Effects of Dose, pH and Osmolarity on Intranasal Absorption of Recombinant Human Erythropoietin in Rats¹⁾

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The effects of dose, pH and osmolarity on the intranasal absorption of a recombinant human erythropoietin (rEPO) solution were studied in male Wistar rats. The intranasal administration of rEPO was evaluated by measuring percentage circulating reticulocytes of red blood cells on a stained blood smear (smear method), and also by measuring residual circulating reticulocyte counts using a microcell counter (sysmex method). Both results suggest that rEPO solution was absorbed through the nasal mucosa of rats without enhancers after a single intranasal administration. The pharmacological availabilities of rEPO after intranasal administration compared with intravenous administration were about 7% and 4%, when estimated by smear method and sysmex method, respectively. The pharmacological activity was enhanced in low pH and hypotonic mannitol solution.

Key words erythropoietin; intranasal administration; glycoprotein; circulating reticulocyte; bioavailability

Erythropoietin (EPO) is a glycoprotein with a molecular weight of about 30000, about 40% of which is ascribed to sugar moiety.²⁾ EPO is produced mainly in the kidney and stimulates proliferation and differentiation of erythroid precursor cells to red blood cells (RBC).³⁾ Recombinant human erythropoietin (rEPO) is produced on a large scale by recombinant DNA technology⁴⁾ and has been proven effective for the treatment of renal anemia.⁵⁾ rEPO, like most other protein medicinal drugs, is clinically administered through an intravenous injection 2–3 times a week. In addition, clinical investigations with subcutaneous or intraperitoneal injection were reported.⁶⁾ However, the injection causes pain in patients, hence, an alternative route of administration is desirable. Although various routes have been investigated, the nasal route seems a suitable alternative for proteins and peptides which are usually enzymatically degraded in the gastrointestinal tract after oral administration.

Absorption through the nasal mucosa has been reported in the literature for a number of proteins and peptides. It was reported that insulin with a molecular weight of about 6600 was absorbed transnasally⁷⁾ and that human fibroblast interferon with a molecular weight of about 22000 was delivered across the nasal mucosa when co-administered with absorption enhancers.⁸⁾ There have, however, been few reports of nasal administration of highly glycosylated hydrophilic peptides with molecular weight as large as 30000. The purposes of this study were to evaluate the pharmacological activity of intranasally administered rEPO in the absence of absorption enhancer and to investigate the effects of dose, pH and osmolarity on the pharmacological activity of rEPO after intranasal administration.

MATERIALS AND METHODS

Materials rEPO used in this study was epoetin β , a preparation from Chugai Pharmaceutical Co., Ltd., Japan, with a mean specific activity of 1.8×10^5 IU(international unit)/ml as determined by *in vivo* polycythemic mouse

assay. One hundred and eighty IU corresponds to 1 μ g polypeptide equivalent of rEPO. rEPO exhibits multiple isoelectric focusing bands in the range of pI 2.80 to 4.55, which could be ascribed to microheterogeneity due to sialic acids at the end of carbohydrate chain. Human serum albumin (HSA, Sigma Chemical Co., U.S.A.) was used to prevent adsorption of rEPO in intravenous preparations. The other chemicals employed in the preparation of buffer solutions or in adjusting osmolality of solutions were of analytical or reagent grade.

Preparation of rEPO Solutions For the dose-dependency study on intravenous administration, rEPO preparation was serially diluted with 0.9% sodium chloride solution containing 0.05% HSA to make 1000, 300 and 100 IU/ml solutions.

For the dose-dependency study on intranasal administration, rEPO preparation was diluted with phosphate buffered saline solution (PBS, pH 7.4) to make 90000, 45000, 22500, and 16665 IU/ml solutions just before administration.

For the pH effect study on intranasal administration, rEPO preparation was diluted with isotonic 0.263 M citric acid–0.123 M disodium phosphate buffer, ranging in pH from 3.29 to 4.27, or isotonic 2.33% potassium phosphate–1.44% sodium bicarbonate buffer, ranging in pH from 6.23 to 8.03 to make 45000 IU/ml solutions.

For the osmolarity effect study on intranasal administration, rEPO preparation was desalted using CentriconTM 10 (Amicon Corp., U.S.A.). Aliquots of the desalted rEPO preparation were diluted with sodium chloride or mannitol solutions in 1/150 M phosphate buffer of pH 7.3 to give hypotonic (about 150 mOsm), isotonic (about 300 mOsm) or hypertonic (about 600 mOsm) solutions. The desalted rEPO preparation was also diluted with 1/150 M phosphate buffer of pH 7.3 to give a hypotonic (about 0 mOsm) solution. The concentration of the resulting solutions was about 200000 IU/ml.

Administration of rEPO Solutions in Rats Male Wistar rats (Saitama Experimental Animal Supply Co., Japan; 10–11 weeks old, 250–350 g) were used in this study.

