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Correlation of erythropoietin and haematocrit levels in the anaemias of chronic kidney diseases: A study in federal medical centre, Umuahia, Nigeria

[1] Obeagu Emmanuel Ifeanyi, [2] Obeagu Gertrude Uzoma

[1] Diagnostic Laboratory Unit, Health Services Department, Michael Okpara University of Agriculture, Umudike, Abia State, Nigeria.
[2] Department of Nursing Science, Ebonyi State University, Abakaliki, Nigeria.

Abstract
The study was done to correlate the levels of erythropoietin (Epo) and haematocrit in some patients diagnosed with renal diseases in Federal Medical Centre, Umuahia, Abia State. A total of one hundred and eighty (180) subjects (90 males and 90 females) were recruited for the study. One hundred and twenty (120) subjects were recruited from those confirmed with renal diseases from the Urology Department of the hospital. Sixty (60) subjects (30 and 30 females) were predialysis chronic kidney disease patients, sixty (60) subjects (30 and 30 females) were chronic kidney disease patients on dialysis (D), and sixty (60) subjects (30 and 30 females) were the apparently healthy controls. Venous blood samples were collected from the subjects using aseptic standard method from the antecubital fossa.Serum samples were used for erythropoietin assay and EDTA anticoagualted blood used for Packed Cell Volume tests. Erythropoietin was determined by sandwich ELISA method and PCV was analysed by microhaemtocit method. The results were statistically analysed using student t-test and Pearson Product Moment method and statistical significance set at $P<0.05$.The result showed significant decrease ($P<0.05$) in Erythropoietin and PCV levels of predialysis subjects (23.3±3.2 iu/l, 16.0±1,4%) compared to Controls (48.6±24.0 iu/l, 43.8±4.9%) respectively and showed significant increase ($P<0.05$) in erythropoietin level of patients on dialysis (71.4±14.7 iu/l) compared to controls (48.6±24.0 iu/l) and significant decrease ($P<0.05$) in PCV level of dialysis subjects (27.0±7.0%) compared to control subjects (43.8±4.9%). The result also showed significant decrease ($P<0.05$) in Erythropoietin and PCV levels of predialysis subjects (23.3±3.2 iu/l, 16.0±1.4%) compared to dialysis subjects (71.4±14.7 iu/l, 27.0±7.0%) respectively. The correlation of erythropoietin and PCV levels showed perfect correlation in predialysis subjects and positive correlation in dialysis subjects. The study shows that Epo and haematocrit differ in predialysis patients. The difference could be due to the administration of recombinant erythropoietin to dialysis patients.

Keywords: Correlation, Erythropoietin, Haematocrit, Anaemia, Chronic kidney disease

Introduction

Erythropoietin (Epo), is a glycoprotein hormone that regulates erythropoiesis.It is a cytokine for erythrocyte precursors in the bone marrow. Human erythropoietin has a molecular weight of 30.4 kDa (Siren *et al.,* 2001) [23].

It is produced by interstitial fibroblasts in the kidney in close association with peritubular capillary and tubular epithelial tubule. It is also produced in perisinusoidal cells in the liver. While liver production predominates in the fetal and perinatal period, renal production is predominant during adulthood. In addition to erythropoiesis, erythropoietin also has other known biological functions. For example, it plays an important role in the brain's response to neuronal injury (Siren *et al.,*2001). EPO is also involved in the wound healing process (Haroon *et al.*, 2003) [11].

Exogenous erythropoietin is produced by recombinant DNA technology in cell culture. Several different pharmaceutical agents are available with a variety of glycosylation patterns, and are collectively called erythropoiesis-stimulating agents (ESA). The specific details for labelled use vary between the package inserts, but ESAs have been used in the treatment of anemia in chronic kidney disease, anemia in myelodysplasia, and in anemia from cancer chemotherapy. Boxed warnings include a risk of death, myocardial infarction, stroke, venous thromboembolism, and tumor recurrence. Exogenous erythropoietin has been used illicitly as a performance-enhancing drug; it can often be detected in blood, due to slight differences from the endogenous protein, for example, in features of posttranslational modification.

