Acta Medica Okayama

Volume 55, *Issue* 6 2001 *Article* 6

DECEMBER 2001

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Mete Akisu[*] Sevgi Tuzun[†] Sertac Arslanoglu[‡]

Mehmet Yalaz[**] Nilgun Kultursay[††]

[*]Egu University Medical School,
[†]Ege University,
[‡]Egu Univershity Medical School,
[**]Egu University Medical School,
[††]Egu University Medical School,

Effect of recombinant human erythropoietin administration on lipid peroxidation and antioxidant enzyme(s) activities in preterm infants.*

Mete Akisu, Sevgi Tuzun, Sertac Arslanoglu, Mehmet Yalaz, and Nilgun Kultursay

Abstract

In the present investigation, we studied the effect of recombinant human erythropoietin (r-HuEPO) on serum malondialdehyde (MDA) as an index of lipid peroxidation, related to iron-catalyzed free radical reaction and erythrocyte superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX) activities in very-low-birth weight (VLBW) infants. Forty premature infants, at gestational ages were less than 33 weeks and birthweights were less than 1,500 g, were enrolled in the study. The study population was randomly divided into 2 groups. Twenty infants in Group 1 (treatment group) were given r-HuEPO, and 20 infants in Group 2 served as the control. r-HuEPO treatment (750 U/kg a week) was initiated on the 10th day of life and continued for 6 weeks. Preterm infants given erythrocyte transfusions during the study were excluded from the results. Serum ferritin and MDA levels, and erythrocyte superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX) activities were analyzed at the end of the first week of life (at the beginning of the study). Subsequently, serum ferritin, and MDA levels were measured at the end of the 3rd and the 6th week. SOD, CAT, and GPX activities in the hemolysate were analyzed at the end of the 4th week. Six infants in the control group and 1 infant in the r-HuEPO group received transfusions through the end of the study, and these infants were excluded from the results. Significantly decreased serum ferritin concentrations were found in the r-HuEPO group compared to those in the control group both at the end of the 3rd and the 6th week ($P < 0.05$, and $P < 0.01$, respectively). In addition, serum MDA levels were also significantly reduced in Group 1 compared to control both at the end of the 3rd and the 6th week ($P < 0.01$ and $P < 0.05$, respectively). A good correlation was found between serum MDA and ferritin levels in Group 1. When the 2 groups were compared with respect to activities of SOD, CAT, and GPX at the end of the 4th week, no differences were observed. Our findings in this study show that administration of r-HuEPO significantly decreases lipid peroxidation, but does not affect erythrocyte antioxidant

*PMID: 11779098 [PubMed - indexed for MEDLINE]

enzyme(s) activities in preterm infants. The mechanism responsible for the r-HuEPO-induced decrease in lipid peroxidation may concern inhibition to iron-catalyzed free radical reactions.

KEYWORDS: anemia of prematurity, erythropoietin, lipid peroxidation, superoxide dismutase, catalase, glutathione peroxidase

Acta Med. Okayama, 2001
Vol. 55, No. 6, pp. 357-362

Acta Medica Okayama
http://www.lib.okayama-u.ac.jp/www/acta/

Original Article

Effect of Recombinant Human Erythropoietin Administration on Lipid Peroxidation and Antioxidant Enzyme(s) Activities in Preterm Infants

Mete Akisu*, Sevgi Tuzun, Sertac Arslanoglu, Mehmet Yalaz, and Nilgun Kultursay

Departments of Pediatrics and Biochemistry, Ege University Medical School, Izmir 35100, Turkey

