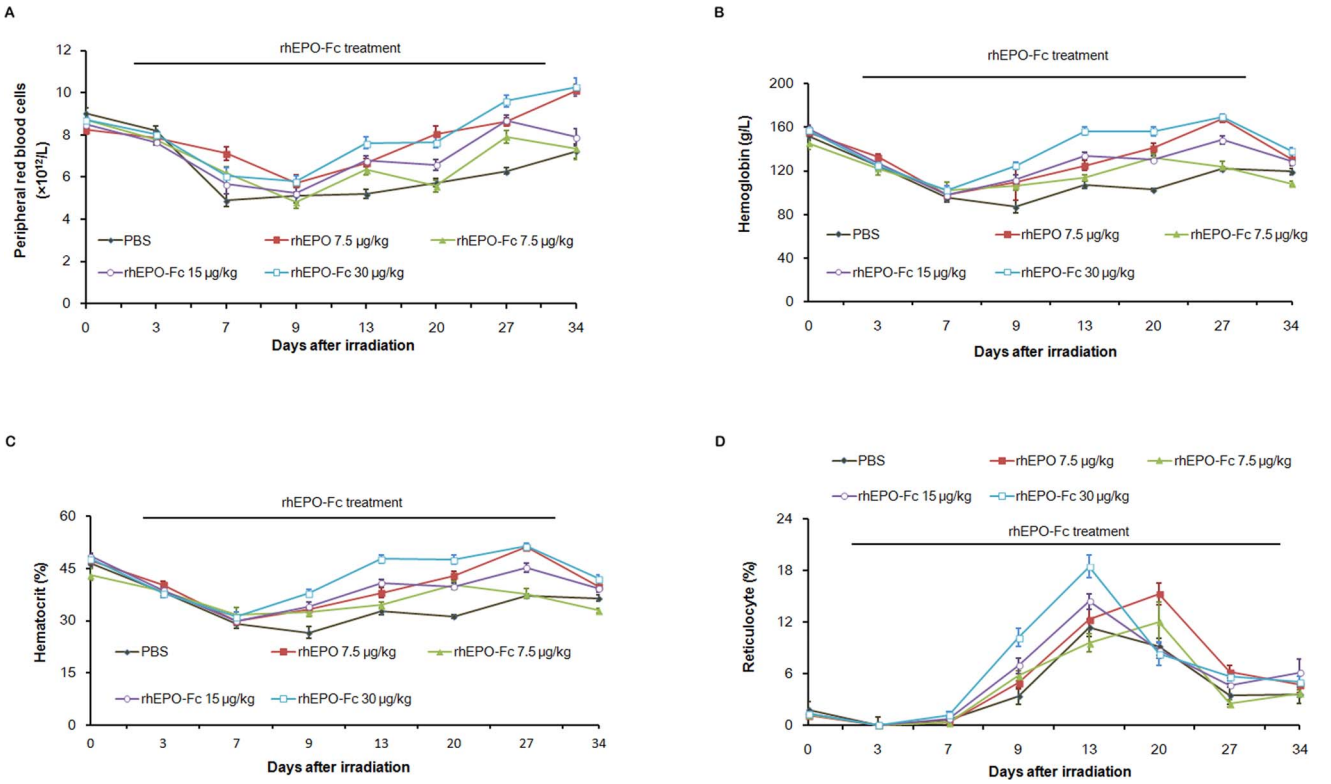

Figure 2. rhEPO-Fc attenuated anemia induced by irradiation in mice. Blood samples were taken at day 0, 3, 7, 9, 13, 20, 27 and 34 to determine RBC (Fig. 2A), hemoglobin (Fig. 2B), hematocrit (Fig. 2C), and reticulocyte (Fig. 2D). Data were presented as Mean±SD.
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30.0 μg/kg on day 9 and day 13 as compared with those in rhEPO-treated mice) ($p \leq 0.05$) (Fig. 2D).

