Research Article

Neocytolysis in Hemodialysis Patients: Effects of Erythropoietin Treatment and Withdrawal

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Abstract
Background: Neocytolysis is the selective destruction of the youngest circulating red cells. Erythropoietin is a growth factor for endothelial cells as well as for erythroid cells. In contrast to their proliferative response to physiological levels of erythropoietin, endothelial cells may respond to decreased levels by triggering a process called neocytolysis. **The aim:** to investigate neocytolysis markers in chronic renal failure patients, who maintained on hemodialysis and treated with erythropoietin. **Material and Methods**: 40 patients maintained on regular hemodialysis were assessed and 10 healthy subjects as a control. We assessed the expression of E-cadherin and vWF in blood serum and CD36 on RBCs membrane before and 6 months after erythropoietin treatment. Urea, creatinine, Na^+ & K^+ were estimated. The correlation between the expression of neocytolysis markers and renal function and electrolytes in those patients was performed. **Results:** There was a statistically significant higher level of E-Cadherin, CD-36 and vWF in patients than control. These findings suggest that there was increase in neocytolysis in the patients than the healthy controls (P value was 0.0001, 0.0001 and 0.004 respectively). The level of E-Cadherin and vWF was significantly increased after stoppage of Erythropoietin treatment for 7 days compared to baseline level (P value = 0.01, 0.03 respectively). Whereas, the level of CD36 was significantly lower after stoppage of Erythropoietin (P= 0.0001). Abrupt withdrawal of erythropoietin treatment from patients maintained on regular dialysis, leads to significant decrease in haemoglobin level, P=0.0001. **Conclusion:** The use of small doses of erytropoietin in continuous fashion will lead to stability of the level of the hormone in the blood, and this in turn will prevent destruction process neocytolysis which contributes to the occurance of anemia in these patients.

Keywords: E-Cadherin – neocytolysis - Von willbrand factor – CD-36 –Erythropoietin

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Introduction
Erythropoietin is the major hormone regulator of erythrocyte production promoting the survival, as well as the differentiation and maturation, of erythroid progenitor cells[1]. Erythropoietin is synthesized in the kidney. It is deficient in the majority of patients with advanced kidney disease thereby predisposing them to anaemia[2,3]. In addition to these well-characterized effects, it appears that an erythropoietin-responsive non-erythroid mechanism also mediates the selective destruction of young circulating erythrocytes (neocytes) when red cell mass becomes excessive – a process termed 'neocytolysis'[4,5,6]. Neocytolysis is the selective destruction of the youngest circulating red cells, which may be prompted by endothelial cells communicating with macrophages to stimulate phagocytosis of this unusual cell subset. It is speculated that this is due to decreased production by endothelial cells of the macrophage-deactivating transforming growth factor-β[7]). The resulting proinflammatory phenotype may include macrophage production of thrombospondin, which forms bridges between adhesion molecules selectively expressed on young red cells (CD-36) and the CD-36/αvβ3 complex on macrophages that

triggers phagocytosis[8]. Alternatively, inflammatory mediators secreted by endothelial cells and macrophages during erythropoietin withdrawal may signal young red cells to expose phosphate-dylserine, which would mark them for elimination via the normal pathway for aged red cell destruction[9]. Astronauts, when they return from space, have been found to be anemic as a result of red cell destruction. During space travel, however, instead of destroying the oldest red cells as it usually does, the body selectively destroys the youngest red cells, a process called "neocytolysis"[10]. Neocytolysis is a physio-logic process which negatively regulates the red cell mass by selectively hemolyzing young circulating red blood cells. This allows fine control of the number of circulating red blood cells under steady-state conditions and relatively rapid adaptation to new environments. Neocytolysis is initiated by a fall in erythropoietin levels[9], so this hormone remains the major regulator of red cell mass both with anemia and with red cell excess[11].