In the present investigation, we studied the effect of recombinant human erythropoietin (r-HuEPO) on serum malondialdehyde (MDA) as an index of lipid peroxidation, related to iron-catalyzed free radical reaction and erythrocyte superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX) activities in very-low-birth weight (VLBW) infants. Forty premature infants, at gestational ages were less than 33 weeks and birthweights were less than 1,500 g, were enrolled in the study. The study population was randomly divided into 2 groups. Twenty infants in Group 1 (treatment group) were given r-HuEPO, and 20 infants in Group 2 served as the control. r-HuEPO treatment (750 U/kg a week) was initiated on the 10th day of life and continued for 6 weeks. Preterm infants given erythrocyte transfusions during the study were excluded from the results. Serum ferritin and MDA levels, and erythrocyte superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX) activities were analyzed at the end of the first week of life (at the beginning of the study). Subsequently, serum ferritin, and MDA levels were measured at the end of the 3rd and the 6th week. SOD, CAT, and GPX activities in the hemolysate were analyzed at the end of the 4th week. Six infants in the control group and 1 infant in the r-HuEPO group received transfusions through the end of the study, and these infants were excluded from the results. Significantly decreased serum ferritin concentrations were found in the r-HuEPO group compared to those in the control group both at the end of the 3rd and the 6th week ($P < 0.05$, and $P < 0.01$, respectively). In addition, serum MDA levels were also significantly reduced in Group 1 compared to control both at the end of the 3rd and the 6th week ($P < 0.01$ and $P < 0.05$, respectively). A good correlation was found between serum MDA and ferritin levels in Group 1. When the 2 groups were compared with respect to activities of SOD, CAT, and GPX at the end of the 4th week, no differences were observed. Our findings in this study show that administration of r-HuEPO significantly decreases lipid peroxidation, but does not affect erythrocyte antioxidant enzyme(s) activities in preterm infants. The mechanism responsible for the r-HuEPO-induced decrease in lipid peroxidation may concern inhibition to iron-catalyzed free radical reactions.

Key words: anemia of prematurity, erythropoietin, lipid peroxidation, superoxide dismutase, catalase, glutathione peroxidase

Preterm infants are at high risk of developing a significant fall in hemoglobin and hematocrit values after birth. These infants frequently receive erythrocyte transfusions to correct anemia and improve oxygen

Received December 26, 2000; accepted June 18, 2001.
*Corresponding author. Phone:+90-232-342-69-90; Fax:+90-232-342-69-90
E-mail: makisu@med.ege.edu.tr (M. Akisu)

transport. Very-low-birth weight (VLBW) infants can require multiple erythrocyte transfusions. Several factors seem to be important in the development of anemia of prematurity, including blood losses, decreased erythrocyte life span, and increased free oxygen radicals [1, 2]. Recently, both clinical and laboratory investigations have established that inadequate production of erythropoietin (EPO) is the major factor in anemia of prematurity [2-7].

Advances in neonatology have markedly improved survival for premature infants, though long-term sequelae due to excessive use of oxygen have increased. Serious disorders such as bronchopulmonary dysplasia (BPD), retinopathy of prematurity (ROP), necrotizing enterocolitis (NEC), and intraventricular haemorrhage (IVH) are thought to be different presentations of a single disease process resulting from free oxygen radical production [8-10].

Iron is considered to be an important catalyst for free radical reactions. Specifically, iron catalyzes the formation of the highly reactive and hazardous hydroxyl radical ($OH^{\cdot}$) from hydrogen peroxide in the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\cdot} + OH^{-}$) [10-12]. If the production of these highly reactive molecules is increased, or if, alternatively, the organism fails to quench their production due to inadequate antioxidant defenses, cell injury, resulting from the initiation of lipid peroxidation, and protein (enzyme) and nucleic acid oxidation, occurs [11]. Lipid peroxidation not only causes damage to the cell membrane, but also leads to injury of the intracellular elements by aldehyde generation. The best known aldehyde generated during peroxidation is malondialdehyde (MDA) [8-11].

Controlled trials have demonstrated that administration of recombinant human erythropoietin (r-HuEPO) to preterm infants significantly diminishes erythrocyte transfusion requirements [2-7]. A rapid decline in serum iron and ferritin concentrations has been reported to be associated with treatment with r-HuEPO, despite iron supplementation [5, 13-15]. The concomitant fall in serum iron and ferritin levels reflects active erythropoiesis.

We hypothesized that the administration of r-HuEPO to preterm infants reduces the body's iron load and leads to inhibition of iron-catalyzed free radical reactions. The present study was designed to evaluate the effect of r-HuEPO on lipid peroxidation and the activities of erythrocyte antioxidant enzymes in VLBW preterm infants.

Materials and Methods

Forty premature infants at gestational ages of less than 33 weeks and birthweights less than 1,500 g were enrolled in the study. The exclusion criteria were birth weight lower than the 10th percentile for gestational age, presence of hemolytic disease, platelet count more than 750,000/mm^3, systemic blood pressure exceeding 90 mmHg, need for mechanical ventilation for more than 7 days, and presence of major congenital abnormalities and cyanotic heart diseases. The study population was randomly divided into 2 groups, consisting of 20 (Group 1) and 20 (Group 2) preterm infants, respectively. The 20 infants in Group 1 (treatment group) were given r-HuEPO, and the remaining 20 infants served as the control (Group 2). r-HuEPO treatment was initiated on the 10th day of life and continued for 6 weeks. Preterm infants requiring erythrocyte transfusions during the study were excluded from the results. The study was approved by the Ethical Committee of the Medical Faculty of Ege University, and was performed at the Neonatal Intensive Care Unit, University of Ege.

