啓明醫大學術誌 第21券 1號
Keimyung Med J
Vol. 21, No. 1, June, 2002

Effect of High Concentration of Uremic Toxin on Erythropoiesis in Acute Blood Loss*

Kyung Dae Park, M.D.* and Kyo Cheol Mun, M.D.

Departments of Internal Medicine, and Biochemistry, Keimyung University School of Medicine, Taegu, Korea*

Abstract : During advanced renal failure, and particularly in patients with end-stage renal diseases (ESRD), proteins are carbamylated and lose biological activity as the result of a reaction with cyanate which is derived from urea. The effect of cyanate on the erythropoietic activity of erythropoietin (EPO) after acute blood loss in rats was studied. EPO was incubated with cyanate at 37℃. The erythrocytes count, hemoglobin concentration, hematocrit and leukocytes were measured after the subcutaneous injection of either normal saline, cyanate, EPO, or incubated EPO with cyanate. Normal saline or 1 M cyanate injected controls and the incubated EPO with cyanate injected animals demonstrated decrease from day zero in erythropoietic parameters. The parameters maintained in EPO injected group. These results support that EPO exposed to cyanate *in vitro* demonstrates diminished biologic activity in acute blood loss. This effect suggest that EPO is inhibited in the presence of cyanate under the stimulatory condition for EPO, and it may contribute to the suboptimal erythropoietic response to EPO therapy associated with high urea levels, especially related to inadequate dialysis.

Key Words : Acute blood loss, Anemia, Cyanate, End-stage renal diseases, Erythropoietin,

Introduction

Anemia is a common feature of end-stage renal disease (ESRD)[1]. Many studies were done about the mechanism of decreased production of erythropoietin (EPO)[2] including the presence of EPO inhibitors[2,3]. Although several factors including iron deficiency, aluminium intoxication and bone marrow fibrosis are known as the important causes of resistance to EPO[1], BUN level is also related to the EPO resistance[4-6].

In aqueous solution there is partial and

* The present research has been conducted by the Grant of Graduate School, Keimyung University, Taegu, Korea in 2001.

spontaneous decomposition of urea to ammonia, carbonate and cyanate[7]. Cyanate then subsequently reacts irreversibly with the N-terminal groups of amino acids, peptides and many proteins by a process known as carbamylation[8-10]. Under physiological conditions, 0.8% of the molar concentration of urea is spontaneously converted to cyanate at equilibrium at physiologic pH and body temperature[11]. Cyanate can react with proteins and carbamylates proteins including hemoglobins[11-13]. In patients with ESRD, proteins are known to be easily carbamylated and lose biologic activity as a result of reactions with urea-derived cyanate due to high levels of cyanate concentration in the plasma[11-14]. EPO is also known to be carbamylated by cyanate and may lose its biologic activity in normal rats[15]. However, it is not clear whether EPO is inhibited by cyanate in a condition under the active state of EPO is active such as acute blood loss. If we make the experiment with ESRD model in rats, several uremic toxins other than cyanate can also affect to EPO activity. We made the experimental model of acute blood loss model in rats.

In this paper, the role of cyanate, which is known as one of the uremic toxins, at high concentration as an inhibitor of EPO was studied in the rats after acute blood loss to explain the EPO resistance in patients with high levels of BUN due to inadequate dialysis.

Materials & Methods

1. Reagent

Recombinant EPO, Epokine prefilled-injection, was purchased from Cheiljedang Co. (Seoul, Korea). Cyanate was purchased from Acros Organics (New Jersey, USA). All other chemicals were of the highest purity commercially available.

2. Incubation of EPO with cyanate

Normal male rats of the Sprague-Dawley strain, weighing between 320 and 350g, were used in this experiment. To evaluate the effect of cyanate on the biologic activity of EPO, all the experimental groups, with 5 rats in each group, were divided as follows: 1) normal control group (Group 1); 0.3 mL of 0.9% NaCl was injected subcutaneously. 2) cyanate group (Group 2); 0.3 mL of 1 M cyanate was injected subcutaneously. 3) EPO group (Group 3); 2,000 units of EPO was dissolved in 1.0 mL of 0.9% NaCl, and 0.3 mL which is equivalent to 2,000 units per kg, was injected subcutaneously. 4) Incubated EPO with cyanate group (group 4); 2,000units of EPO was dissolved in 1.0mL of 1M cyanate by incubation at 37 ℃ for 6 hours, then 0.3 mL of incubated EPO with cyanate was injected subcutaneously.

With light ether anesthesia, 2 mL of blood was drawn from right jugular vein to make the state of acute blood loss at the day zero. The care of the animals is consistent with the NIH Guides for the Care and Use of Laboratory Animals. All animals were maintained on commercial pellets purchased from Sam Yang Food Co.