It has been suggested that neocytes are identified as targets for phagocytosis in the spleen by the expression of cell-surface adhesion molecules characteristic of their specific stage of differentiation [10]. Various candidate surface proteins are currently being investigated, including CD35, CD36, CD44 (HCAM), CD47, CD49e (VLA4), CD55 (DAF), cadherin, von Willebrand factor, ICAM 4, chondroitin sulphate, and phosphatidylserine[11]. The expression of these surface proteins is highest on erythroblasts and gradually declines as cells mature in the peripheral blood. Erythro-blasts and normoblasts express E-cadherin, but mature erythrocytes do not[12]. Lymphoid and all the other myeloid cell lineages do not express E-cadherin at any developmental stage[13].

However, neocytolysis may also have therapeutic implications for the manage-ment of anaemia in patients with chronic renal failure. The administration of erythro-poietic agents to alleviate anaemia can lead to undesirable fluctuations in serum erythropoietin levels that may trigger unwanted neocytolysis, leading to a decrease in treatment efficacy[14]. Indeed, an understanding of neocytolysis and its relationship to erythropoietin kinetics is an important consideration in establishing optimal erythropoietic agents and administration schedules for treatment of patients with anaemia[15].

Material and Methods

This study was conducted in Internal medicine department El-minia university hospital during the period from June 2012 to April 2014. The study included (40) patients with chronic renal failure maintained on regular haemodialysis and ten healthy volunteers were selected randomly of matched age and gender to the study group as a control group. Informed consent was taken from all patients and control. Approval of the Ethical Medical Committee at Minia Faculty of Medicine was taken.

The **main selection criteria** were: Chronic renal failure maintained on regular hemodialysis, Anemic, Middle age group, not associated with any other systemic disease. **Exclusion criteria** include patients with advanced Heart, liver, Pulmonary or hematological problem, Extremes of age. The patients were chosen from the dialysis unit. Ten additional healthy volunteers were selected of matched age and gender to the patients as a control group.

Full history was taken and clinical examination (including blood pressure) of the patients and control was done. The weight, height and body mass index was calculated as weight (kg) /(Height (m))^2 . Routine laboratory investigations were done including urea, creatinine calcium, phosphorus, uric acid, electrolyes, blood sugar and complete blood picture for both patients and control.

Blood sample were taken from all patients and control and applied to flow cytometry using 488 nm wavelength laser excitation and monitoring emitted fluorescence with a detector optimized to collect peak emissions at 515 - 545 nm. searching for CD36 as a marker for young RBCS (Neocyte) and serum was taken from all patients and controls and analysed for E-

cadherin by an assay which employs the quantitative sandwich enzyme immune-assay technique and von-Willibrand factor by assay which is a sandwich ELISA technique. Subsequently, The patients were given 2000 U Epo every other day for six months then stopped, after 7 days of stopping erythropoietin another sample of blood were taken from the patient and tested for CD-36, E-Cadherin and Von-willibrand factor. Flow cytometric studies were done at baseline (just before starting treatment), then one week after the last rhEpo dose. Hemoglobin, hematocrit levels, was monitored every month during injections and upon Epo withdrawal. Blood sample were taken from all patients and applied to flow cytometry searching for CD-36 as a marker for young RBCS (Neocyte) and serum was taken from all patients and analysed for cadherin and von-Willibrand factor subsequently. Samples were taken and examined for detection of the occurance of neocytolysis.

Statistical analysis

All data were tabulated and expressed on EXCEL® software using PC computer, then SPSS® software package for social statistical analysis, USA, version 18 was used for statistical analysis. Univariate analysis was done. Numerical data were expressed as mean and standard deviation (SD). The categorical data were expressed as number and percent. On comparison of numerical data the following tests were used: 1- Between groups: Unpaired t-test. 2- Within each group: Paired t-test was used. On comparison of categorical data the following tests were used: Between groups: Chi-square test was used., Within each group: Fischer's exact test was used., P-value was calculated after each comparison, and the statistical significance was expressed as follows; Non significant if p-value > 0.05, Significant if p-value < 0.05.