Table 1 presents the demographic characteristics of the study population. Group 1 infants were administered r-HuEPO (Recormon; Boehringer-Mannheim, Germany) subcutaneously, 3 times a week, totaling 750 U/kg a week. Because iron supplementation is necessary for optimal erythropoiesis, all infants were given oral $FeSO_4$ supplements (Ferro Sanol drops-Adeka, Istanbul, Turkey) at a dose of 3 mg/kg/d [2, 5-7]. Additionally, polyvitamin supplements (Dapta drops, Wyeth, CA, USA), vitamin D (Devit 3 drops-Deva, Turkey), and folate (Folbiol-ibrahim Ethem, Istanbul, Turkey) at a dose of 1 mg each were given to all of the infants. Five babies were fed only breast milk with human milk fortifier (Eoprotin-Milupa, Koln, Germany); 10 babies were fed

Table 1 Demographic characteristics of the study groups

	Group 1 (r-HuEPO)	Group 2 (Control)
N	19	14
Gestational age (week)	29.9 ± 1.3	31.0 ± 1.4
Birth weight (grams)	1245 ± 170	1315 ± 163
Gender (F/M)	10/9	6/8
Type of delivery		
Cesarian section	12	9
Normal spontaneous	7	5

a preterm formula (Prematil-Milupa, Koln, Germany); and 5 babies were fed both breast milk and the preterm formula.

Group 2 babies (control) were given the same polyvitamin supplements; vitamin D and folate at a dose of 1 mg each starting on the 10th day of life. $FeSO_4$ was not administered until the 6th week of life due to its oxidant nature. Eight babies were fed only breast milk with the same human milk fortifier; 8 babies were fed the same preterm formula; and 5 babies were fed both breast milk and the same preterm formula.

Blood was obtained by venipuncture for the examination of serum ferritin and MDA levels at the beginning of the study (pretreatment), and again after 3 and 6 weeks. Erythrocyte superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX) activities were measured at entry and after 4 weeks. The complete blood cell counts, with reticulocyte counts, were obtained at entry and after 2, 4, and 6 weeks. Serum ferritin concentration was examined by radioimmunassay.

Malondialdehyde (MDA) measurement. MDA, the most important product of peroxidation, was measured in serum samples by the method of Okhawa *et al.* [16] as a substrate reacting with thiobarbituric acid, and the results were expressed as nmol/L.

Measurement of erythrocyte antioxidant enzyme activities. Venous blood samples were taken into tubes containing EDTA and centrifuged. After separation of plasma, the packed erythrocytes were washed 2 times with 9 g/L NaCl solution then hemolysed with ice-cold water. The samples were stored at − 20 °C until use.

Activities of SOD, CAT, and GPX were evaluated in the hemolysate. SOD activity was measured by the method of Misra and Fridovich [17], based principally upon the inhibition of epinephrine autooxidation with SOD at 480 nm by LKB Ultraspect spectrophotometry. The enzyme activity inhibiting 50% of the autooxidation of epinephrine was defined as 1 U. CAT activity was measured by the method described in Aebi [18]. The enzyme level decompensating 1 μmol H_2O_2 in 1 min was defined as 1 U CAT activity. GPX activity in the hemolysate was measured by the method of Paglia and Valentine [19], using Cumen hydroperoxide as substrate (Ransel, GPX kit; Randox Laboratories Ltd., Manchester, UK). Enzyme activities were expressed as U/g Hb.

All values were presented as mean ± SD. The statistical analyses were performed by using Epistat and Instat computer programs. All laboratory data (means) of the 2 groups were compared using the unpaired Student's t test. Fischer's exact test was used to assess differences in the number of infants given transfusion and the number of transfusions between groups. The association between serum ferritin and serum MDA levels was compared using Pearson's correlation analysis. Statistical significance was defined at $P < 0.05$.

