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The relationship between the dose of continuous erythropoietin receptor activator and oxidative stress in hemodialysis patients

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Abstract. *At present, there have been no reports on the dose-dependent effects of continuous erythropoietin receptor activator (CERA) therapy on oxidative stress and red blood cell membrane lipid peroxidation parameters in hemodialysis (HD) patients.*

The aim of our work was to evaluate whether the dose of CERA treatment affected lipid peroxidation and antioxidant system in HD patients.

Methods. 38 HD patients were included in this single-center cross-sectional observational study. The study protocol was approved by a local Ethics Committee and all patients provided signed informed consent. The patients were stratified into quartiles (≤25% and ≥75%) according to the average dose of continuous erythropoietin receptor activator (CERA) and grouped in the following way: Group I (> 6 months of CERA treatment in a low dosage ≤ 50 μg/month, n = 20) and Group II (> 6 months of CERA treatment in a high dosage ≥ 125 μg/month, n = 18). Along with the standard diagnostic methods, we defined the content of malondialdehyde levels in the serum (MDAs) and erythrocytes (MDAe) spectrophotometrically as an indicator of lipid peroxidation. Such parameters as the concentration of ceruloplasmin (CP) and transferrin (TR) in the blood and total peroxidase activity (TPA) in erythrocyte were studied as the indicators of antioxidant system. In addition, we calculated the percentage of hemolysis, the RBC membrane permeability and oxidation coefficient.

Results. We obtained heterogeneous results in assessing oxidative stress parameters. The significantly higher levels of CP ($p = 0.007$) and TR ($p = 0.0003$) were found in the patients treated with a high dosage CERA. TPA activity in erythrocyte in the patients of Group II was statistically higher compared to Group I ($p = 0.02$). Moreover, we determined a statistically high percentage of hemolysis ($p = 0.03$) and RBC membrane permeability ($p < 0.0001$) in the patients who were treated with CERA in a dose ≥ 125 μg/month compared to other patients. Using the probit regression model, we established the dose-dependent effect of CERA on the level of RBC membranes permeability: $\chi 2 = 21$; $p =< 0.0001$.

Conclusions. We demonstrated that administration of CERA in a dose more 125 μg/month improved the antioxidant status in HD patients. But, at the same time, it increased the hemolysis and RBC membranes permeability. Our preliminary data pointing to the dose-dependent effect of CERA on the RBC membrane lipid peroxidation parameters require further confirmation.

Key words: *hemodialysis patients,continuous erythropoietin receptor activator, oxidative stress.*

Conflict of interest statement: all the authors declared no competing interests.

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Взаємозв'язок між дозою тривалого активатора рецепторів еритропоетину та оксидативним стресом у хворих, які лікуються методом гемодіалізу

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Резюме. *Наукові дані щодо дозозалежного впливу тривалого активатора рецепторів еритропоетину (ТАРЕ) на показники оксидативного стресу та перекисного окислення ліпідів мембран еритроцитів у хворих на хронічну хворобу нирок V, які лікуються гемодіалізом (ГД), є обмеженими.*

Метою нашої роботи було оцінити взаємозв'язок між застосованою дозою ТАРЕ та інтенсивністю оксидативних процесів у ГД пацієнтів.

Матеріал та методи. 38 ГД пацієнтів були включені до одномоментного обсерваційного дослідження. Протокол дослідження був схвалений локальною етичною комісією ДУ «Інститут нефрології НАМН України». Усі пацієнти надали письму інформовану згоду на участь у досліджені. Пацієнти були стратифіковані за квартилями (≤ 25% та ≥ 75%) відповідно до середньої дози ТАРЕ і розподілені наступним чином: I групу склали хворі, які отримували ТАРЕ більше 6 місяців у дозі ≤ 50 мкг/міс (n = 20), до II групи увійшли хворі, у яких доза ТАРЕ склала ≥ 125 мкг/міс (n = 18). Поряд із стандартними діагностичними методами досліджували концентрацію малонового діальдегіду у сироватці (МДА) й еритроцитах (МДАе), вміст у крові церулоплазміну (ЦП) та трансферину (ТР). Крім того, визначали загальну пероксидазну активність (ЗПА) у еритроцитах, проникність еритроцитарних мембран (ПЕМ), підрахували відсоток гемолізу.