(Wonju, Korea).

3. Erythropoietic parameters

The levels of RBC, hemoglobin, hematocrit and leukocytes were measured using CELL-DYN 1,300 model from Abbott Diagnostics (Il, USA) before subcutaneous injection (day zero), and 72 hours after subcutaneous injection (day three).

All animals were maintained on commercial pellets purchased from Sam Yang Food Co. (Wonju, Korea).

4. Statistical analysis

Values were expressed as mean ± SD. Statistical evaluation of the difference between before and after subcutaneous injection was performed with paired t-test.

Results

The RBC counts in groups 1, 2 and 4 were decreased at the three days of the subcutaneous injections of saline, cyanate and incubated EPO with cyanate, respectively (Fig. 1). The RBC counts ($x10^6/\mu L$) decreased from 7.4 ± 0.3 to 6.1 ± 0.3 in the group 1 (normal control group, $P<0.01$), decreased from 7.4 ± 0.2 to 6.0 ± 0.2 in the group 2 (cyanate group, $P<0.01$), decreased from 7.7 ± 0.2 to 6.6 ± 0.3 in the group 4 (incubated EPO with cyanate group, $P<0.05$). However, it didn't decrease in the group 3 (EPO group).

The hemoglobin concentration (g/dL) is paralleled with those of the RBC counts (Fig. 2). It decreased from 14.6 ± 0.3 to 12.6 ± 0.1 in the group 1 ($P<0.001$), decreased from 14.3 ± 0.2 to 11.8 ± 0.2 in the group 2 ($P<0.01$), decreased from

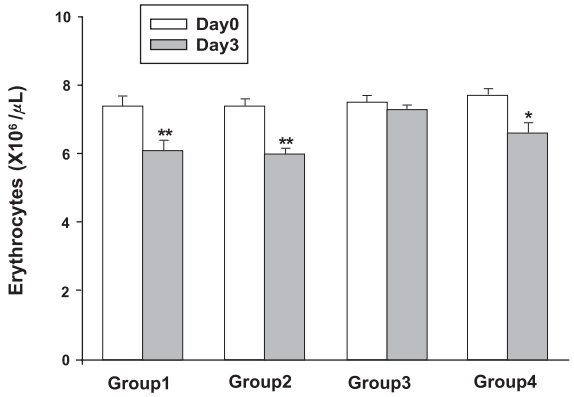

Fig. 1. Erythrocytes counts after carbamylated erythropoietin injection in rats. Group 1: normal control, Group 2: 1.0 M cyanate was injected, Group 3: erythropoietin was injected, Group 4: erythropoietin was injected after incubation with cyanate. *: $P<0.05$ vs. day 0, **: $P<0.01$ vs. day 0.

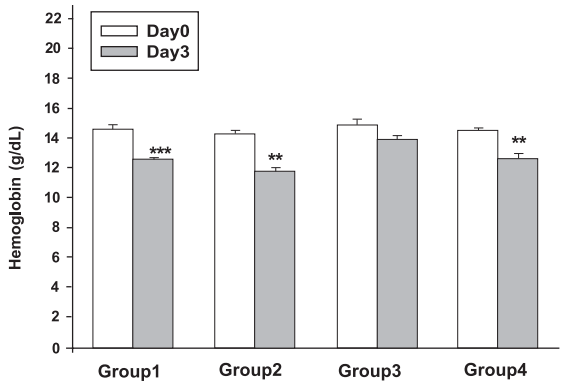

Fig. 2. Hemoglobin levels after carbamylated erythropoietin injection in rats. Group 1: normal control, Group 2: 1.0 M cyanate was injected, Group 3: erythropoietin was injected, Group 4: erythropoietin was injected after incubation with cyanate. **: $P<0.01$ vs. day 0, ***: $P<0.001$ vs. day 0.

14.6 ± 0.1 to 12.7 ± 0.3 in the group 4 (P<0.01). However, it didn't decrease in the group 3.

The parallelism continued with the hematocrit (%) results (Fig. 3). It decreased from 42.3 ± 1.1 to 33.4 ± 1.3 in the group (P<0.001), decreased from 42.5 ± 1.2 to 33.6 ± 1.1 in the group 2 (P<0.001), decreased from 42.1 ± 0.4 to 36.4 ± 1.2 in the group 4 (P<0.05), and decreased without significance from 43.3 ± 1.0 to 43.6 ± 0.8 in the group 3.

Leukocyte count was unchanged in all experimental groups (Fig. 4).