These data demonstrated that rhEPO-Fc dose-dependently attenuated reduction of RBC, hemoglobin and hematocrit, rapidly elevated levels of reticulocyte in anemia induced by TBI in mice.

rhEPO-Fc alleviates anemia induced by partial renal ablation

The erythropoietic effect of rhEPO-Fc was also evaluated in anemia induced by partial renal ablation in rats. The mean RBC count decreased to the lowest level (5.3×10^{12}/L, 76% of baseline) on day 19 after partial nephrectomy, and remained at this level throughout the entire experiment (Fig. 3A). However, when treated with rhEPO-Fc (5.4, 10.7, 21.4 μg/kg weekly), on day 19, RBC counts significantly increased (7.9×10^{12}/L, 7.8×10^{12}/L, 8.5×10^{12}/L) in animals treated with all three doses of rhEPO-Fc. In anemic rats treated with rhEPO-Fc at doses of 10.7 and 21.4 μg/kg, the RBC counts were restored to the sham operation levels, significantly higher than those of PBS-treated anemic rats ($p \leq 0.05$) in the course of treatment. At the end of the treatment, RBC counts of rats treated with rhEPO-Fc (21.4 μg/kg weekly) reached similar levels, as compared with those in mice treated with rhEPO (5.4 μg/kg, 3 times a week).

Similar dose-dependent stimulating effects on hemoglobin and hematocrit were also observed (Fig. 3B and 3C). During rhEPO-Fc therapy with weekly doses of 10.7 and 21.4 μg/kg, hemoglobin and hematocrit levels were restored to the sham operation level. The effects of rhEPO-Fc (21.4 μg/kg) were similar to those of rhEPO treatment (5.4 μg/kg, 3 times a week).

After the partial renal ablation surgery, reticulocytes of PBS-treated rats rapidly increased and changed in fluctuation. At the dose of 21.4 μg/kg, rhEPO-Fc exerted the strongest effect on reticulocyte (243% of baseline at day 12, $p \leq 0.05$, *vs* PBS), similar to those of rhEPO therapy (5.4 μg/kg, 3 times a week) (Fig. 3D).

The partial renal ablation also resulted in high levels of BUN in the rats and neither rhEPO nor rhEPO-Fc treatment affected BUN (Fig. 3E). Although administration of rhEPO-Fc did not improve impaired kidney function, it effectively corrected anemia induced by partial renal ablation.

rhEPO-Fc attenuates anemia induced by cyclophosphamide in rhesus monkeys

The *in vivo* erythropoietic effect of rhEPO-Fc was further investigated in rhesus monkeys after anemia induction with cyclophosphamide. As shown in Fig. 4A, the mean RBC count of PBS-treated monkeys decreased to the lowest level (3.6×10^{12}/L, 61% of the baseline) on day 7 after cyclophosphamide administration and then recovered slowly to the baseline level. rhEPO-Fc dose-dependently alleviated reduction of RBC counts. On day 7, rhEPO-Fc treatment (5.0, 10.0 μg/kg weekly) increased RBC counts to 5.4×10^{12}/L, and 5.6×10^{12}/L and then restored RBC counts to the baseline level. The effect of rhEPO-Fc (10.0 μg/kg weekly) was similar to that of rhEPO (2.5 μg/kg, 3 times a week). Similar effects were also observed on hemoglobin and hematocrit upon rhEPO-Fc treatment (Fig. 4B and 4C).

The reticulocyte count reduced to zero on day 3 and increased rapidly to the highest (3.4%) on day 9 and then dropped to baseline level on day 21 in PBS-treated anemic rhesus monkeys. Upon rhEPO-Fc treatment, there was some increase of reticulocytes (4.27% for 5.0 μg/kg on day 7, 4.27% for 10.0 μg/kg on day 12), but no significant difference was observed among the monkeys treated with rhEPO, rhEPO-Fc or PBS (Fig. 4D).