Результати. У ГД пацієнтів, які лікувались ТАРЕ у дозі ≥ 125 мкг/міс визначено підвищення концентрації ЦР (p = 0,007) та ТР (p = 0,0003), тоді як ЗПА була статистично значуще зниженою порівняно з II групою (p = 0,02). Крім того, У пацієнтів I групи встановлено вищий відсоток гемолізу (p = 0,03) та ПЕМ (p < 0,0001). Використовуючи модель пробіт-регресії, ми визначили дозозалежний вплив ТАРЕ на рівень ПЕМ: $\chi^2 = 21$; p < 0,0001.

Висновки. Таким чином, застосування ТАРЕ у дозі понад 125 мкг на місяць покращує антиоксидантний статус ГД хворих, але разом з тим, підвищує гемоліз еритроцитів та ПЕМ. Отримані нами дані потребують подальшого підтвердження.

Ключові слова: *гемодіаліз, тривалий активатор рецепторів еритропоетину, оксидативний стрес.*

Introduction. Oxidative stress (OS) is a constituent of the inflammatory mechanisms that contributes to anemia in hemodialysis (HD) patients. The intensity of OS is closely correlated with anemia and associated with poor clinical outcomes [1]. One of the possible causes of anemia association with oxidative stress is considered red blood cell (RBC) membrane lipid peroxidation due to chronic hemolysis in HD patients [2].

Nowadays, the prescribing of erythropoiesis-stimulating agents (ESA) plays an indispensable role in clinical practice for the treatment of anemia. Today, regular supplements of intravenous iron and ESA are standard therapies in the treatment of anemia in HD patients [3, 4].

There are a lot of current studies devoted to the effects of ESA on oxidative status in HD patients [5-9]. But, the results of these studies are contradictory.

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Several clinical reports have demonstrated the positive impact of ESA on OS status in HD patients [7, 8] while other scientists have found no antioxidant effects of ESA [9, 10].

Continuous erythropoietin receptor activator (CERA) is a newer, longer acting ESA based on its lower frequency of administration [11]. However, at the present time, there have been no reports on the dose-dependent effects of CERA therapy on oxidative stress and red blood cell membrane lipid peroxidation parameters in HD patients.

Objectives. Therefore, the aim of our work was to evaluate whether the dose of CERA treatment affected lipid peroxidation and antioxidant system in HD patients.

Materials and methods. Study Design and Subjects. 38 HD patients were included in this single-center cross-sectional observational study which was conducted at State Institution «Institute of Nephrology of the National Academy of Medical Sciences» in Kyiv, Ukraine. The study protocol was approved by a local Ethics Committee and all patients provided signed informed consent.

The enrolment criteria were: patients aged >18 years who were at least six months on HD treatment, with a stable clinical condition and normally functioning arteriovenous fistula. We excluded the patients with erythropoietin-resistant anemia, systemic disease, diabetes mellitus, malignancy, acute inflammation processes, immunosuppressive treatment and active hepatitis.

The patients were stratified into quartiles (≤25% and ≥75%) according to the average dose of CERA and grouped in the following way: Group I (> 6 months of CERA treatment in a low dosage≤ 50μg/month, n = 20) and Group II (> 6 months of CERA treatment in a high dosage ≥ 125 μg/month, n = 18).

Dialysis prescription. All patients were routinely dialyzed three times a week, 4 h per session with bicarbonate based dialysate, volumetric ultrafiltration control, single use synthetic (polysulphone) dialyzers and heparin as a standard anticoagulant. Dialysis prescription was guided by a goal of achieving a value of Kt/V≥1.2.

