

# Antierythropoietin Antibodies in Hemodialysis Patients Treated with Recombinant Erythropoietin

## *Rekombinant Eritropoietin ile Tedavi Edilen Hemodiyaliz Hastalarında Antieritropoietin Antikorlar*

### ABSTRACT

**OBJECTIVE:** Erythropoietin resistance is a serious problem in patients treated with recombinant erythropoietin. Antierythropoietin antibodies are considered to be one of the causes of this resistance.

**MATERIAL and METHODS:** We investigated antierythropoietin antibodies in chronic hemodialysis patients and compared the results with healthy controls by means of establishing an ELISA method. A total of 121 chronic hemodialysis patients receiving recombinant erythropoietin were included in the study. The patients were subdivided according to the type of recombinant erythropoietin (erythropoietin- $\alpha$  or erythropoietin- $\beta$ ) they had been treated with in the last six months.

**RESULTS:** The absorbance values of patients were compared with the absorbance values of the control group by a specific and reproducible method. LOD (limit of detection) and LOQ (limit of quantitation) values were also calculated. The difference in the absorbance values between the therapy and control groups was statistically significant.

**CONCLUSION:** Both erythropoietin- $\alpha$  and erythropoietin- $\beta$  induce production of antibodies against erythropoietin. Anti rh-EPO antibodies may play a role in EPO resistance.

**KEY WORDS:** Erythropoietin, Erythropoiesis stimulating agents, Recombinant erythropoietin, Antierythropoietin antibody, Hemodialysis, Renal anemia

### ÖZ

**AMAÇ:** Eritropoietin direnci rekombinant eritropoietin ile tedavi edilen hastalarda ciddi bir sorundur. Antieritropoietin antikorlar, bu direncin nedenlerinden biri olarak kabul edilir.

**GEREÇ ve YÖNTEMLER:** Kronik hemodiyaliz hastalarında ELISA yöntemi oluşturmak suretiyle antieritropoietin antikorları araştırıldı ve sonuçları sağlıklı kontrollerle karşılaştırıldı. Rekombinant eritropoietin alan 121 kronik hemodiyaliz hasta çalışmaya alındı. Hastalar bu son altı ay içinde tedavi edilmiş rekombinant eritropoietinin (eritropoietin -  $\alpha$  ya da eritropoietin -  $\beta$ ) tipine göre ayrıldı .

**BULGULAR:** Hastaların absorbans değerleri spesifik ve çoğaltılabilir bir yöntem ile kontrol grubunun absorbans değerleri ile karşılaştırıldı. LOD (saptanma limiti) ve LOQ (niceliklendirme sınır) değerleri de hesaplandı. Tedavi ve kontrol grubu arasında absorbans değerleri farkı istatistiksel olarak anlamlı idi.

**SONUÇ:** Hem eritropoietin- $\alpha$  hem de eritropoietin- $\beta$  eritropoietine karşı antikorların üretimini başlatabilir. Anti rh-EPO antikorları EPO direncinde rol oynayabilir.

**ANAHTAR SÖZCÜKLER:** Eritropoietin, Eritropoez uyarıcı ajanlar, Rekombinant eritropoietin, Antieritropoietin antikor, Hemodiyaliz, Renal anemi

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## INTRODUCTION

Erythropoietin (EPO) is an erythropoiesis stimulating agent (ESA), composed of a glycosylated protein consisting of 165 amino acids and four carbohydrate side chains with a molecular weight of 30400 DA (1). The gene responsible for the synthesis of EPO is localized on the 7<sup>th</sup> chromosome (2). It is synthesized in renal fibroblasts and secreted to the blood circulation. The physiological role of EPO is to increase the oxygen transport capacity of blood and all forms of oxygen deprivation act as a stimulus for EPO production (3). The erythropoietin receptor is placed on progenitor cells membrane at the bone marrow. It stimulates erythroid progenitor cells for differentiation and proliferation. EPO receptors have tyrosine kinase activity (4).

The main cause of anemia observed in patients with renal failure is EPO deficiency and the type of anemia is normochromic normocytic (5). New treatment opportunities have prolonged the survival of chronic kidney disease (CKD) patients and anemia has therefore become a more serious problem.