Results

At baseline (i.e. beginning of this study) there was a statistically significant higher level of E-Cadherin, CD-36 and vWF in patients than control. These findings suggest that there was increase in neocyto-lysis in the patients than the healthy controls (P value was 0.0001, 0.0001 and 0.04 respectively) (Table 1)**.** No significant correlations were found between the expression of E-Cadherin and any of the studied laboratory parameters. E-Cadherin level was significantly increase after stoppage of Erythropoietin treatment for 7 days compared to baseline level (P value = 0.01). So while the E-Cadherin level was 14.49±5.72 ng/mL at baseline, it increased to 21.26±9.83 ng/mL after stoppage of Erythropoietin treatment. (Fig.1). Serum level of von Willibrand factor was significantly higher after stoppage of Erythropoietin treatment compared to baseline (P=0.03) (Fig. 2). The level of CD-36 was significantly lower after stoppage of Erythropoietin (p value= 0.0001) (Fig. 3). Abrupt withdrawal of erythropoietin treatment from patients maintained on regular dialysis, leads to significant decrease in haemoglobin level P=0.0001. Figure (4) demonstrated the difference of haemoglobin level in patients who were treated for six months with erythropoietin and then 7 days after stoppage of the treatment.

Table 1: Comparison between the expression of neocytolysis markers at baseline between patients and controls

Variable	Patients	Controls	P value
E-Cadherin (ng/ml)			
range	5.7-23.9	3.9-8.2	
Mean±SD	14.5±**5.7**	5.9±1.4	0.0001
CD-36			
range	6-8.5	1.3-1.6	
Mean±SD	6.575±0.9	1.5±0.1	0.0001
vWF (IU/dl)			
rang	59-145	21-137	
Mean±SD	99.5±32.9	72±39	0.004

Table 2: Correlations between E-Cadherin expression and laboratory parameters at baseline

Variable	r value	P value
Albumin	0.002	0.99
ALT	0.14	0.55
AST	0.1	0.67
Urea	0.27	0.25
Creatinine	0.34	0.14
Phosphorus	0.14	0.56
Calcium	-0.02	0.9
Sodium	-0.11	0.64
Potassium	0.15	0.52

Table: (3) E-Cadherin level at baseline and 7days after stoppage of Erythropoietin treatment.

Variable	baseline	7 days after stoppage	P value
E-cadherin level (ng/ml) Mean±SD	14.49±5.72	21.26±9.83	0.01

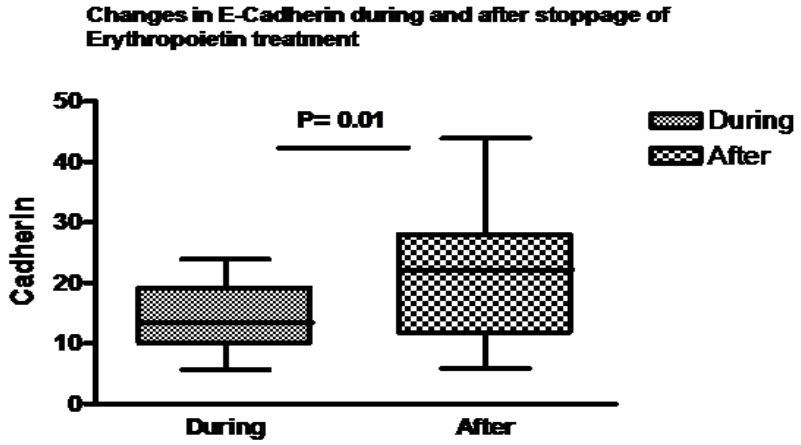

Figure (1) E-Cadherin level during and 7days after stoppage of Erythropoietin

Table: (4): von Willibrand factor level at baseline and 7 days after stoppage of Erythropoietin treatment

	baseline	7days after stoppage	P
vWF level (IU/dl) Mean ±SD	99.55±32.93	120.7±31.3	0.03