Results

Six infants in the control group and 1 infant in the r-HuEPO group required transfusions during course of the study ($P < 0.05$). These erythrocyte transfusions were administered to the infant in the r-HuEPO group, compared with 10 erythrocyte transfusions administered to infants in the control. These infants given erythrocyte transfusions were excluded from the results. Hemoglobin, hematocrit, and reticulocyte values during the study period are presented Table 2. Hemoglobin and hematocrit values were significantly higher in the r-HuEPO group than in the control at 2, 4, and 6 weeks after the treatment ($P < 0.05$, $P < 0.01$, and $P < 0.01$ respectively). A significantly higher reticulocyte count in the r-HuEPO group was noted from the 2nd to the 6th week of the study period ($P < 0.01$).

No significant difference was observed in the level of serum ferritin between the 2 groups before the therapy

Table 2 Hemoglobin, hematocrit, and reticulocyte values during the study period

	Group I (r-HuEPO)	Group 2 (Control)	*P*
Hemoglobin (g/dl)			
Pretreatment	9.8 ± 0.9	9.4 ± 0.7	NS
On 2nd week	9.3 ± 1.4	8.1 ± 0.9	< 0.05
On 4th week	10.1 ± 0.8	8.5 ± 0.5	< 0.01
On 6th week	10.8 ± 1.0	8.9 ± 0.6	< 0.01
Hematocrit (%)			
Pretreatment	29.7 ± 4.5	28.8 ± 3.9	NS
On 2nd week	29.8 ± 3.7	27.0 ± 2.8	< 0.05
On 4th week	31.6 ± 3.5	28.1 ± 2.5	< 0.01
On 6th week	32.4 ± 2.7	28.9 ± 2.2	< 0.01
Reticulocyte (%)			
Pretreatment	1.4 ± 1.1	1.5 ± 1.3	NS
On 2nd week	4.4 ± 2.7	2.1 ± 0.9	< 0.01
On 4th week	5.3 ± 2.3	2.6 ± 1.5	< 0.01
On 6th week	4.6 ± 2.6	2.7 ± 1.0	< 0.01

($P > 0.05$). At the end of the 3rd and the 6th week, the mean serum ferritin level showed a profound decline in Group 1 ($P < 0.05$) (Table 3). Serum MDA levels were similar in the 2 groups before initiation of the study ($P > 0.05$); a striking decline was observed in the levels in Group 1 at the end of the 3rd and the 6th week ($P < 0.01$ and $P < 0.05$, respectively) (Table 3).

In Group 1, a good correlation was found between serum MDA and ferritin levels at the end of the 3rd and the 6th week ($r = 0.6299$ $P < 0.05$ and $r = 0.7149$ $P < 0.01$, respectively). In Group 2, however, serum MDA levels did not correlate with serum ferritin levels at the end of the 3rd or the 6th week ($r = 0.2867$ $P > 0.05$ and $r = 0.3318$ $P > 0.05$, respectively). In addition, no correlation between serum MDA levels and serum ferritin concentrations was observed before the therapy in Group 1 or in Group 2 ($r = 0.3921$ $P > 0.05$ and $r = -0.2113$ $P > 0.05$, respectively).

When the 2 groups were compared with respect to the activities of SOD, CAT, and GPX, the activities of the respective enzymes were similar between groups before the treatment ($P > 0.05$). At the end of the 4th week no difference in activities of these enzymes was observed between groups ($P > 0.05$) (Table 4)

Table 3 Serum Ferritin (ng/ml) and MDA levels (nmol/L) of the study groups before and after the treatment (mean ± SD)

	Group I (r-HuEPO)	Group 2 (Control)	P
Ferritin			
Pretreatment	210 ± 103	218 ± 100	NS
On 3rd week	140 ± 52	198 ± 93	< 0.05
On 6th week	109 ± 68	163 ± 85	< 0.05
MDA			
Pretreatment	38.3 ± 7.1	36.1 ± 6.6	NS
On 3rd week	24.9 ± 5.7	31.3 ± 5.1	< 0.01
On 6th week	17.6 ± 4.9	21.8 ± 7.8	< 0.05

Table 4 Erythrocyte SOD, CAT, and GPX activities (U/gram Hb) of the study groups before and after the treatment (mean ± SD)

	Group I (r-HuEPO)	Group 2 (Control)	P
SOD			
Pretreatment	2946 ± 783	3120 ± 1417	NS
On 4th week	3188 ± 921	3203 ± 1016	NS
CAT			
Pretreatment	9660 ± 2914	10911 ± 3142	NS
On 4th week	9989 ± 2888	11876 ± 2903	NS
GPX			
Pretreatment	11.6 ± 3.3	10.9 ± 2.2	NS
On 4th week	12.1 ± 2.9	12.8 ± 2.4	NS

Discussion

Anemia of prematurity is a hyporegenerative, normochromic, normocytic type of anemia that is particularly profound in VLBW infants at 3–4 weeks of life, typically leading to frequent blood transfusions at 4–12 weeks of life. Recent studies have shown that r-HuEPO treatment increases postnatal red cell production in VLBW infants [2–7]. In this study, administration of r-HuEPO led to a significant rise in reticulocyte counts. This data provides evidence that administration of r-HuEPO enhances the rate of erythropoiesis. Our results have demonstrated and supported the relevant literature data that r-HuEPO administration is very useful for the prevention of anemia and for decreasing the need for blood transfusions in preterm infants.

