Effective Administration of Erythropoietin for Renal Anemia

Hiroaki MASUNAGA, Masatugu UEDA, Tadanori SAWAI, and Gosei KAWANISHI
Research Institute of Life Science, Snow Brand Milk Products Co., Ltd., 519 Ishibashi, Shimotsuga-gun, Tochigi 329-05, Japan
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ABSTRACT. The erythropoietic effects of erythropoietin (EPO) have been investigated in the different administration schedule and injection routes. EPO was intravenously, intramuscularly or subcutaneously injected to partially nephrectomized anemic rats in 3 types of prescriptions (300 units of EPO per kg of body weight was respectively given in a dose at the first day, in 4 divided doses every 4 days, and in 7 divided doses every 2 days for 2 weeks). Repeated injections of EPO in divided doses caused stronger erythropoiesis than single injection. Especially, seven repeated injections promoted the strongest erythropoiesis. The serum iron concentration and reticulocyte counts suggested that erythropoiesis was continued less than 2 weeks after single injection but erythropoiesis in repeated injection groups had been accelerated for 2 weeks. Intravenous injections were less effective than either intramuscular or subcutaneous injections at 2 weeks after 7 repeated injections of 300 units of EPO per kg of body weight in 7 divided doses. Erythropoietic effects of EPO on the same total dose are dependent on the frequency of EPO injection and the durability of serum EPO concentration. On EPO usage, one bolus intravenous injection of excessive dose is considered to be wasteful and the repeating injections to maintain plasma EPO concentration is expected for the rational treatment of uremic anemia.—KEY WORDS: erythropoietin, nephrectomy, rat, renal anemia, serum iron.


Erythropoietin (EPO) has been known to be a humoral sialoglycoprotein which is a primary inducer of erythrocyte differentiation and to regulate erythrocyte production. It promotes the growth of late erythroid precursor cells and their maturation into proerythroblasts. It also effects on the turn over of plasma iron [11].

A large amount of EPO has been provided by recombinant DNA technology as the drug for anemia. Previously, we showed a suitable model to examine the improvement of anemia by EPO and reported that urinary and recombinant EPOs improved anemia by stimulating erythropoiesis [6, 7, 8]. The detail studies of administration schedule and injection route of EPO have been required to know the rational treatment of renal anemia.

MATERIALS AND METHODS

Erythropoietin: Recombinant EPO was produced by the mammalian cell BHK-21 introduced with an expression vector containing a human genomic EPO gene and was isolated in electrophoretically pure form from the cultured media [4, 12]. EPO activity was determined by the in vivo $^{59}$Fe incorporation assay using starved rat [3].

Animals: Male Wistar rats were used and maintained in a constant temperature on a standard diet and water ad libitum for this examination. Partially nephrectomized rats were prepared as follows. Rats (6 weeks old) were anesthetized with ether and the upper and lower poles of the left kidney were removed leaving the pelvis and hilus intact. A week later, the right kidney was removed [6]. At 3 weeks after the opera-
tions, rats showing anemic conditions (described in results) were used for this study.

**Hematological examinations:** Hematological examinations were done at the first and last day of the two weeks experimental period. Blood samples were collected from retro-orbital sinus of rats. Hematocrit was measured by the microcapillary method. Hemoglobin concentration was measured using Hemoglobin B-Test (Wako Pure Chemical, Osaka). Reticulocytes count was determined by Brecher’s method [2]. Red blood cell (RBC) count was measured using a microcell counter (Sysmex CC-120). At the last day of the experimental period, the blood was collected from the inferior vena cava after the sampling for the hematological examinations and serum was separated. Serum iron and unsaturated iron binding capacity (UIBC) were measured by the direct method with 2-nitroso-5-(N-propyl-N-sulfopropylamino) phenol (Nitro-PSAP; Wako Pure Chemical).