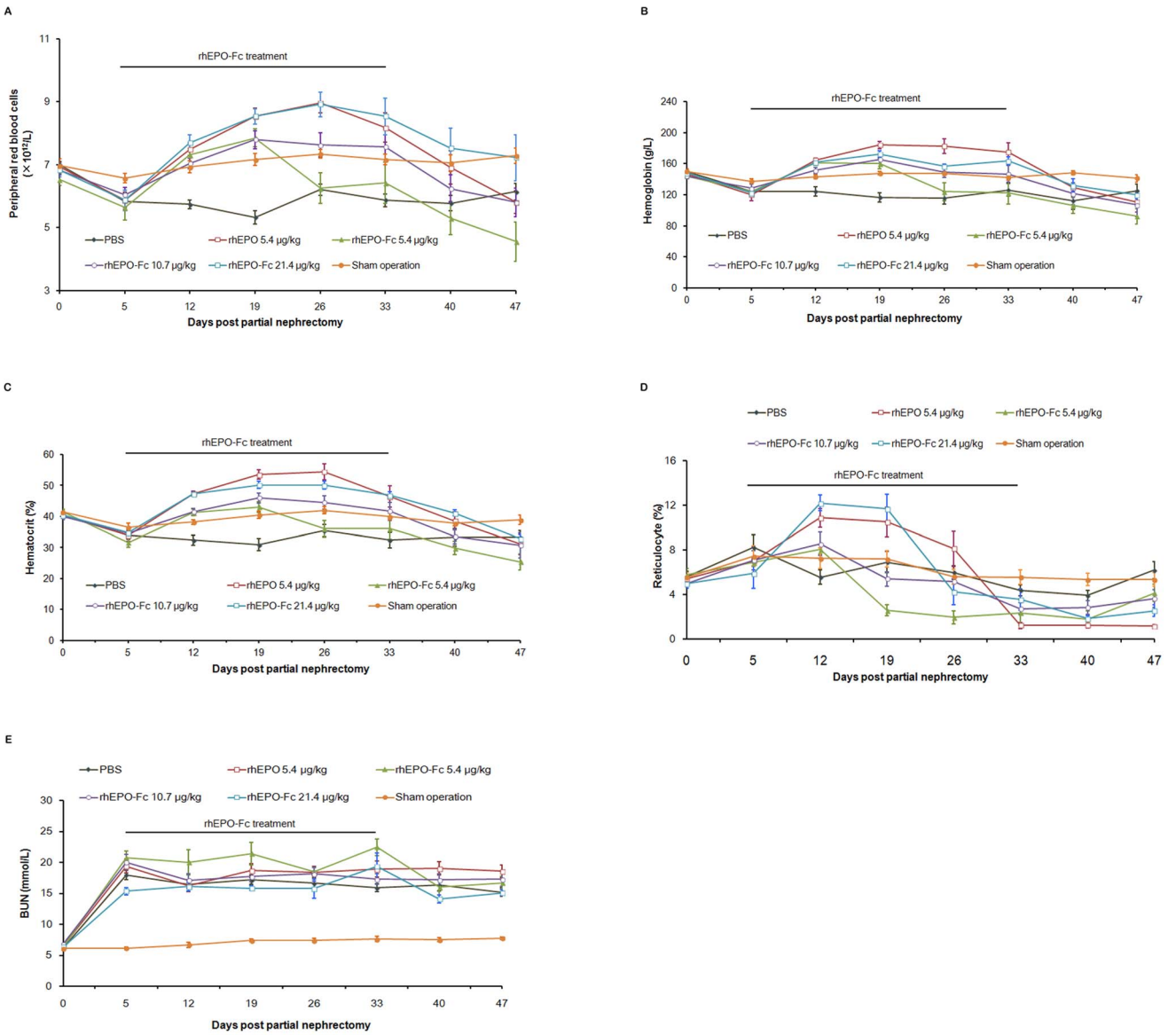

Figure 3. rhEPO-Fc attenuated anemia induced by partial renal ablation in rats. Blood samples were collected at the indicated time points (0–4 week, and additional two weeks after the treatment ended). RBC (Fig. 3A), hemoglobin (Fig. 3B), hematocrit (Fig. 3C), reticulocyte (Fig. 3D) and BUN (Fig. 3E) were shown. Data were presented as Mean±SD.
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Cyclophosphamide also reduced blood platelet and leukocyte counts, which gradually recovered to the baseline levels in PBS-treated rhesus monkeys. Interestingly, rhEPO-Fc (5.0, 10.0 μg/kg weekly) showed some stimulating effects on platelet and leukocyte recovery. However, no significant difference was observed in blood platelet and leukocyte counts in animals treated with rhEPO, rhEPO-Fc or PBS as shown in Fig. 4E and 4F.