The primary role of erythropoietin is an essential hormone for red cell production. Without it, definitive erythropoiesis does not take place. Under hypoxic conditions, the kidney will produce and secrete erythropoietin to increase the production of red blood cells by targeting CFU-E, proerythroblast and basophilic erythroblast subsets in the differentiation. Erythropoietin has its primary effect on red blood cell progenitors and precursors by promoting their survival through protecting these cells from apoptosis.

Erythropoietin has a range of actions including vasoconstriction-dependent hypertension, stimulating angiogenesis, and inducing proliferation of smooth muscle fibers. It can increase iron absorption by suppressing the hormone hepcidin (Ashby *et al.,* 2010) [3].

Multiple studies have suggested that erythropoietin improves memory. This effect is independent of its effect on hematocrit (Miskowiak *et al.,* 2007; Miskowiak *et al.,* 2007) [16, 17]. Rather, it is associated with an increase in hippocampal response and effects on synaptic connectivity, neuronal plasticity, and memory-related neural network (Adamcio *et al.,* 2008; Adamcio *et al.*, 2010) [1]. Erythropoietin may have effects on mood (Miskowiak *et al.*, 2007) [16, 17].

Erythropoietin has been shown to exert its effects by binding to the erythropoietin receptor (EpoR) (Livnah *et al.,* 1998; Middleton *et al.,* 1999) [14, 15].

Erythropoietin is highly glycosylated (40% of total molecular weight), with half-life in blood around five hours. Erythropoietin's half-life may vary between endogenous and various recombinant versions. Additional glycosylation or other alterations of erythropoietin via recombinant technology have led to the increase of erythropoietin's stability in blood (thus requiring less frequent injections). Erythropoietin binds to the erythropoietin receptor on the red cell progenitor surface and activates a JAK2 signaling cascade. Erythropoietin receptor expression is found in a number of tissues, such as bone marrow and peripheral/central nervous tissue. In the bloodstream, red cells themselves do not express erythropoietin receptor, so cannot respond to erythropoietin. However, indirect dependence of red cell longevity in the blood on plasma erythropoietin levels has been reported, a process termed neocytolysis.

Erythropoietin levels in blood are quite low in the absence of anemia, averaging at around 10 mU/ml. However, in hypoxic stress, erythropoietin production may increase 1000-fold, reaching 10,000 mU/ml of blood. Erythropoietin is produced mainly by peritubular capillary lining cells of the renal cortex, which are highly specialized, epithelial-like cells. It is synthesized by renal peritubular cells in adults, with a small amount being produced in the liver. Regulation is believed to rely on a feedback mechanism measuring blood oxygenation (Jelkam, 2007). Constitutively synthesized transcription factors for erythropoietin, known as hypoxia-inducible factors, are hydroxylated and proteosomally digested in the presence of oxygen.

Erythropoietin (Epo) is a complex molecule, which regulates red blood cell production in the bone marrow. Recombinant human erythropoietin (rHuEPO) is commercially available and is widely used for the treatment of anemia. In recent years, additional nonerythropoietic tissue/organ protective properties of erythropoietin have become apparent, in particular for kidneys

Erythropoietin is a 30.4 kD glycoprotein and class I cytokine consisting of 165 amino acids (Mocini *et al.,* 2007). Erythropoietin has four acidic oligosaccharide side chains (3 N-linked and 1 O-linked) and contains up to 14 sialic acid residues. Its carbohydrate portion contributes 40% of its molecular weight (Mocini *et al.*, 2007) [18]. The N-linked polysaccharide side chains appear to be important for the biosynthesis and secretion of erythropoietin, enhance its stability in blood, and limit hepatic clearance, thus facilitating the systemic transit of erythropoietin from kidney to bone marrow (Obeagu, 2015) [22].