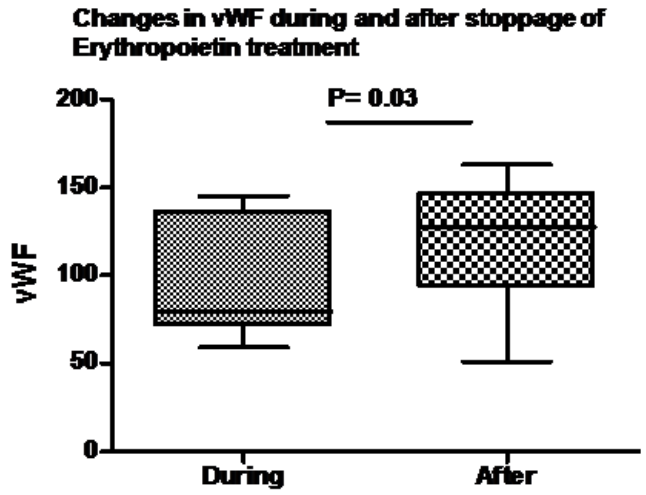

Figure (2): Van Willibrand factor level during intake and 7 days after stoppage of Erythropoietin

Table: (5) CD-36 during and 7 days after stoppage of Erythropoietin treatment

CD-36	During	After	P value
Mean ±SD	6.575±0.872	2.055±0.437	0.0001

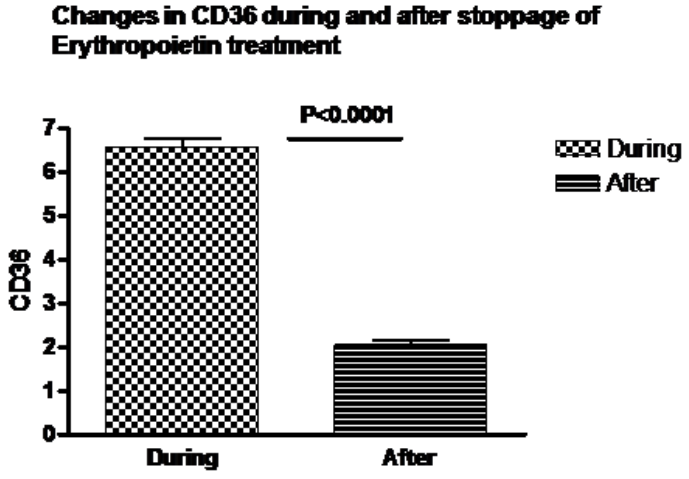

Fig: (3) CD36 during intake and 7 days after stoppage of Erythropoietin treatment

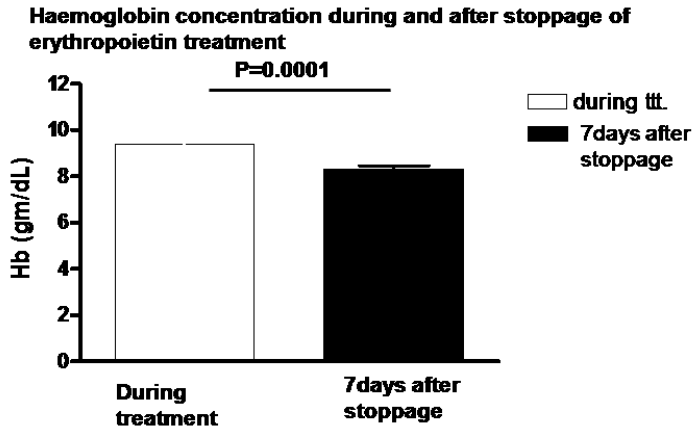

Figure (4): Haemoglobin concentration during and 7 days after stoppage of erythropoietin treatment.

Discussion

Deficiency of EPO, as occurs in patients with CKD, retards maturation of red blood cells from progenitor cells into normoblasts and reticulocytes[16]. Furthermore, deficiency of EPO decreases the survival of these immature red blood cells, a process known as neocytolysis, thereby resulting in anemia[17,18].

We investigated neocytolysis in chronic renal failure patients with anemia, who maintained on hemodialysis and treated with erythropoietin. Effect of termination of erythropoietin treatment abruptly on neocytolysis markers was studied. In our study we used changes in the serum level of E-cadherin, vWF and the changes in expression of CD36 on the surface of RBC as markers of neocytolysis. Our results showed that at baseline (i.e. beginning of this study) there was a statistically significant higher level of E-Cadherin, CD-36 and vWF in the patients than control (P value was 0.0001, 0.0001 and 0.004 respectively) which can result from neocytolysis and support the role of neocytolysis in the etiology anaemia of chronic kidney disease. This is in agreement with Alfrey and Fishbane[5].