Anemia treatment. Treatment of anemia was carried out in accordance with the clinical protocol of medical care "Treatment of patients with chronic kidney disease stage V with anemia" approved by the Ministry of Health of Ukraine [12]. Erythropoietin was prescribed via a standardized algorithm. All of the 38 HD patients received subcutaneous CERA (methoxy polyethylene glycol-epoetin beta) and intravenous iron replacement therapy. The dose of CERA was adjusted to maintain the individual patient's Hb within a range of ±10 g/L of the reference hemoglobin (Hb) concentration to achieve a hemoglobin value of 110-120 g/l. The iron dose was adjusted to reach ferritin and transfer saturation (TSAT) levels of 300-400 ng/ml and 30-40%, respectively. 100 mg of iron sucrose was administered in a dilution with 100 ml saline as 30 minute intravenous infusion at the end of HD session. Iron supplementation was temporarily discontinued in the patients with serum ferritin >800 ng/mL or TSAT>50% until serum ferritin decreased to <800 ng/mL and TSAT to <50% [12].

Methods. All measurements were performed after an overnight fast between 7.00–9.00 a.m. during a midweek non-dialysis day. The blood samples were processed immediately after sampling. Along with the standard diagnostic methods, we defined the content of malondialdehyde levels in the serum (MDAs) and erythrocytes (MDAe) spectrophotometrically as an indicator of lipid peroxidation. Such parameters as the concentration of ceruloplasmin (CP) and transferrin (TR) in the blood and total peroxidase activity (TPA) in erythrocyte were studied as the indicators of antioxidant system. In addition, we calculated the percentage of hemolysis AND the RBC membrane permeability.

Preparation of red blood cells: red cell suspensions for blood were taken with an anticoagulant (heparin) and centrifuged for 10 minutes at 3000 g. Red cell mass was washed three times with isotonic sodium chloride and centrifuged for 10 minutes at 3000 g. Here is the list of the reagents used in this study: 2,4-dinitrophenylhydrazine, 1,2-phenylenediaminedihydrochloride, Indigo Carmine, (Sigma-Aldrich, USA), transferrin (Fluka), urea, sodium fluoride, sodium acetic, ammonium iron(III) citrate, potassium iodide, tris, trichloroacetic and thiobarbituric acid (Merck, Germany).

In order to estimate MDA, we used serum and red cell suspensions for blood which was drawn using vials containing of 3.8% sodium citrate and centrifuged for 10 minutes at 3000 g [13].

TPA concentration in erythrocyte: the reaction mixture contained 0.5 mL hemolysate of erythrocyte hemolysate (1:1000) sample, 1 mL of 0.2 M acetic buffer solution (pH 4.9), 1mL 1ml 0.05 mM solution of Indigo Carmine solution. After 5 minutes incubation at 30 C, it was added 0.5 ml 0.03 M solution hydrogen peroxide to the samples. The control samples were added 0.5 ml of distilled water. The reaction was stopped 2 min after the addition of 3 ml of 20% sulfuric acid. The absorbance was measured at 670 nm.

For the purpose of studying CP and TR, it was used 2.5 mL of whole blood which was taken from a vein and centrifuged at 2500 r.p.m. for 5 minutes. CP concentration: the reaction mixture contained 0.05 mL sample, 4 mL of 0.4M acetic buffer solution (5.5) and 0.5 mL of 0.5% aqueous solution of 1.2 phenylenediamine dihydrochloride. After 1h incubation at 37 C, it was added 1 mL 3% aqueous solution of sodium fluoride, and, absorbance was measured at 530 nm.

TR concentration: the reaction mixture contained 0.1 mL sample, 1 mL of 0.2% solution of ammonium iron (III) citrate (5.5-5.8). After 30 minutes incubation at room temperature, absorbance was measured at 440 nm. As a standard, a TR solution was used [13].

The rate of hemolysis was calculated based on the measurement of Hb released from the cells relatively to the total amount of Hb in the RBC suspension. Free Hb concentration was determined by cyanmethemoglobin method using Drabkin's reagent. The percentage of hemolysis was calculated using the formula described by K. Janatpour et al [14]. A supernatant volume was calculated from Ht.

Determination of RBC membrane permeability (%) was performed by the method, which is based on the detection of differences in the osmotic stability of erythrocytes in a mixture with different concentrations of isotonic solutions of sodium chloride and urea [15].