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For intravenous administration, 1 ml/kg of each rEPO solution (corresponding to 100, 300 or 1000 IU/kg) was injected through the tail vein and animals were returned to cages for further blood collections. For intranasal administration, rats were anesthetized by intraperitoneal injection of sodium pentobarbital (Nembutal®, Abbot Laboratories) at a dose of 50 mg/kg, and 200 μ l/kg of each rEPO solution (corresponding to 18000, 9000, 4500 or 3333 IU/kg for the dose-dependency study, 9000 IU/kg for the pH effect study, and 40000 IU/kg for the osmolarity effect study) was applied into a nostril with an Eppendorf pipette. The animals were kept in supine position for 1 min after the administration and then returned to cages for further blood collections.

Collection of Blood Samples Blood (10 or 20 μ l) was collected from the dorsal metatarsal vein before and on 2, 4 and 7 d after administration of rEPO solutions. Blood was also collected from untreated rats as a control group.

Measurement of Percentage Circulating Reticulocytes of RBC on a Blood Smear (Smear Method) Twenty μ l of 10% tripotassium edetate (Wako Pure Chemical Industries, Ltd., Japan) aqueous solution and 800 μ l of Brecher's new methylene blue solution (Muto Pure Chemicals, Ltd., Japan) were mixed just before use. Twenty μ l of the mixture was placed in each microplate well (Cell Wells™; Corning Glass Works, U.S.A.). Collected blood (10 μ l) was immediately put into the microplate well, mixed gently and allowed to stand 10–20 min for supravital staining with new methylene blue. Three μ l of the stained blood was placed on a slide glass and a smear was prepared. The smears were further treated with Giemsa's solution (E. Merck A. G., Germany). Numbers of reticulocytes and RBC were counted on the stained blood smear microscopically using a Miller ocular disc until total RBC count in the small square reached over 400. Percentage circulating reticulocytes of RBC was calculated as follows: $100 \times (\text{reticulocyte count in large squares}) / (\text{RBC count in small squares} \times 9)$.⁹⁾

Measurement of Residual Circulating Reticulocyte Counts by a Microcell Counter (Sysmex Method) Collected blood (20 μ l) was hemolyzed using 100 μ l of stromatolizing agent, Quicklizer (Sysmex, Toa Medical Electronics, Japan), after diluting 500 fold with Cellpack (Sysmex). The stromatolized blood cells were counted using an automatic microcell counter (Sysmex F-500) and a cell monitor (Sysmex CM-5) 10–15 min after Quicklizer treatment. The difference in numbers counted at discriminator levels 1 and 5 with sensitivity level 4 of the cell monitor was calculated as the residual circulating reticulocyte counts.¹⁰⁾

Statistical Evaluation Statistical evaluation was performed using Student's *t*-test or Welch test.

RESULTS AND DISCUSSION

Although EPO levels can be determined by radioimmunoassay (RIA),¹¹⁾ this does not seem to be the first choice for evaluating intranasally administered EPO, because intranasally administered proteins and peptides might be degraded by enzymes in the nasal cavity¹²⁾ and RIA does not necessarily distinguish the active native

form from the degraded forms such as inactive asialoform.¹³⁾ Doses of EPO reportedly result in an increase of reticulocyte first and an increase of RBC later. Pharmacological effects of EPO can be assessed by the increase in reticulocyte, hematocrit value, hemoglobin content and RBC counts. In the latter three, however, time is required to observe a significant rise. On the other hand, the maximum reticulocyte number is known to occur on the third to fourth day after a single injection of EPO, and the relatively immature reticulocytes to markedly increase on the second day. Therefore, the increase in reticulocytes can be a useful index for the early assessment of pharmacological effects of EPO.

Counting of reticulocytes (percentage of RBC) using a Miller ocular disc on smears stained with new methylene blue and Giemsa solution, the smear method, has been proposed as an *in vivo* bioassay method.⁹⁾ Kawamura¹⁰⁾ *et al.* recently reported a new simple *in vivo* bioassay method for EPO based on the measurement of immature reticulocytes using an automatic microcell counter in normal mice which were injected subcutaneously for 3 successive days with EPO. To evaluate intranasally administered rEPO, we applied the smear method as well as the sysmex method, a modified method of Kawamura *et al.*, where normal rats received a single administration intravenously or intranasally.