No significant correlations were found between the expression of the studied markers and gender, HCV status, any of the studied laboratory parameters or the electrolytes. The level of E-Cadherin and vWF was significantly increased after stoppage of Erythropoietin treatment for 7 days compared to baseline level (P value = 0.01, 0.03 respectively). CD-36 is an adhesion molecule that is selectively expressed on young red cells[10]. In our study, the level of CD-36 was significantly lower after stoppage of Erythropoietin than during treatment. Haemoglobin level showed progressive increase in chronic renal failure patients who maintained regular haemodialysis combined with continuous erythropoietin treatment [19].

As expected, abrupt withdrawal of erythropoietin treatment from patients maintained on regular dialysis, leads to significant decrease in haemoglobin level. Anemia is common in patients who have chronic kidney disease [20]. The main causes of anemia are deficient production of erythropoietin (EPO), iron deficiency, and chronic disease with endogenous EPO resistance[21]. EPO has been successfully used for over a decade to treat anemia in patients with chronic kidney disease[22]. Anemia was twice as prevalent in people with CKD (15.4%) as in the general population (7.6%) and the prevalence of anemia increased with the stage of CKD[23] and renal function as measured by eGFR was the strongest predictor of anaemia. There is significant association between anaemia and renal function and as the glomerular filtration rate increase, the risk to be anaemic will decrease dramatically[24].

Neocytolysis can contribute to the anaemia of renal disease and explain some unresolved issues about such anaemia**.** Moreover the current pharmacologic approach for the treatment of anaemia in chronic kidney disease may cause neocytolysis and could keep therapy from reaching its full potential[25]. Understanding neocytolysis and its relationship to fluctuating serum erythropoietin levels might help to better understand optimal treatment with erythropoietic agents **[15, 26].** In the present study there was a statistically significant higher level of E-Cadherin, CD-36 and vWF in the patients than the healthy controls at the base line (P value was 0.0001, 0.0001 and 0.004 respectively) which can result from neocytolysis and support the role of neocytolysis in the aetiology anaemia of chronic kidney disease. Our study showed that the level of E-Cadherin was significantly higher after withdrawal of Erythropiotein treatment (p value= 0.01) and the serum level of Von Willibrand factor was significantly higher after stoppage of Erythropiotin (p=0.03), while the level of CD-36 was significantly lower after stoppage of Erythropiotein (p=0.001). The aforementioned findings indicate that neocytolysis occur in the patient after stoppage of Erythropiotein treatment and this is in accordance with the study done by Rice and Alfrey[11] as our results are in accordance with the results of their study. In the study done by Alfrey et al., [27], nine inhabitants of Cerro de Pasco, Peru, living at 14,500 feet, with baseline hematocrits of 65-75%. On descent to sea level, the predicted EPO suppression and red cell decline occurred, along with evidence from differential isotope labelling studies that the young red blood cells had been selectively hemolyzed. The same happens in space flights [28].

There was no difference in the expression of the neocytolysis markers between hepatitis C virus positive and negative patients (P=0.1). This will exclude the effect of viral status on the neocytolysis measured by the used markers**.** Similarly correlations between the percent of change in the expression of neocytolysis marker before and after stoppage of the erythropoietin treatment and age, blood pressure, sex, HCV status, different laboratory parameters and electrolytes were studied and no significant statistical correlations had been found.

Effects of Erythropiotein treatment with-drawal on Hb level was studied by Collins et al.,[29] and the same findings were observed by Chung-Che chang et al., [30] where they found that abrupt withdrawal of erythropoietin, leads to significant decrease in haemoglobin level (P=0.0001). Our results are in accordance with their results and also they verified that young RBCs are preferentially destroyed on rhEpo withdrawal[30].

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