Statistical analysis. Analysis and all graphs were performed using MedCalc (Belgium). The average means (M) and standard deviations (SD) or the median (Me) and interquartile ranges [Q25 - Q75] were calculated according to a normal distribution. For the statistical analysis, we used the Student's t-test and nonparametric (U-test) Mann-Whitney. Categorical variables were expressed as proportions, and, chi-square tests were used for the comparison of 2 Groups.

The dose dependence assessment was performed using the probit regression model. Odds ratios (OR) and 95% confidence intervals (CI) were calculated using logistic regression. Correlation analysis was performed using the Spearman non-parametric criterion (r).

Results. The demographic, biochemical and clinical characteristics of the HD patients both with and without CERA treatment are presented in Table 1.

Table 1

Demographic and clinical characteristics of the HD patients

Parameter	Group I (n = 20)	Group II (n = 18)	p
Gender (m/f, %)	60/40	44/56	0.71
Age, years	52.6 ± 4.4	55.1 ± 3.3	0.07
Duration of HD (months)	46.8 ± 14.3	52.8 ± 16.2	0.23
Kt/V	1.31 ± 0.3	1.36 ± 0.8	0.8
Hb (g/L)	109.4 ± 6,8	112.2 ± 3,4	0.12
TSAT (%)	45.6 ± 15.5	52.4 ± 12.02	0.17
Ferritin (ng/mL)	695 [402-725]	723 [425-806]	0,76
CERA dose (μg/month)	50 [25-75]	125 [100-150]	0.0001
iPTH (pg/ml)	527.4 [380-750]	593.4 [390-810]	0.8
Albumins (g/L)	36.1 ± 3.8	37.8 ± 3.1	0.14
P (μmol/L)	1.82 ± 0.7	2.2 ± 1.1	0.2
Ca (μmol/L)	2.21 ± 0.3	2.11 ± 0,7	0.56
CRP (mg/L)	6.8 ± 2.9	6.1 ± 4.01	0.54

Data are presented as mean ± standard deviation, or median [25th–75th percentile], or proportions; Ca: calcium; CERA: continuous erythropoietin receptor activator; CRP: C-reactive protein; iPTH: intact parathyroid hormone; Hb: hemoglobin; P: phosphate; TSAT: transferrin saturation.

Accordingly, the data given in Table 1 represented the evidence that CERA therapy with different dosages showed non-significant difference in most of measured demographic and clinical parameters.

In assessing oxidative stress parameters, we obtained heterogeneous results. The evaluated parameters in the study groups are shown in Table 2.

Table 2

Oxidative stress parameters in the HD patients depending on the monthly overage dose of CERA

Parameter	Group I (n = 20)	Group II (n = 18)	p
MDAs, , μmmol/L	476 [411-565]	540 [437-604]	0.06
MDAe, , μmmol/L	604 [411-772]	617 [450-639]	0.27
CP, g/L	0.13 [0.11-0.17]	0.15 [0.16-0.2]	0.007
TR, g/L	2.3 [1.9-2.2]	2.7 [2.4-2.9]	0.0003
TPA, μmol/min/g Hb	196 [175-216]	204 [179-261]	0.02
Hemolysis (%)	4.79 ± 3.05	7.07 ± 4.3	0.03
RBC membrane permeability (%)	9 [4-13]	21.6 [16.6-28]	< 0.0001

Data are presented as mean ± standard deviation or median [25th–75th percentile] or proportions; CP: ceruloplasmin; MDAs: serum malondialdehyde; MDAe: erythrocytes malondialdehyde; RBC: red blood cells; TR: transferring; TPA: total peroxidase activity.

The data displayed in Table 2 demonstrated the significantly higher levels of CP ($p = 0.007$) and TR ($p = 0.0003$) in the patients on high CERA dosage treatment. TPA activity in erythrocyte of Group II patients was statistically higher compared to Group I ($p = 0.02$).

But, the results of our study indicated not only the positive antioxidant effects of high doses of CERA in the HD patients. We determined a statistically high percentage of hemolysis ($p = 0.03$) and RBC membrane permeability ($p < 0.0001$) in the patients who were treated with CERA in a dose ≥ 125 μg/month compared to other patients.