The variable nature of the sialic acid content gives rise to erythropoietin isoforms with differences in charge. As the number of sialic acid groups on the carbohydrate portion of erythropoietin increase, so does its serum half-life, whereas receptor-binding capacity decreases (Cartlin *et al.,* 2002; Elliot *et al.*, 2004; Middleton *et al.,*1999; Weidemann and Johnson, 2009) [7, 15, 24]. Clearance, however, appears to have a stronger influence on *in vivo* activity than receptor-binding affinity.

Each erythropoietin molecule has two erythropoietin receptor (EpoR) binding sites. There are two affinities of the EpoR for erythropoietin in solution: one of high and one of low affinity (needs 1,000 times the concentration of erythropoietin for activation) (Weidemann and Johnson, 2009) [24].

Diseases of the kidney are diverse, but individuals with kidney disease frequently display characteristic clinical features. Common clinical conditions involving the kidney include the nephritic and nephrotic syndromes, renal cysts, acute kidney injury, chronic kidney disease, urinary tract infection, nephrolithiasis, and urinary tract obstruction.Many other disease cases have adverse effects on the kidney which is the major site of production of erythropoietin such hypertension,diabetes, HIV/AIDS. Various cancers of the kidney exist; the most common adult renal cancer is renal cell carcinoma. Cancers, cysts, and some other renal conditions can be managed with removal of the kidney, or nephrectomy. When renal function, measured by glomerular filtration rate, is persistently poor, dialysis and kidney transplantation may be treatment options..

The kidneys secrete a variety of hormones, including erythropoietin, and the enzyme renin. Erythropoietin is released in response to hypoxia in the renal circulation. It stimulates erythropoiesis in the bone marrow.

The anemia of chronic kidney disease (CKD) is, in most patients, normocytic and normochromic. It is principally due to reduced renal erythropoietin (EPO) production and, to a lesser degree, to shortened red cell survival and decreased responsiveness to the hormone.

Anemia can develop well before the onset of uremic symptoms due to end-stage renal disease (ESRD). Although anemia due to renal dysfunction generally develops when the glomerular filtration rate (GFR) declines to <30 mL/min, it can also be observed in those with markedly higher GFRs (such as 60 mL/min) and tends to occur at higher levels of GFR in African Americans than whites.

If left untreated, the anemia of chronic kidney disease is associated with several abnormalities. These include deterioration in cardiac function, decreased cognitive function and mental acuity, fatigue, and other signs and symptoms. There are also associations with an increased risk of morbidity and mortality, principally due to cardiac disease and stroke.

Justification of the Study

Renal diseases are increasing at alarming rate causing a great damage on the renal function.The damage caused by these diseases can affect the level of erythropoietin which in turn affects erythropoiesis. This might have contributed to increase in anaemia seen in many hospitals these days.

There is an increasing prevalence of renal insufficiency diseases in Umuahia as seen in the Urology Department of Federal Medical Centre, Umuahia which if nothing is done may cause increase in mortality and morbidity among the patients and results to loss of much manpower to the society and cost of managing them. The cost of care includes not only the direct cost of dialysis and transplant services but also indirect cost like man hours lost at the workplace. A study to determine the levels of Erythropoietin and haematocrit has not been done in this part of the world which call for the study.

Aim

The aim of this study is to correlate erythropoietin and haematocrit levels in Patients with kidney diseases seen at the Federal Medical Centre, Umuahia.

Materials and Methods

Study area

The study was conducted at the Federal Medical Centre, Umuahia.Umuahia is the capital of Abia State, with a population of 264,662 and covers a land area of about 245KM^2. It lies on Latitude 5.52627 (decimal degree) North and Longitude 7.48959 (decimal degree) East with an elevated alttitude of 152 meters (NPC, 2006).

Umuahia is inhabited by the three major tribes of Nigeria but dominated by the Igbo speaking people. They are more of traders, Technical workers, civil servants and farmers. About two Universities are located there. They have few government hospitals which the most respected is Federal Medical Centre, Umuahia. They patronise drug dealers without consulting medical doctors, take a lot of NSAIDS, use herbal medicines, high prevalence of UTI and have few markets to buy better food stuffs.