Recombinant human EPO (rh-EPO) a therapeutic choice since the late 1980's, and has resulted in significant reduction of blood transfusion requirements in renal failure patients. At present, ESAs are routinely used as long-term treatment in the vast majority of patients with renal failure. Pure red cell aplasia (PRCA) may be observed in some patients during ESA treatment. This is a dilemma and a specific humoral immune response against rh-EPO is accused as the reason of this phenomenon (6). Endogenous EPO and rh-EPO have different patterns of glycosylation, which primarily involve the sialic acid composition of oligosaccharide groups. EPO- $\alpha$  (Johnson & Johnson, Manati, Puerto Rico) and EPO- $\beta$  (Roche, Mannheim, Germany) are produced by recombinant methods and have slight differences in glycosylation. rh-EPO  $\alpha$  has more sialic acid residues than rh-EPO- $\beta$ . Endogen EPO and rh-EPO have different electrophoretic mobility patterns (7).

Detection of antibodies against EPO is an important part of the diagnosis of PRCA. While there are several available tests for the detection of anti-EPO antibodies, each with its own advantages and disadvantages, none of these assays has any extra advantages to be preferred over others (8). Although radioimmunoprecipitation assay (RIPA) seems to be the most accurate test for detecting anti-EPO antibodies, it is not standardized and while enzyme linked immunosorbent assays (ELISAs) are more widely available, they appear to have lower sensitivity and specificity than RIPA (9, 10).

The aim of this study was to establish an ELISA method for detection of anti-EPO antibodies and to compare the sera of patients on rh-EPO treatment with those of a healthy group.

## MATERIAL and METHOD

**Patients Groups:** A total of 121 (55 females and 66 males) hemodialysis patients were included in the study.

Mean duration of hemodialysis was  $44.28 \pm 42.85$  (min: 3, max: 216) months. The CKD etiology was hypertension in 39, diabetic nephropathy in 38, polycystic kidney disease in 3, nephrolithiasis in 3, pyelonephritis in 1, Alport syndrome in 1, familiar Mediterranean fever in 1, chronic obstructive disease in 1 and chronic glomerulonephritis in 11 patients. The etiology of CKD was unknown in 23 patients.

Patients with any kind of malignancy, autoimmune disease or any disease that might affect immunity or those using any kind of immunosuppressive drugs were excluded from the study. Data on the use of rh-EPO were obtained from clinical records. The dose of rh-EPO, 75-150 IU/kg per week, was applied subcutaneously according to the patients' requirement based on red blood cell counts and iron metabolism parameters.

Patients were divided into three groups according to their last rh-EPO type which they had been administered for the last six months.

- Group A (71 patients) treated only with rh-EPO- $\alpha$
- Group B (22 patients) treated only with rh-EPO- $\beta$
- Group C (28 patients) treated with both rh-EPO- $\alpha$  and rh-EPO- $\beta$
- Fifteen female and 18 male healthy volunteers were selected as controls (Group D). They were apparently normal without any renal or hematological pathology.

**Sampling:** Fasting peripheral blood samples were taken in the morning and collected into gel separator tubes and centrifuged. The rotor was operated for 15 minutes at room temperature and 1500 g. The sera were stored at -20 °C for three-months.

**Reactive and Chemicals:** All chemicals were reagent grade. Uncoated ELISA plaques were obtained from Nagle Nuch Company. Antigens: Rh-EPO- $\alpha$  (Eprex) was obtained from Santa-Farma and rh-EPO- $\beta$  (NeoRecormon) from Roche. Bovine serum albumin (BSA) was obtained from Sigma (product no: A3803). Conjugate was obtained from Sigma (product no: A8792) and substrate was obtained from Trinity Biotech Company.

**Carbonate-Bicarbonate Buffer:** Solution A consisting of  $\text{Na}_2\text{CO}_3$  10.6 g/L (Merck), and solution B consisting of  $\text{NaHCO}_3$  8.4 g/L (Merck) were prepared and 270 ml of solution A was mixed with 730 ml of solution B to prepare 1 liter carbonate-bicarbonate buffer at pH 9.5 (11).

**Phosphate Buffered Saline (PBS):** For 1 liter buffer, NaCl 8 g (Merck), KCl 200 mg (Merck),  $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$  4.22 g (Merck)  $\text{KH}_2\text{PO}_4$  240 mg (baker) were dissolved in deionized water, the pH was adjusted into 7.4 and completed into 1 liter (12).