**Administration schedule of EPO:** Anemic rats were divided into 10 groups of 5 or 6 rats each. One is the control group and subcutaneously received saline 7 times every 2 days for 2 weeks. Other 9 groups were injected with EPO. According to three kinds of the administration schedule, rats were received EPO by three injection routes, 300 units of EPO per kg of body weight in one dose intravenously (group A), intramuscularly (group B), and subcutaneously (group C) at the fist day of the experimental period. Three hundred units of EPO per kg body weight in 4 divided doses were given to the rats 4 times intravenously (group D), intramuscularly (group E), and subcutaneously (group F) every 4 days. Three hundred units of EPO per kg body weight in 7 divided doses were given to the rats 7 times intravenously (group G), intramuscularly (Group H), and subcutaneously (group I) every 2 days.

**Analysis of serum EPO titers after the injection of EPO:** Each group of 6 partially nephrectomized rats was injected with 300 units of EPO per kg body weight in one dose intravenously, intramuscularly, and subcutaneously. Blood samples were collected at 1, 2, 4, 8, and 24 hr after the injection of EPO. Serum EPO titer was determined by the radioimmunoassay [9].

**RESULTS**

**Condition of anemic animals:** Partially nephrectomized rats showed immediate decrease in hematocrit, hemoglobin and RBC. At 3 weeks after operations, their hematocrits, hemoglobin and RBC respectively fell down from 46%, 15 g/dl and 850 × 10⁴/mm³ to 35%, 12 g/dl and 640 × 10⁴/mm³ (Fig. 2). However, their reticuloocyte counts were equal to or lower than those of sham operating rats.

**Erythropoietic effects of EPO injection on anemic animals:** Anemic rats injected intravenously with 0 to 1000 units of EPO per kg body weight were examined at 2 weeks after the injection, as in Fig. 1. The groups that received more than 200 units of EPO per kg body weight showed significant increases in hematocrits, hemoglobin and RBC. From these results, following detailed examinations were carried out using 300 units of EPO per kg body weight, that is considered to promote the erythropoiesis.

Fig. 2 shows the changes of hematological parameters before and after the EPO injections. In all groups received EPO, erythropoiesis was recognized, compared with that in control group received saline subcutaneously. The groups (G, H, I) received EPO in 7 divided doses showed stronger erythropoiesis than those (D, F, E) received EPO in 4 divided doses (P<0.05). Those groups (D, F, E) also showed stronger erythropoiesis than the groups (A, B, C) received EPO in one dose (P<0.05). Among the groups received EPO in 7
divided doses, the intravenous injection (G) promoted weaker erythropoiesis than both the intramuscular (H) and the subcutaneous (I) injections at the level of P<0.05. Reticulocyte counts of the groups having repeated injections of EPO showed a marked increase, though those of the groups injected with EPO in one dose fell below the initial value. Reticulocyte counts of the groups (G, H, I) injected with EPO in 7 divided doses increased significantly higher than those of the groups (D, F, E) injected with EPO in 4 divided doses (P<0.05). On the other hand, the effect of EPO injection on iron metabolism was also investigated (Table 1). Total iron binding capacity (TIBC) of all anemic rats decreased com-
pared with that of sham operating rats. The single EPO injection groups showed the same values of serum iron as that of control group. However, the serum iron values of the repeated injection groups were lower than that of control group.

The changes of serum EPO titer after injection were shown in Fig. 3. After intravenous injection of 300 units of EPO per kg body weight, the serum EPO titer rapidly decreased. On the other hand, both rats injected with EPO intramuscularly and subcutaneously showed gradual increase in serum EPO titers. At 4 to 8 hr after the injection, equal levels of serum EPO titers were observed among 3 groups. At 24 hr after the intravenous injection, EPO titers (97.2±3.8 mU/ml) were still higher than

Fig. 1. Relation between EPO doses and hematological parameters of partially nephrectomized rats at 2 weeks after the single intravenous injection of EPO. Data represents the mean±SE of 5 rats. Significant difference from the saline control group (0U/kg): **p<0.01, *p<0.05.