A direct correlation was observed between the rate of hemolysis and TSAT ($r = 0.43$, $p = 0.008$; Fig. 1).

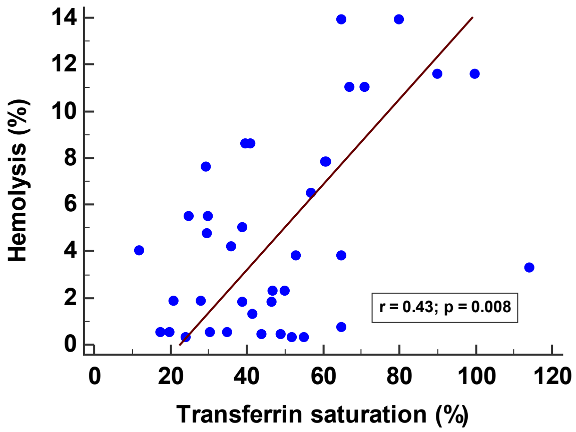

Fig. 1. The correlation between the rate of hemolysis and the serum transferrin saturation level in the HD patients.

Moreover, the level of TPA had a significant negative correlation with the RBC membrane permeability (r = -0.4; p = 0.01; Fig. 2). .

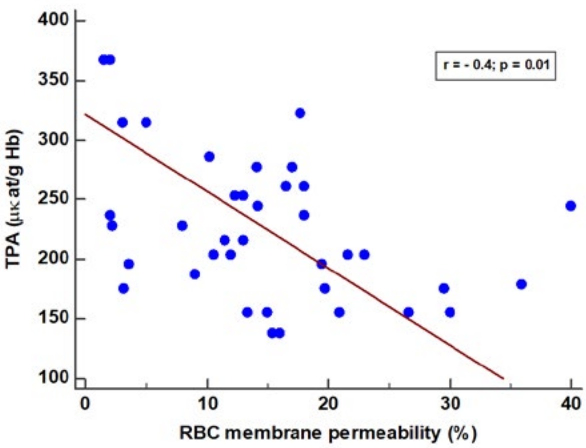

Fig. 2.The correlation between the TPA level and the RBC membrane permeability in the HD patients.

Using the probit regression model, we established the dose-dependent effect of CERA on the level of RBC membranes permeability: $\chi^2 = 21$; p = < 0.0001 (Fig. 3).

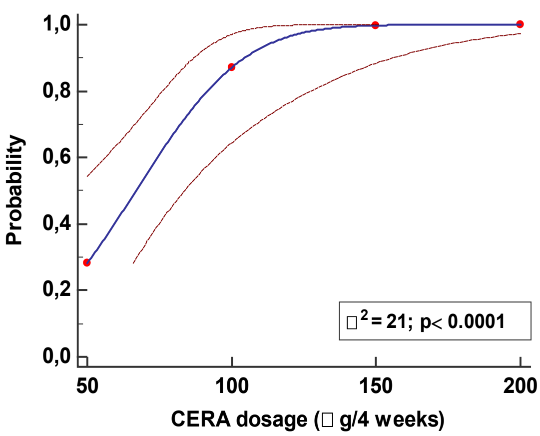

Fig.3. Dose-response plot of a CERA dosage and RBC membrane permeability in the HD patients.

That is, the higher a CERA dosage was applied to achieve the target Hb level in the HD patients, the higher RBC membrane permeability was observed: OR = 17.9, (95% CI 3.03 to 106).

Discussion. There are numerous evidence suggesting excessive oxidative stress in HD patients which can result from loss of antioxidants during dialysis procedures and accumulation of oxidative products [1, 16-18]. Focusing on the association between oxidative stress and anemia, it can be noted that increasing oxidative stress is frequently declared in HD patients who fail to respond to ESA administration [16, 19]. In accordance with these data, we confirmed this position in our study: a tendency of increasing serum MDA level was found in the patients requiring a higher dose of CERA.

Moreover, previous evidence has shown that ESA therapy has an antioxidant effects in HD patients [1, 7, 8, 20, 21]. In this study, we also showed and proved the positive association between antioxidant parametrs and a CERA dosage. CP, TR and TPA blood concentration were significantly higher in the HD patients who were treated with a CERA dose ≥ 125 μg/month.