**Wash Solution:** 2% tween 20 (Merck) in PBS.

**Washing Procedure:** The automated ELISA plaque washer (ELX-50) were used to wash micro wells with the 350 ml wash solution and this step was repeated 5 times

**ELISA Procedure:** We have determined optimal concentrations of samples and conjugate via the Gaetano Castelli et al study (8). Step 1: Coating the plaques: Rh-EPO's were diluted into 500 IU/ml with carbonate-carbonate buffer and 200 ml pipetted into micro wells. Plaques were incubated for 24 hours at room temperature and were washed. Artifacts of plastic were blocked with BSA to prevent nonspecific bindings. Coated plaques were incubated with 5 % BSA (200 ml per well) for 24 hours at room temperature again. Plaques were ready to use. Step 2: Applying samples: All samples (patient and control) were diluted 1/10 with wash solution and pipetted into 200 ml wells. They were incubated for 24 hours at room temperature and washed. Step 3: Applying conjugate: Horseradish peroxidase labeled antihuman Ig G was used as conjugate. We diluted conjugate 1/2500 with PBS. 200 ml conjugate solution was pipetted into each micro well. Plaques were incubated for three hours at room temperature and were washed. Step 4: Applying substrate: Tetramethylbenzidine (TMB) was used as substrate (ready for use), and pipetted 200 ml into each microwell. Plaques were incubated 30 minutes at room temperature. Step 5: Applying stop solution: The reaction was stopped with 1.5 N 200 ml H<sub>2</sub>SO<sub>4</sub>. Step 6: Reading. The absorbance of color appearing at the end of the procedure was read at 450 nm with the ELX-800 ELISA reader.

All samples were studied both with rh- EPO- $\alpha$  and rh-EPO- $\beta$  coated plaques.

**Statistics:** The obtained data were evaluated with the SPSS 13 program. In the absence of a positive calibrator, we could not calculate concentrations of antierythropoietin antibodies. We used absorbance values for statistical calculations in this study. All plaques were prepared in pairs and all samples were analyzed twice. The samples with an absorbance value difference greater than 5% between two analyses were excluded from the study.

All groups were compared with ANOVA analysis and than we applied other statistical tests to determine which group was different. There was an adequate number of samples in group A and the control group so we used Student's t test. However, we used a non parametric test for comparing groups B and C with the control group. We applied the Bonferroni correction and selected a p value of 0.0125 for the four groups. The 95% confidence interval was chosen for all statistical analyses.

**RESULTS**

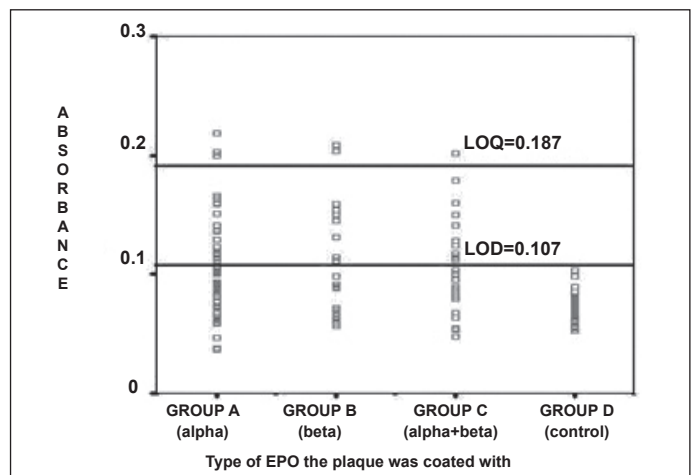
Table I gives the demographical and biochemical values of the patient and control groups. The absorbance values of each individual are presented in Figure 1 and 2. In the absence of positive external calibrator, we tried to detect the possible anti-EPO antibodies positive patients by calculating LOD and LOQ values (11, 13).

**LOD:** It is the detection limit of any analytical procedure for which analysis is just feasible. A series of blank (negative)

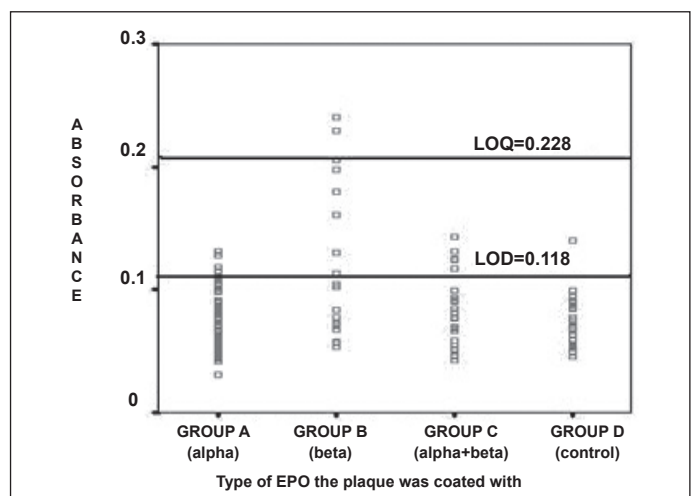
**Table I:** The demographic and biochemical values of the patient and control groups.