MDA is a product of lipid peroxidation and is traditionally accepted as an indicator of lipid peroxidation. Lipid peroxidation in preterm infants is always much more active than in full term infants because of the frequent exposure to oxygen and immature/inadequate antioxidant systems in the former [10, 20]. We previously showed that serum MDA levels in preterm infants were higher than those in term infants [21].

Iron is an important catalyst for free oxygen radicals and lipid peroxidation reactions. We have hypothesized that administration of r-HuEPO mobilizes non-heme iron, and inhibits iron-catalyzed reactions. As shown in this study and previous studies, r-HuEPO treatment administered with additional iron still resulted in a significant decline in ferritin levels [5, 13–15]. The concomitant fall in serum ferritin levels reflects active erythropoiesis in preterm infants. The antioxidant effect of the iron chelator deferoxamine has been demonstrated in several animal models of free radical-mediated tissue injury [22, 23]. In addition, nutritional iron deficiency has been shown to be protective against several types of free radical-mediated injuries [24]. Inder *et al.* [25] reported that the frequency and severity of retinopathy of prematurity were correlated with high free iron levels, whereas Grifiths *et al.* [26] showed that prevalence of bronchopulmonary dysplasia declined with the administration of r-HuEPO.

Recently, Bany-Mohammed *et al.* [27] have shown that administration of r-HuEPO inhibits lipid peroxidation and reduces the severity of alveolar damage in prematurely born rabbits exposed to a high concentration of oxygen. In addition, we have recently demonstrated that oxygen-derived free radicals are involved in the genesis of necrotizing enterocolitis, and that administration of rHuEPO significantly decreased lipid peroxidation in hypoxia-induced intestinal injury [28]. In that study, we suggested that decrease of the free iron in circulation and intestinal tissue leads to the inhibition of Fenton reaction, and this inhibition prevents the formation of OHo radicals, which cause the most harmful and highly active lipid peroxidation. In the present study, we demonstrated that administration of r-HuEPO significantly inhibits MDA generation in circulating blood. Because we found a good correlation between serum MDA and ferritin levels in r-HuEPO group, the mechanism responsible for the r-HuEPO-induced decrease in lipid peroxidation may involve mobilization of non-hem iron, with less iron available for catalyzing lipid peroxidation.

In a healthy human, formation and inactivation of free oxygen radicals is balanced at a level at which the compounds can play their physiological role without any toxic side effects. This balance can be unstable in the perinatal period following rapid changes in the antioxidant defense enzyme activities [29]. Cellular defenses against oxidant injury include the antioxidant enzymes SOD, CAT, and GPX. The activities of these enzymes both in the erythrocyte and in the serum are low in preterm infants, and show an increase along with the gestational age [30]. However, in several studies, antioxidant enzyme activities have been shown to increase in response to oxidative stress [31, 32]. Rosenfeld *et al.* [29] showed that plasma SOD activities in preterm infants with respiratory distress syndrome, who were exposed to high oxygen tensions for prolonged periods, progressively increased in the first week of life. These studies indicate that preterm infants have the ability to induce antioxidant enzymes in response to oxidative stress. In this study, administration of r-HuEPO led to a significant rise in reticulocyte, hemoglobin, and hematocrit values. This treatment condition may contribute to increased antioxidant enzyme activities in the circulation. In the present study, however, we did not find any significant increase in the antioxidant enzyme activities in the r-HuEPO group as compared with the control.

In conclusion, the present study demonstrates that r-HuEPO treatment reduces the need for erythrocyte transfusions in VLBW infants. This study also shows that administration of r-HuEPO significantly decreases lipid peroxidation, though it does not affect erythrocyte antioxidant enzyme(s) activities in preterm infants. The possible mechanism responsible for the r-HuEPO-induced decrease in lipid peroxidation may be involve the inhibition of iron-catalyzed free radical reactions.

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