Pharmacological Evaluation of Intranasally Administered rEPO by the Smear Method Changes in percentage circulating reticulocytes of RBC were evaluated by the smear method after a single intravenous and intranasal administration of rEPO. Results are shown in Fig. 1. Percentage circulating reticulocytes in rats which received rEPO intravenously on day 0 rose significantly on day 2 in comparison with those in untreated rats, showed a dose-dependent rise on day 4, and returned to pre-administration level on day 7. Similar profiles were obtained after intranasal administration of rEPO. Percentage circulating reticulocytes also rose on day 2, showed a

Fig. 1. Effects of a Single Administration of rEPO on Percentage Circulating Reticulocytes of Red Blood Cells in Rats as Evaluated by the Smear Method

(a) Intravenous administration at doses of 1000 (○), 300 (△) and 100 (□) IU/kg/rat. (b) Intranasal administration at doses of 18000 (●), 9000 (▲), 4500 (■) and 3333 (▼) IU/kg/rat. Data are given as mean $\pm$ S.E. for 3–4 rats with the level of significance between treated and untreated (∇) groups (*, $p < 0.05$; **, $p < 0.01$).

Fig. 2. Regression Analysis of Dose-Response of rEPO on Percentage Circulating Reticulocytes Four Days after a Single Intravenous (○) or Intranasal (●) Administration in Rats

$Y = 4.67 \times \log X - 7.93$ and $Y = 4.67 \times \log X - 13.4$ for intravenous and intranasal administration, respectively. Data for untreated (▽) group is given at 0 IU/kg/rat. Data are given as mean $\pm$ S.E. for 3–4 rats.

Fig. 3. Effects of a Single Administration of rEPO on Circulating Reticulocyte Counts in Rats as Evaluated by the Sysmex Method

(a) Intravenous administration at doses of 1000 (○), 300 (△) and 100 (□) IU/kg/rat. (b) Intranasal administration at doses of 18000 (●), 9000 (▲), 4500 (■) and 3333 (▼) IU/kg/rat. Data are given as mean $\pm$ S.E. for 3–4 rats with the level of significance between treated and untreated (▽) groups (*, $p < 0.05$; **, $p < 0.01$).

dose-dependency on day 4, and returned to day 0 level on day 7. This suggests that part of the intranasally administered rEPO was absorbed across the nasal mucosa in pharmacologically active form.

Regression analysis of dose-response of rEPO is shown in Fig. 2. The peak percentage circulating reticulocytes of RBC by the smear method, namely, data on day 4, correlated well with logarithm of dose (IU/kg) in both intravenous and intranasal administrations of rEPO. Pharmacological availability was about 7% when calculated by the parallel-line method and the fiducial limits of error ($p = 0.95$) were 3–16%.

Pharmacological Evaluation of Intranasally Administered rEPO by the Sysmex Method Changes in residual circulating reticulocyte counts after single intravenous and intranasal administrations of rEPO as evaluated by the sysmex method are shown in Fig. 3. Residual circulating reticulocyte counts in rats which received rEPO intravenously on day 0 rose significantly and in a dose-dependent manner on day 2 in comparison with those in

untreated rats, and returned to pre-administration level on days 4 and 7. Residual circulating reticulocyte counts after intranasal administration of rEPO showed a similar profile, also rising on day 2 in a dose-dependent manner, and returning to day 0 level on days 4 and 7. This supports that part of the intranasally administered rEPO was absorbed transnasally in pharmacologically active form.

Regression analysis of dose-response of rEPO is shown in Fig. 4. The peak residual circulating reticulocyte counts by the sysmex method, namely, data on day 2, correlated well with logarithm of dose (IU/kg) in both intravenous and intranasal administration of rEPO. Pharmacological availability was 4% when calculated by the parallel-line method and the fiducial limits of error ($p = 0.95$) were 2–7%.

Correlation between the Smear Method and the Sysmex Method Data on day 4 by the smear method and those on day 2 by the sysmex method of individual rat are plotted in Fig. 5. A good linear correlation was observed between the smear method and the sysmex method after adminis-

Fig. 4. Regression Analysis of Dose-Response of rEPO on Circulating Reticulocyte Counts Two Days after a Single Intravenous (○) or Intranasal (●) Administration in Rats

$Y = 370 \times \log X - 742$ and $Y = 370 \times \log X - 1256$ for intravenous and intranasal administration, respectively. Data for untreated (▽) group is given at 0 IU/kg/rat. Data are given as mean $\pm$ S.E. for 3–4 rats.

Fig. 5. Correlation of Evaluations in Rats between the Smear Method and the Sysmex Method

Untreated (Δ), intravenous (○) or intranasal (●) administration.

tration of rEPO in rats ($Y = 61.3 \times \log X - 84.2$, $r = 0.919$). Both methods count reticulocytes, however, at different stages of maturation. The good correlation between the two methods reflects the due maturation of reticulocytes to RBC. Counting of reticulocytes and RBC on smears tends to vary from one person to another and is taxing for eyes. Counting of reticulocytes by sysmex is carried out semi-automatically. Additionally, the sysmex method requires 2 d to get a dose-dependent response, while the smear method requires 4 d. The sysmex method therefore seems preferable in evaluating pharmacological effects of rEPO.