Discussion

The novel fusion protein, rhEPO-Fc, reported in the present study has its unique advantages. First, no mutation was introduced into the EPO molecule itself. Although the mutation on a disulfide bond of EPO structure has been reported to improve its pharmacokinetics and hematopoietic effects [25], some mutations on EPO could change its erythropoietic activity. For instance, EPO mutant S100E induces a significantly lower hematocrit increase than natural EPO while provides similar protection from progressive photoreceptor degeneration [26,27]. To maintain its erythropoietic property and to avoid unpredicted risk of immunogenicity in clinical application, no mutation was introduced into EPO in our rhEPO-Fc fusion protein.

Second, the Fc region of modified IgG2 with diminished CDC and ADCC function was linked to the EPO molecule. Fusion proteins consisting of the Fc fragment of human IgG have been shown to have significantly longer *in vivo* half-lives while retaining their biological and therapeutic properties [20,28]. Several fusion proteins comprising an Fc fragment have been successfully developed for clinical application and approved by FDA for treatment of rheumatoid arthritis and chronic plaque psoriasis [29,30]. In this study, the Fc region of IgG2 was selected to link to the EPO molecule for the following reasons. The IgG2 molecule does not bind to $Fc_{\gamma}R$, which can activate antibody dependent

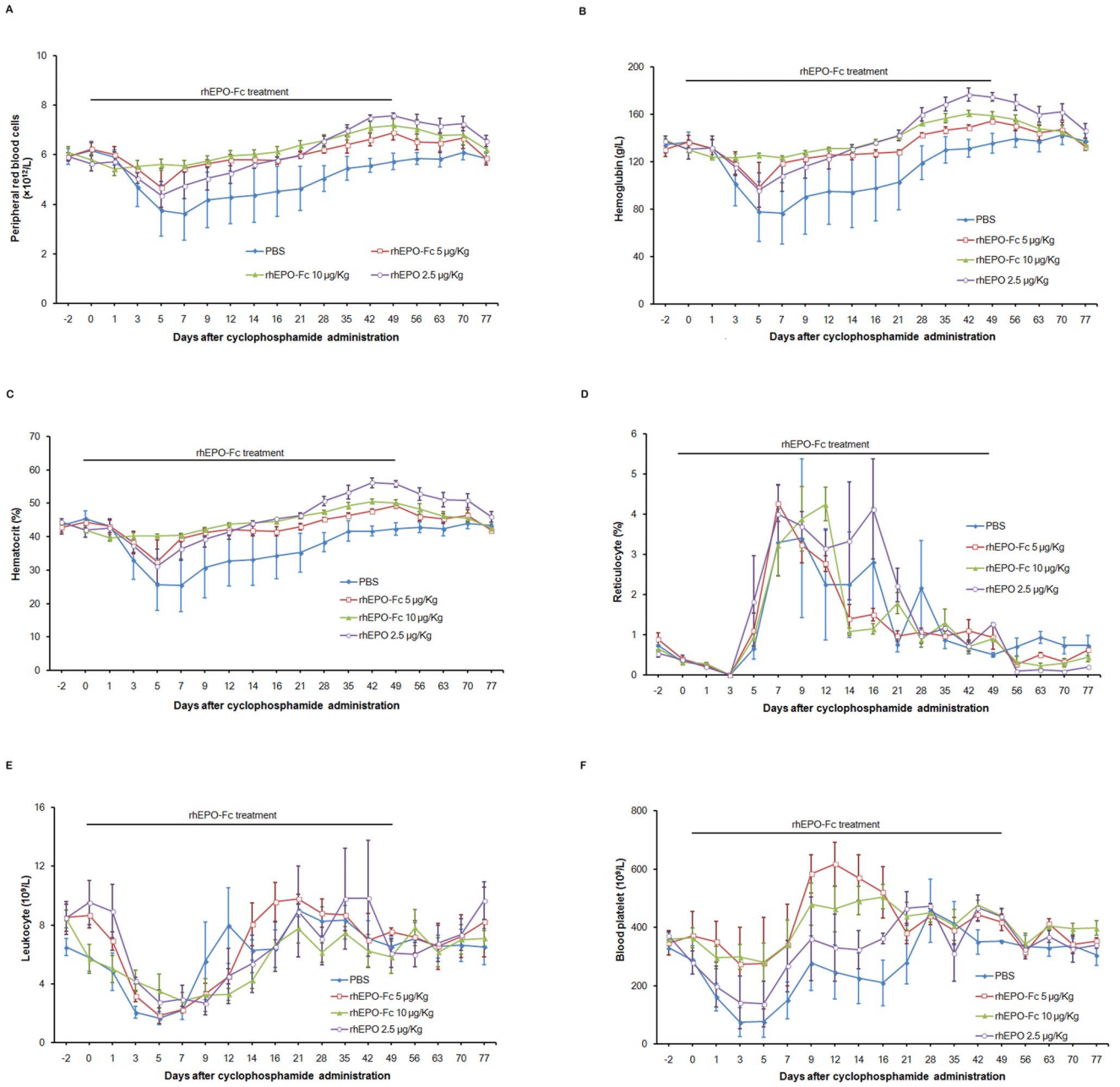

Figure 4. rhEPO-Fc attenuated anemia induced by cyclophosphamide in rhesus monkeys. Blood samples were collected at 0–49 days during treatments and additional 2 times weekly after the treatment ended. RBC (Fig. 4A), hemoglobin (Fig. 4B), hematocrit (Fig. 4C), reticulocyte (Fig. 4D), leukocyte (Fig. 4E) and blood platelet (Fig. 4F) were determined. Data were presented as Mean±SD.
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cellular cytotoxicity (ADCC). In addition, IgG2 is particularly resistant to proteases, which might reduce the clearance of the fusion protein mediated by autoantibody [31]. Furthermore, a site mutation (Pro331Ser mutation) had been made near the carboxyl-terminus of the CH2 domain of human IgG that appears to be important for both FcγR and C1q binding. So the Fc variant should have less complement-activating activity than the natural Fc fragment while remain as a non-binder to FcγR [19]. Thus the novel rhEPO-Fc molecule was expected to have a extended half-life for *in vivo* applications.