Advocacy, Pre-survey contacts and ethical considerations

With a letter of introduction from the Department, the secretary, Health Research and Ethical Committee of the Health Institution was met with a well detailed research proposal, after which an ethical approval was obtained for the research work.

Study population and enrolments

The formular of Araoye (2004) was used to determine the sample size.

$$n=\frac{Z^2 XP(q)}{d^2}$$

where n =sample size

z=confidence interval 95% (1.96)

p=prevalence rate

q=1-p

d=degree of freedom 5% (0.05)

Prevalence rate 7.7% according to Egbi *et al*. (2014).

$$n=\frac{(1.96)^2 \times 0.077 \times 0.923}{(0.05)^2}$$

Minimum sample size=100 but for the study sample size of 180 was used.

The study group (patients) were recruited from the Urology Department of Federal Medical Centre, Umuahia by the help of some nurses who helped in retreiving the patients' folders after they gave their consents. A total of one hundred and eighty (180) subjects were recruited for the study whose age ranges from 35-71 years.Sixty (60) subjects (30 males and 30 females) were chronic renal disease patietnts on predialysis, sixty (60) subjects (30 males and 30 females) were chronic kideny subjects on dialysis. Also, sixty (60) subjects (30 males and 30 females) were the controls who were apparently healthy individuals.

Inclusion criteria

Written and oral consents were obtained from the subjects and other information which was helpful for the study. The subjects were selected based on those who were confirmed of having different renal diseases such as chronic kidney disease on predialysis and chronic kidney disease on dialysis.

The control subjects were selected on ground that they were apparently healthy.

Exclusion criteria

For the study group,the subjects showing normal renal function, HIV/AIDS patients, patients with urinary tract infection, hypertensive patients, those on nephrotoxic drugs such NSAIDS were excluded from the study.Subjects with the following symptoms were excluded:

- Generalized weakness or malaise, easy fatigability
- Generalized body aches, or myalgias
- Orthostatic symptoms (eg, lightheadedness, dizziness)
- Syncope or near-syncope
- Decreased exercise tolerance
- Chest discomfort
- Palpitations
- Cold intolerance
- Sleep disturbances
- Inability to concentrate
- Loss of appetite
- Skin - Pallor
- Neurovascular - Decreased cognitive ability
- Eyes - Pale conjunctivae
- Cardiovascular - Orthostatic hypotension, tachyarrhythmias
- Pulmonary - Tachypnea
- Abdomen - Ascites, hepatosplenomegaly and those abnormal urea,creatinine in serum and protein in urine were equally excluded for the study.

For the controls,subjects showing any clinical conditions of any disease at all were excluded for the study.

Sample Collection

About 6ml of venous blood was aseptically collected from the antecubital vein of each subject by standard technique. About 4.5ml was dispensed into plain tubes for Erythropoietin assay and the remaining was dispensed into an EDTA bottle for packed cell volume (PCV) test. The blood samples for serum were allowed to clot for 2 hours at room temperature before centrifugation for 20 minutes at approximately 1000Xg. EDTA whole blood was used for PCV test.

Laboratory investigations

All reagents and kits were commercially purchased from reputable company whose standard operating procedures were strictly followed. Human Epo (Erythropoietin) ELISA kit was purchased from Elabscience with catalog No**:**E-EL-H0066c. The erythropoietin was bought from Elabscience Biotechnology Co. Ltd,Wuhan.

Principle of Erythropoietin (Sandwich-Elisa Method) of Elabscience

The ELISA kit uses sandwich-ELISA as the method. The micro ElISA plate provided in this kit was coated with an antibody specific to Erythropoietin.Standard or samples are then added to the appropriate micro ELISA plate wells and combined to the specific antibody. Then a biotinylated detection antibody specific for Erythropoietin and Avidin-Horseradish Peroxidase (HRP) conjugate is added to each micro plate well and incubated.Free components are washed away. The substrate solution is added to each well. Only those wells that contain Erythropoietin, biotinylated detection antibody and Avidin-HRP conjugate will appear bluein colour. The enzyme-subtrate reaction is terminated by the addition of a sulphuric acid solution and the colour turns yellow. The optical density (OD) is proportional to the concentration of Erythropoietin. You can calculate the concentration of Erythropoietin in the sample by comparing the OD of the sample to the standard curve.