It is well known that the oxidative stress promotes ESA resistance by causing lipid peroxidation of RBC membranes [22]. Erythrocytes are particularly prone to the action of free radicals because they are a potential source of reactive oxygen species. In addition, in order to prevent peroxidation reactions, the oxidative stress depletes the protective mechanisms of RBC [23]. Based on these facts, we found it interesting to compare RBC membrane lipid peroxidation parameters in the HD patients depending on a CERA dose. To our knowledge, studies on the dose-dependent effect of CERA on RBC membrane lipid peroxidation parameters in HD patients are few. It should be noted that our study is not the first to observe the impact of ESA on erythrocyte's oxidative status. But, actually, we are the first to demonstrate a significantly negative effect of a higher CERA dose on RBC membrane lipid peroxidation parameters.

The main finding of the present study was the strong association of a higher CERA dose (≥ 125 μg/month) in the HD patients with the increasing level of RBC hemolysis and membrane permeability levels. As the patient groups with different CERA doses had similar demographic and clinical characteristics in our study, the differences encountered between them with respect to hemolysis and RBC membrane permeability levels could not be attributed to varying degrees of anemia, treatment duration, dialysis quality or inflammation.

In point of fact, there is no current consensus as to the impact of ESA in general and CERA specifically on the intensity of oxidative stress and RBC membrane lipid peroxidation parameters in HD patients. The results we obtained are contrary to the most of existing data. Thus, in a recent experimental study, Aizawa K et al has demonstrated that the intravenous administering of the adequate CERA dose (0.6 μg/kg every 2 weeks during 5 weeks) improves erythrocyte quality (deformability and life-span) in rats [24]. Zorica M. Dimitrijevic with her colleagues has suggested that long term of ESA administration attenuates the lipid peroxidation process and restores the levels of antioxidants [8]. In an earlier study, Galluci M at al has indicated that the ESA therapy is related to a decrease in RBC membrane oxidative damage [25].

On the other hand, Pawlak at al has showed no effect of one-year ESA therapy on oxidative stress markers in patients undergoing regular HD [9]. Moreover, E. Tutal at al has indicated a significantly higher intensity of oxidative stress in HD patients requiring high doses of EPO. The patients with poor ESA responses had a significantly higher level of MDA and lower levels of plasma superoxide dismutase [26].The authors have concluded that increased oxidative stress has a strong influence on ESA response in HD patients, and, therefore, it can also be a potential explanation of the data we received.

Thus, according to our findings, we observed that the patients with higher CERA requirements showed a higher antioxidant status simultaneously with higher percents of hemolysis and RBC membrane permeability.

We acknowledged some limitations in our study. First, in this study, the oxidative stress markers were measured just a once. In this way, cross-sectional design of our study could not provide definite information about cause-effect relationship between CERA treatment and oxidative stress. Second, it was a small sample size study performed in a single center; therefore, our findings only revealed associations. Third, we did not take into account the iron dose which could also affect the results. Finally, there was a high probability of changes in the intensity of oxidative processes in the HD patients with a high level of the comorbidity index.

Despite its limitations, the strong association observed in the present study has indicated the potential impact of a monthly CERA dose ≥ 125 μg in the violating of RBC membrane lipid peroxidation. The larger scale well-planned studies are needed for further confirmation of our findings.

Conclusions. In conclusion, we demonstrated that administration of CERA in a dose more 125 μg/month improved the antioxidant status in HD patients. But, at the same time, it increased the hemolysis and RBC membrane permeability. Our preliminary data pointing to the dose-dependent effect of CERA on the RBC membrane lipid peroxidation parameters require further confirmation.

Disclosure Statement. The authors declare no conflict of interest.

Authors' contributions.

N. Stepanova: analyzed and interpreted the patient data, a major contributor in writing the manuscript.

L. Korol: performed the biochemical examination in the blood samples, analyzed and interpreted the patient data.

V. Novakivskyy: interpreted the data.

M. Kolesnyk: idea and management of the research.

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