Fig. 2. Changes in hematocrit, hemoglobin, red blood cell and reticulocytes counts before (shaded column) and after (open column) the injections of 300 unit/kg EPO (total dose) to partially nephrectomized rats. Data represents the mean±SE of 5~6 rats.
Table 1. Effects of EPO injections on serum iron, UIBC and TIBC in the anemic rats

<table>
<thead>
<tr>
<th>Dose(U/kg)×Time</th>
<th>Routes</th>
<th>n</th>
<th>Serum iron(μg/dl)</th>
<th>UIBC(μg/dl)</th>
<th>TIBC(μg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>300×1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>i.v (A)</td>
<td>6</td>
<td></td>
<td>207.7±10.8a)</td>
<td>247.3±21.6</td>
<td>455.0±19.5</td>
</tr>
<tr>
<td>i.m (B)</td>
<td>5</td>
<td></td>
<td>202.4±26.2</td>
<td>228.8±25.7</td>
<td>431.2±42.8</td>
</tr>
<tr>
<td>s.c (C)</td>
<td>5</td>
<td></td>
<td>189.2±25.1</td>
<td>207.2±26.2</td>
<td>396.4±47.9</td>
</tr>
<tr>
<td>75×4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>i.v (D)</td>
<td>5</td>
<td></td>
<td>138.4±8.5</td>
<td>249.4±28.0</td>
<td>387.8±28.9</td>
</tr>
<tr>
<td>i.m (E)</td>
<td>6</td>
<td></td>
<td>163.5±7.1</td>
<td>252.3±18.2</td>
<td>415.8±21.8</td>
</tr>
<tr>
<td>s.c (F)</td>
<td>6</td>
<td></td>
<td>141.0±7.0</td>
<td>263.5±18.0</td>
<td>404.5±24.5</td>
</tr>
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<td>42×7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>i.v (G)</td>
<td>6</td>
<td></td>
<td>135.8±16.3</td>
<td>248.5±12.9</td>
<td>367.7±25.0</td>
</tr>
<tr>
<td>i.m (H)</td>
<td>6</td>
<td></td>
<td>119.3±5.7**b)</td>
<td>314.8±20.0</td>
<td>434.2±17.8</td>
</tr>
<tr>
<td>s.c (I)</td>
<td>5</td>
<td></td>
<td>115.0±10.2**</td>
<td>269.8±14.6</td>
<td>384.8±18.2</td>
</tr>
<tr>
<td>Saline×7</td>
<td>s.c</td>
<td>6</td>
<td>172.0±13.4</td>
<td>223.5±17.2</td>
<td>395.5±27.3</td>
</tr>
<tr>
<td>Sham ope.</td>
<td>9</td>
<td></td>
<td>203.8±16.1</td>
<td>417.9±15.5**c)</td>
<td>621.8±14.4**</td>
</tr>
</tbody>
</table>

a) Mean±S. E.
b) Significant difference from the saline control group (**: P<0.01).
c) Significant difference from the partially nephrectomized control rats (***: P<0.01).

Serum EPO titers of intramuscular and subcutaneous injection group were respectively 251.3±15.5 mU/ml and 183.3±13.3 mU/ml at 24 hr after the injection. These values were significantly higher than those in intravenous injection group (P<0.01).

DISCUSSION

As described in the previous report [7], partially nephrectomized rats showed the uremic anemia, and their serum EPO titer and reticulocyte count was not increased in spite of progress of anemia. These rats also showed the lower serum value of iron and total iron binding capacity (TIBC) than those of normal rats (Table 1). It has already been documented that the human patients with renal anemia were in the same conditions described above [5]. These facts indicate that these animals are available for investigating the effects of EPO injection on the improvement of renal anemia.