Pharmacological availabilities of 7% and 4% by the smear method and the sysmex method, respectively, do not necessarily mean that 4–7% of rEPO was absorbed transnasally. It has been reported that bioavailability (14–49%) of subcutaneously administered EPO is low in comparison with that of intravenous administration in human healthy volunteers or patients.⁶⁾ On the other hand, however, it was indicated that smaller doses (50–60%) are required to obtain the same therapeutic efficacy with

subcutaneous administration as with intravenous administration.¹⁴⁾ Therefore, bioavailability is not simply correlated with the pharmacological effects of EPO depending on administration route. Serum level of rEPO after intranasal administration in rats will be reported later to show the correlation.

The nasal route was reportedly suitable for efficient rapid delivery of many molecules with molecular weight less than 1000, and this limit could be extended to at least 6000 and possibly much higher with the use of adjuvants.¹⁵⁾ It was also reported, however, that nasal absorption was 2.8% for dextran with a molecular weight of 70000 and that aqueous channel mechanisms was inferred for the nasal absorption of water-soluble compounds.¹⁶⁾ Although the molecular weight of rEPO is about 30000, rEPO is water-soluble, since about 40% of its molecular weight is accounted for by oligosaccharides. Therefore, rEPO is speculated to be absorbed partly through water channels of small pore size. The pore sizes of the aqueous channels were 0.39–0.84 nm in nasal membrane of Wistar rats,¹⁷⁾ while the Stokes radius of rEPO was calculated at 2.02 nm from the molecular weight and partial specific volume.¹⁸⁾ So it is believed that rEPO can be absorbed through the aqueous channels not in intact active form, and that the pore sizes or the radius might change for the passing of an rEPO molecule. It is also conceivable that rEPO could pass through the nasal membrane by another mechanism such as passive diffusion, endocytosis or transport in vesicles.¹⁹⁾

Effects of pH Effects of pH of the solution on intranasal administration of rEPO are shown in Fig. 6. Pharmacological effects of rEPO were enhanced in acidic pH. Sialic acids attached at the end of the carbohydrate chain of rEPO and nasal mucosa are electrically rather neutral, and the lack of electric repulsion between rEPO and the mucous membrane might facilitate the transnasal absorption of rEPO. Acidic pH might also affect the integrity of the nasal membrane and damage enzymes at the mucosal surface of the membrane resulting in the enhancement of the absorption of rEPO.

Fig. 6. Effects of pH on Pharmacological Activity of rEPO after a Single Intranasal Administration in Rats at a Dose of 9000 IU/kg as Evaluated by (a) the Smear Method and (b) the Sysmex Method

Data are given as mean $\pm$ S.E. for 3–4 rats with the level of significance as compared to pH 8.03 group (*, $p < 0.05$; **, $p < 0.01$).

Fig. 7. Effects of Osmolarity on Pharmacological Activity of rEPO after a Single Intranasal Administration in Rats at a Dose of 40000 IU/kg as Evaluated by (a) the Smear Method and (b) the Sysmex Method

Osmolarity was adjusted with NaCl (●), mannitol (○), unadjusted (Δ). Data are given as mean $\pm$ S.E. for 3–4 rats with the level of significance as compared to unadjusted solution group (*, $p < 0.05$; **, $p < 0.01$).

Effects of Osmolarity Effects of osmolarity on intranasal administration of rEPO are shown in Fig. 7. When osmolarity was adjusted with mannitol, the pharmacological effects of rEPO decreased with increasing osmolarity of the solution. When osmolarity was adjusted with sodium chloride, however, the pharmacological effects were not significantly influenced in either the hypotonic or hypertonic solution. Ohwaki *et al.* reported that the nasal absorption of secretin with a molecular weight of 3055 in rats decreased with increasing osmolarity of the sorbitol solution and that the maximum absorption was observed in a sodium chloride solution.²⁰⁾ They also reported shrinkage of epithelial cells treated with hypertonic sodium chloride, and no lesion with hypertonic sorbitol solution by microscopic observation. The difference in pharmacological effects of intranasally administered rEPO might reflect the difference in effects of mannitol and sodium chloride on the nasal mucosa. In

the case of mannitol the absorption of rEPO might be prevented by water flow from nasal membrane to nasal cavity, thereby neutralizing osmolarity without affecting integrity of the membrane. In the case of sodium chloride the water flow might be affected by shrinkage of epithelial cells treated with hypertonic sodium chloride.

In conclusion, this study revealed that rEPO in solution exerts hematopoietic action in a dose-dependent manner by intranasal administration and that pharmacological effects of intranasally administered rEPO could be improved by modification of pH and osmolarity of the solution formulation. Further study to improve the availability after intranasal administration might lead to a clinically feasible formulation for an alternative route of rEPO administration.

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