	Patient (no=121)	Control (no=33)
Age	51±17	51±14
Hemoglobin (mg/dl)	9.7±1.41	14.74±0.99
Hematocrit (%)	28.94±4.59	42.97±2.73
MCV ( $\mu\text{m}^3$ )	81.71±3.36	84.68±3.43
Creatinine (mg/dl)	8.11±2.79	0.82±0.16
Urea (mg/dl)	146±36	32±16

MCV: Mean corpuscular volume



**Figure 1:** The absorbance values obtained from the rh-EPO- $\alpha$  coated plaque. The absorbance values of each sample in the groups are demonstrated. Calculated LOD and LOQ values are shown.



**Figure 2:** The absorbance values obtained from the rh-EPO- $\beta$  coated plaque. The absorbance values of each sample in the groups are demonstrated. Calculated LOD and LOQ values are shown.

samples (a sample containing no analyte but with a matrix identical to the average sample analyzed) are tested and the mean blank value and standard deviation (SD) are calculated. We calculated the LOD value with the control group because we know that anti-EPO antibody is not a regular parameter detected in normal individuals. LOD= mean of absorbance value of the control group + 3 SD.

**LOQ:** The limit of quantitation (LOQ) or concentration is the quantitative result that can be reported with a high degree of confidence. LOQ can also be calculated by using the control group mean and SD values. LOQ= mean of absorbance value of control

group + 10 SD. The scatter of each patient's absorbance values obtained from the rh-EPO- $\alpha$  coated plaque is demonstrated in Figure 1 and Table II shows the comparison of group absorbance values. The scatter of each patient's absorbance values obtained from the rh-EPO- $\beta$  coated plaque is demonstrated in Figure 2 and Table III shows the comparison of group absorbance values. The absorbance values of groups obtained from the rh- EPO- $\alpha$  coated plaque were significantly different ( $p=0.0004$ ) and the absorbance values of groups obtained from rh-EPO- $\beta$  coated plaque were significantly different ( $p=0.01$ ). The statistical results as a whole are presented in Table IV.

**Table II:** The comparison of group absorbance values obtained from the rh-EPO  $\alpha$  coated plaque.

	Group			
	A	B	C	D
Mean ABS	0.100±0.036	0.107±0.047	0.101±0.035	0.072±0.011
Minimum ABS	0.037	0.057	0.048	0.053
Maximum ABS	0.219	0.209	0.203	0.103
Patients above the LOD value	70 57%	9 40%	10 35%	0 0%
Patients above the LOQ value	3 4.2%	2 9%	1 3.5%	0 0%

**Table III:** The comparison of group absorbance values obtained from rh-EPO  $\beta$  coated plaque.

	Group			
	A	B	C	D
Mean ABS	0.075±0.022	0.111±0.067	0.078±0.027	0.073±0.015
Minimum ABS	0.031	0.054	0.43	0.046
Maximum ABS	0.131	0.240	0.143	0.099
Patients above the LOD value	3 2%	7 31%	4 18%	0 0%
Patients above the LOQ value	0 0%	2 9%	0 0%	0 0%

**Table IV:** The obtained results of statistical analysis for each group.

	Group A	Group B	Group C
Rh-EPO $\alpha$ coated plaques	The difference between mean values is statistically significant ( $p<0.01$ )	$Z=  -2.83  > 2.58$ Group B is different from the control group ( $p<0.01$ )	$Z=  -3.46  > 3.28$ Group B is different from the control group ( $p<0.001$ )
Rh-EPO $\beta$ coated plaques	The difference between mean values is not statistically significant ( $p>0.05$ )	$Z=  -2.26  > 1.96$ Group B is different from the control group ( $p<0.05$ )	$Z=  0.558  < 1.96$ Group B is not different from the control group ( $p>0.05$ )



**Precision of test:** For calculating the coefficient of variance (CV), we prepared three serum pools: 1. Negative pool: From five negative samples. 2. Anti rh-EPO- $\alpha$  positive pool: From two patients giving high absorbance values bigger than the LOQ value with the rh-EPO- $\alpha$  coated plaque. 3. Anti rh-EPO- $\beta$  positive pool: From two patients giving high absorbance values bigger than the LOQ value with the rh-EPO- $\beta$  coated plaque. We studied each pool 10 times and CV values were calculated (Table V).