Assay procedure
All the reagents were allowed to reach room temperature, mixed thoroughly by gently swirling before pipetting.

1. Add sample: 100µL of standard, blank, or sample was added per well. The blank well was added with reference standard and sample Diluent.Solutions were added to the bottom of micro ELISA plate well, mixed gently and covered the plate with sealer and incubated for 90 minutes at 37 ^{0}C.

2. Biotinylated detection antibody: The liquid of each well was removed. 100µL of biotinylated Detection Antibody working solution was added immediately to each well and covered with plate sealer. The plate was gently tap to ensure thorough mixing and then incubated for 1 hour at 37 ^{0}C.

3. Wash: Each well was apirated and washed 3 times. It was washed by filling each well with wash buffer (approximately 350 µL.At the last wash the remaining wash buffer was removed. The plate was inverted and pat against thick clean absorbent.

4. HRP Conjugate: 100 µL of HRP conjugated working solution was added to each well and covered with the plate sealer and incubated for 30 minutes at 37 ^{0}C.

5. Wash: The wash process was repeated 5 times as in step 3.

6. Substrate: 90 µL of substrate solution was added to each well an was and covered with a new plate sealer and was incubated for about 15 minutes at 37 ^{0}C.

7. Stop: 50 µLof stop solution was added to each well and colour turned to yellow immediately.

8. OD Measurement: The optical density (OD) of each well was determined at once using a microplate reader set to 450nm.

(b)Packed Cell Volume (Microhaematocrit method) was used.

Principle of Microhaematocrit Method (Cheesbrough, 2004).
The packed cell volume is that proportion of whole blood occupied by red blood cells when it is packed together, expressed as a ratio (Litre/Litre).
Anticoagulated blood in a glass capillary of specified length, bore size and wall-thickness is centrifuged in a microhaematocrit centrifuge at RCF 12000-15000Xg for 3-5 minutes to obtain constant packing of red cells. A small amount of plasma remains trapped between the packed red cells.The PCV value is read from the scale of a microhaematocrit reader or calculated by dividing the height of the red cell column by the height of total column of blood.

Procedure for PCV (Cheesbrough, 2004)**.**
1. A plain capillary tubes were filled with well mixed EDTA anticoagulated blood.
2. The unfilled ends were sealed with plasticine.
3. The filled capillary tubes were carefully located in numbered slots of the microhaematocrit rotor with the sealed end against the rim gasket.
4. They were centrifuged for 5 minutes at 15,000 xg.
5. The results were read immediately after centrifuging them.

Statistical analysis
The data were presented as mean values and ± standard variation (± SD) in tables. The data were analysed using t-test and Pearson Product moment method.Statistical significance was set at $P<0.05$

Results

Table 1: Comparism of erythropoietin (Epo) and PCV Levels in patients on predialysis and controls

Parameters	PD (60)	Control (60)	Level of significance
Epo(iu/l)	23.3±3.2	48.6±24.0	$P<0.05$
PCV(%)	16.0±1.4	43.8±4.9	$P<0.05$

PD=Predialysis

Table 1 showed significant decrease ($P<0.05$) in Erythropoietin and PCV levels of predialysis subjects (23.3±3.2 iu/l, 16.0±1,4%) compared to Control subjects (48.6±24.0 iu/l, 43.8±4.9%) respectively.

Table 2: Comparism of Erythropoietin (Epo) and PCV in patients with dialysis to controls

Parameters	PD (60)	Control (60)	Level of significance
Epo(iu/l)	71.4±14.7	48.6±24.0	$P<0.05$
PCV(%)	27.0±7.0	43.8±4.9	$P<0.05$

D= Dialysis

Table 2 showed significant increase ($P<0.05$) in Erythropoietin level of dialysis subjects (71.4±14.7 iu/l) compared to control subjects (48.6±24.0 iu/l) and significant decrease ($P<0.05$) in PCV level of dialysis subjects (27.0±7.0%) compared to control subjects (43.8±4.9%).