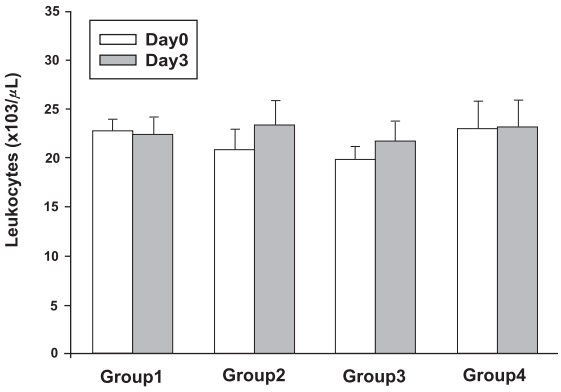

Fig. 4. Leukocytes levels after carbamylated erythropoietin injection in rats. Group 1: normal control, Group 2: 1.0 M cyanate was injected, Group 3: Erythropoietin was injected, Group 4: erythropoietin was injected after incubation with cyanate.

Discussion

Before the availability of recombinant EPO, the mainstays of anemia therapy in patients with ESRD were blood transfusion[1]. The clinical trials of recombinant EPO in patients with ESRD showed a good response in patients with anemia[1]. However, a study shows the suboptimal response to EPO therapy in some patients due to inadequate dialysis[4]. Patients with ESRD contains a material that is inhibitory to erythropoiesis rather than the absence of some substance necessary for normal erythropoiesis[3]. The nature of inhibitory substance of erythropoiesis is just known as a low molecular weight substance and the inhibitor can be removed by dialysis[3]. Moreover, inadequate dialysis assessed by measuring BUN levels is known as the important causes of resistance to EPO[4] and removal of inhibitory substance in some patients by dialysis makes an increase in hematocrit levels[3].

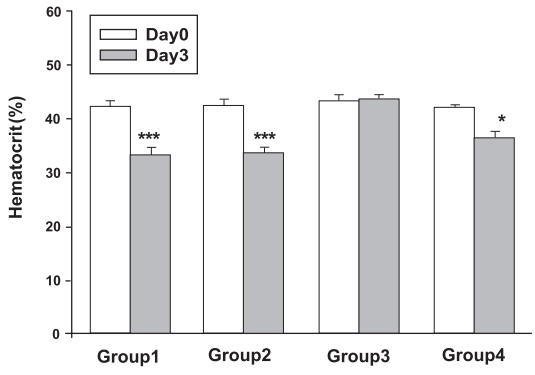

Fig. 3. Hematocrit levels after carbamylated erythropoietin injection in rats. Group 1: normal control, Group 2: 1.0 M cyanate was injected, Group 3: erythropoietin was injected, Group 4: erythropoietin was injected after incubation with cyanate. *: P<0.05 vs. day 0, ***: P<0.001 vs. day 0.

Under physiological conditions, 0.8% of the molar concentration of urea is converted to cyanate at equilibrium in vivo[11]. Cyanate, the molecular weight of which is about 43, can react with proteins and carbamylates proteins including hemoglo-

bins[11-13], low-density lipoprotein[16] and caeruloplasmin[11]. Under the state of ESRD, proteins are known to be easily carbamylated as a result of reactions with urea-derived cyanate due to high levels of cyanate concentration in the plasma[12-14,16]. EPO may also be carbamylated by cyanate from urea and may lose its biologic activity, thus causes anemia in patients with ESRD. According to Mun and Golper[15], EPO was carbamylated by high concentration of cyanate, thus resulted in the loss of its biologic activity in the normal rats. This study was done to evaluate whether cyanate results in the loss of biological activity of EPO or not in the rats with acute blood loss.

To evaluate the biological effect of incubated EPO with cyanate, EPO or incubated EPO with cyanate was injected to the rats. In control groups such as group 1 and 2, RBC, hemoglobin and hematocrit levels were decreased (Fig. 1, 2 and 3). When EPO was injected (group 3), the levels of erythrocytes, hemoglobin and hematocrit were maintained (Fig. 1, 2 and 3). However, when incubated EPO with cyanate was injected (group 4), the levels of these 3 erythropoietic parameters were similar with control groups which indicates the loss of EPO activity due to cyanate. Leukocytes counts, which was used as an another control marker, in all experimental groups showed no significant change (Fig. 4).

Some authors reported that reduction in blood urea nitrogen was significantly correlated with hematocrit level in patients with ESRD[4-5]. And according to Stojimirovic and Grujic[6], continuous ambulatory peritoneal dialysis is more successful for improving renal anemia, and patients on continuous ambulatory peritoneal dialysis show a significantly lower urea value compare to patients on hemodialysis due to better clearance during continuous ambulatory peritoneal dialysis. The data in this experiment may support or correlate these reports.