**Table V:** The precision of established test and calculated coefficient of variance (CV) values are represented.

	Negative pool	Positive pool
rh-EPO- $\alpha$ coated plaque	4.5%	6.9%
rh-EPO $\beta$ coated plaque	4.8%	8.4%

## DISCUSSION

The Kidney Disease Improving Global Outcomes (KDIGO) 2012 guidelines for anemia in chronic kidney disease suggest that PRCA due to anti-EPO antibodies should be evaluated in patients exposed to at least eight weeks of ESA therapy who develop all of the following: 1) decline in hemoglobin level of  $>0.5$  to  $1.0$  g/dL/week or transfusion requirement of at least 1-2 units/week to maintain adequate hemoglobin, 2) normal leukocyte and thrombocyte count, 3) absolute reticulocyte count of  $<10,000/\text{microL}$  (14). Determination of presence of anti-EPO antibodies in such patients is crucial. When the diagnosis of PRCA is confirmed, termination of EPO therapy and initiation of immunosuppressive therapy are recommended (6, 15). The presented study indicates that the absorbance values of patients treated with rh-EPO- $\alpha$  against the plaque coated also with rh-EPO- $\alpha$  are higher than the control group and statistically significantly different (Table II, Table IV). Similarly, Table III and IV indicate that absorbance values of the patients treated with rh-EPO- $\beta$  against the plaque coated also with rh-EPO- $\beta$  are higher than the control group and statistically significantly different. Interestingly while group B gave a response against the rh-EPO- $\alpha$  coated plaque, group A did not give a response to the rh-EPO- $\beta$  coated plaque. Similar cross reaction to EPO- $\alpha$  has been reported in other studies (9, 16). It is possible to think that rh-EPO- $\alpha$  has different epitopes from rh-EPO- $\beta$  but rh-EP- $\beta$  has similar epitopes to rh-EPO- $\alpha$ . This knowledge can be used while planning the EPO resistant anemic patient's further therapy. Antibody clearance time is dependent on the concentration of antibody in the circulation and the half-life of antibody clearance is 5 days, leading to very low antibody concentrations six months after stopping exposure to antigen (17, 18). We therefore did not exclude the patient who used both kind of rh-EPO earlier than the last 6 six months for clearance of possible previous anti-EPO antibodies.

The presence of anti-EPO antibodies should be verified by using another method of detection such as radioimmunoprecipitation or passive gel immuno diffusion. Moreover it is important to investigate the immunogenic part of the EPO molecule. Recently, Mytych et al. (19, 20) tried to develop more sensitive and standard methods for detection and standardization anti-EPO antibody and observed different results when using EPO- $\alpha$  or EPO- $\beta$ . Since these rh-EPOs differ only in their carbohydrate content, the suspected antibodies should be directed against the carbohydrates. This hypothesis should be discussed and experimentally addressed using enzymatically deglycosylated EPO.

On the other hand, there is no direct relationship between the rate of ESA hyporesponsiveness and anti-EPO antibodies in the literature. In the study of Kharagjitsingh et al. (21) using the serum specimens of fifty-seven patients who had an inadequate EPO response, only two were positive for anti-EPO antibodies. Similarly, Stoffel et al. (22) found out only 6 such patients among 536 hypo- and normoresponsive patients treated with epoetins for renal anemia.

Schett et al. (23) have shown anti-EPO antibodies in autoimmune diseases such as systemic lupus erythematosus. Therefore, we can say not only that the foreign side chain of rh-EPO induces antibody production but also that a part of the EPO peptide chain may be immunogenic. Elliot et al. (24) isolated and characterized three different monoclonal and two different polyclonal antibodies against rh-EPO obtained from Chinese hamster ovary at their study.

## CONCLUSION

These findings together with the clear evidence obtained in our experiments, where all healthy donors had absorbance values lower than those of patients treated with rh-EPO, clearly indicate that an EPO-specific immune response is possible and, more importantly, seems to be strictly related to rh-EPO administration. This shows that anti rh-EPO antibodies may play a role in ESA resistance.

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