In this study, the half-life of rhEPO-Fc were29.5 to 38.9 h at doses of 8, 25, 80 μg/kg in rhesus monkeys and 35.5 to 43.5 h at doses of 16, 50, 160 μg/kg in rats. Compared with the reported pharmacokinetics data, the half-life of the EPO-Fc fusion protein was obviously extended [14]. Administration of rhEPO-Fc once a week exerted similar hematopoietic effects to that of rhEPO given 3 times a week in anemia animal models induced by irradiation, partial renal ablation and cyclophosphamide, which verified the possibly extended half-life of rhEPO-Fc. When repeated subcutaneous injections were performed in rhesus monkeys, no EPO protein was detected before the 2nd, 3rd and 4th injections. This indicated that no rhEPO-Fc accumulation in long-term administration. It also supports safe long-term use of rhEPO-Fc as has been reported previously [32].

Reticulocyte is the newly-produced red blood cell and the count is used to assess bone marrow response to an anemic state. Reticulocyte production increases in response to loss of red blood cells. It increases within 2–3 days of a major acute hemorrhage and reaches its peak in 6–10 days [33,34]. In this study, similar responses of reticulocyte in different anemia models were also observed, and the rhEPO-Fc administration clearly enhanced production of reticulocytes, supporting rapid and efficient recovery of anemia induced by irradiation, cyclophosphamide and partial renal ablation.

In summary, the low administration frequency of rhEPO-Fc (once a week) was shown to have similar erythropoietic effects in a variety of rodent and primate anemia models when compared with rhEPO administrated three times a week. The pharmacokinetics and pharmacodynamics profiles of rhEPO-Fc strongly indicate that rhEPO-Fc could be potentially used as clinical treatment for anemia associated with chronic renal failure or chemotherapy, and future clinical trials are warranted.

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Author Contributions

Conceived and designed the experiments: QT DY BNCS MY DJ. Performed the experiments: XS JY HZ JL HH LY MF GH YL. Analyzed the data: LHKS CRYS YL MY PZ. Wrote the paper: XS PZ DJ.

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