This experiment indicate that high concentration of uremic toxin such as cyanate is an inhibitor of EPO in patients with ESRD, and may explain one of the reasons for the suboptimal response to EPO therapy in patients with high levels of BUN due to inadequate dialysis.

Summary

During advanced renal failure, and particularly in patients with end-stage renal disease (ESRD), proteins are carbamylated and lose biological activity as a result of a reaction with cyanate which is derived from urea. The effect of cyanate on the erythropoietic activity of erythropoietin (EPO) after acute blood loss in rats was studied. EPO was incubated with cyanate at 37℃. The erythrocyte count, hemoglobin concentration, hematocrit and leukocytes were measured after the subcutaneous injection of either normal saline, cyanate, EPO, or incubated EPO with cyanate. Normal saline or 1 M cyanate injected controls and the incubated EPO with cyanate injected animals demonstrated decrease from day zero in erythropoietic parameters. The parameters maintained in EPO injected group. These results support

that EPO exposed to cyanate in vitro demonstrates diminished biologic activity in acute blood loss. This effect suggest that EPO is inhibited in the presence of cyanate under the stimulatory condition for EPO, and it may contribute to the sub-optimal erythropoietic response to EPO therapy associated with high urea levels, especially related to inadequate dialysis.

References

1. Remuzzi G, Rossi EC. Hematologic consequence of renal failure. In: Brenner BM, editor. *Brenner and Rector's the Kidney.* 5th ed. Philadelphia: Saunders Company; 1996, p. 2170-86.
2. Morris K, Coulthard M. End-stage kidneys are capable of increased erythropoietin production. *Pediatr Nephrol* 1993; **7**(3): 273-5.
3. Wallner SF, Vautrin RM. Evidence that inhibition of erythropoiesis is important in the anemia of chronic renal failure. *J Lab Clin Med* 1981; **97**(2): 170-8.
4. Ifudu O, Feldman J, Friedman EA. The intensity of hemodialysis and the response to erythropoietin in patients with end-stage renal disease. *N Eng J Med* 1996; **334**(7): 420-5.
5. Opatrna S, Opatrny K Jr, Cejkova P, Sefrna F, Hostasova A, Opatrny K. Relationship between anemia and adequacy of continuous ambulatory peritoneal dialysis. *Nephron* 1997; **77**(3): 359-60.
6. Stojimirovic B, Grujic-Adanja G. The effect of hemodialysis and continuous peritoneal dialysis on renal anemia. *Srp Arh Celok Lek* 1997; **125**(5-6): 163-7.
7. Marier JR, Rose D. Determination of cyanate, a study of its accumulation in aqueous solutions of urea. *Anal Biochem* 1964; **7**(2): 304-14.
8. Knochel JP, Seldin DW. The pathophysiology of uremia. In: Brenner BM, Rector FC, editors. *The Kidney,* Philadelphia: Saunders; 1976, p. 1448-85.
9. Fluckiger R, Harmon W, Meier W, Gabbay KH. Hemoglobin carbamylation in uremia. *N Engl J Med* 1981; **304**(14): 823-7.
10. Kraus LM, Jones MR, Kraus AP Jr. Essential carbamoyl-amino acids formed in vivo in patients with end-stage renal disease managed by continuous ambulatory peritoneal dialysis: isolation, identification, and quantitation. *J Lab Clin Med* 1998; **131**(5): 425-31.
11. Roxborough HE, Millar CA, McEneny J, Young IS. Carbamylation inhibits the ferroxidase activity of caeruloplasmin. *Biochem Biophys Res Commun* 1995; **214**(3): 1073-8.
12. Stim J, Shaykh M, Anwar F, Ansari A, Arruda JA, Dunea G. Factors determining hemoglobin carbamylation in renal failure. *Kidney Int* 1995; **48**(5): 1605-10.
13. Balion CM, Draisey TF, Thibert RJ. Carbamylated hemoglobin and carbamylated plasma protein in hemodialyzed patients. *Kidney Int* 1998; **53**(2): 488-95.
14. Nilsson L, Lundquist P, Kagedal B, Larsson R. Plasma cyanate concentrations in chronic renal failure. *Clin Chem* 1996; **42**(3): 482-3.
15. Mun KC, Golper TA. Impaired biological activity of erythropoietin by cyanate carbamylation. *Blood Purif* 2000; **18**(1): 13-7.
16. Horkko S, Savolainen MJ, Kervinen K, Kesaniemi YA. Carbamylation- induced alterations in low-density lipoprotein metabolism. *Kidney Int* 1992; **41**(5): 1175-81.