In this report, we examined about the administration schedule and injection route of EPO to know the rational treatment of renal anemia. The half life of erythrocytes is about 20 days [10], and the improved state of anemia by EPO injection lasts about 2 weeks [6, 8]. Therefore, hematological examinations were done at the last day of the two weeks experimental period. Repeating injections of EPO in divided doses caused stronger erythropoiesis than single injection. Especially, seven repeated injections promoted the strongest erythropoiesis with respect to the administration schedule of
300 units of EPO per kg body weight. On the other hand, both intramuscular and subcutaneous injections were more effective than intravenous injection concerning the injection route (Fig. 2).

It has been reported that polycythemic rats made by the injection of EPO showed the inhibition in the production of endogenous EPO, the significant depressions of iron incorporation into erythrocyte, and reticulocyte count [1]. When erythropoiesis declined, serum iron was not consumed and its concentration rised, while, accelerated erythropoiesis promoted the reduction of serum iron concentration by the consumption of iron [5]. Reticulocyte count increased at 4 days after EPO injection, but it fell down below the initial value at 2 weeks after the injection (Fig. 1). In the groups that received 300 units of EPO per kg body weight in one dose, reticulocyte count fell down below the initial value and their serum values of iron were higher than that in the control group (Fig. 2, Table 1). Opposite results were observed in the repeated injection groups, indicating that erythropoiesis was continued less than 2 weeks after single injection but erythropoiesis in repeated injections groups had been accelerated for 2 weeks.

The serum EPO titers after the injection were investigated to know the difference of erythropoietic effect among the three kinds of injection routes. When EPO was injected intravenously, the pattern of clearance of EPO titers showed biphasic decrease (an initial phase ranged from 0 to 4 hr and a secondary phase ranged from 4 to 24 hr). Serum EPO titers of other 2 groups (intramuscular and subcutaneous injection) gradually increased after the injection and reached the same level as the intravenous injection group after 4 to 8 hr. At 24 hr after intravenous injection of EPO, serum EPO titers were still maintained higher levels than those of non-treated rats but the titers were significant lower than those of intramuscularly and subcutaneously injected groups at 24 hr after the injection (P<0.01). Though the minimum effective level of serum EPO titer remains to be determined, these results speculate us that the duration of optimal serum EPO titer may be more important for erythropoiesis than its peak level after the EPO injection.

All these results show that repeated injections of EPO in divided doses promote stronger erythropoiesis under the fixed amounts of EPO. One bolus intravenous injection of excessive dose is considered to be wasteful because erythropoietic effect is dependent on the duration of serum EPO level which contributes to erythropoiesis. Therefore, for the clinical applications, it is necessary to determine the optimum prescription of EPO according to the degree of anemia.

REFERENCES


要　約

腎性貧血におけるエリスロポエチンの効果的投与法の検討：升永博明・上田正次・沢井忠則・川西悟生（雪印乳業株式会社生物科学研究所）——腎性貧血ラットを用いエリスロポエチン（EPO）の投与間隔と投与経路による造血効果の違いについて検討した。すなわちラットに体重1 kg 当たり300ユニットのEPOを全量1回で投与する群、4 回に分けて4日毎に投与する群、7 回に分けて2日毎に投与する群の3投与スケジュールで、静脈内、筋肉内、皮下投与の3投与経路からそれぞれ投与し、2週間後の貧血改善効果を比較した。各投与経路とも単回より4回、7回と低用量高頻度になるに従い強い造血を示したが、7回投与において静脈内投与群は、筋肉内、皮下投与群に比べ効果が弱かった。血清鉄濃度と網状赤血球数の所見から連続投与群では、2週間目の時点で未だ造血機能の亢進が維持されていたが、単回投与群では既に停止していることが示唆された。EPOの造血作用は最高血中濃度より有効血中濃度の持続時間に依存することが推察され、臨床応用に際し、一時的に大量のEPOを静脈内に投与するより、適正な用量を繰り返し投与し有効血中濃度を長期間維持することが腎性貧血の治療に効果的であると考えられた。