Table 3: Comparism of Erythropoietin and PCV in Patients on predialysis and on dialysis

Parameters	PD (60)	D(60)	Level of significance
Epo(iu/l)	23.3±3.2	71.4±14.7	$P<0.05$
PCV(%)	16.0±1.4	27.0±7.0	$P<0.05$

PD=Predialysis

D=Dialysis
Table 3 showed significant decrease ($P<0.05$) in Erythropoietin and PCV levels of predialysis subjects (23.3±3.2 iu/l, 16.0±1.4%) compared to dialysis subjects (71.4±14.7 iu/l, 27.0±7.0%) respectively.

Table 4: Correlation of EPO and PCV values in the renal Insufficiencies

		PCV(PD)	PCV(D)
EPO	Pearson	1.000**	0.9986*
	2 tailed	.2732	.2732
	N	60	60

PD=Predialysis

D=Dialysis
Table 4 showed correlation of erythropoietin and PCV values in the renal diseases which showed perfect relationship in

predialysis subjects (1.000) and positive relationship (0.9986) in patients on dialysis.

Discussion

The study was done to correlate the levels of erythropoietin and haematocrit in anaemias of renal diseases at the Federal Medical Centre, Umuahia.Anaemia is one of the clinical and laboratory manifestations of chronic kidney disease.Relatively little is known about the development and progression of anaemia in patients with chronic kidney disease.As kidney function declines and in patients with more advanced chronic kidney disease stages, the incidence and prevalence of anemia increased. There is an exponential relationship between glomerular filteration and anaemia.

Table 1 showed significant decrease (P<0.05) in Erythropoietin and PCV levels of predialysis subjects (23.3±3.2 iu/l, 16.0±1,4%) compared to Control subjects (48.6±24.0 iu/l, 43.8±4.9%) respectively. A relative lack of erythropoietin (Epo) is considered to be the main cause of the development of renal anaemia seen with low PCV. The main stimulus for elevated synthesis of erythropoietin is tissue hypoxia, which normally leads to an exponential increase in serum erythropoietin levels. This feedback is affected in patients with pathological condition involving the kidneys and the developing anaemia is not adequately compensated by a sufficient increase in the erythropoietin production (Nangaku and Eckaradt, 2006) [19]. The kidneys of the subjects on predialysis might have been affected to cause the reduction in the erythropoietin level when compared to the control subjects who were apparently healthy but the level can still sustain the subject although,the subject will be anaemic. The result obtained here is in accordance with research done by Fehr *et al*. (2004) [10] which showed low erythropoietin in the study group.

Table 2 showed significant increase (P<0.05) in Erythropoietin level of dialysis subjects (71.4±14.7 iu/l) compared to control subjects (48.6±24.0 iu/l) and significant decrease (P<0.05) in PCV level of dialysis subjects (27.0±7.0%) compared to control subjects (43.8±4.9%). This might be as a result of gradual improvement in the damaged fibroblasts of the renal cortex particularly the renal medulla at the outer layer between the basolateral membrane of the proximal tubules and peritubular capillaries.Hybridisation techniques have confirmed that erythropoietin is not produced for storage,but an increase in its production related to hypoxic stimulus is caused by exponential growth in the number of speciallised cells (Lacombe *et al.,*1988). This observation could be attributed to many of the dialysis patients are always palced on recombinant erythropoietin therapy. The feedback mechanism is highly sensitive because an incraese in the activity of specific mRNA can be demonstrated within two hours of the onset of hypoxic stimulus (Epstein, 1997) [8].

Normochromic normocytic anaemia can occur in various chronic diseases, and it appears that dysregulation of iron homoeostasis and inflammatory processes acta s the main mediators (Ezekowitz *et al.,* 2003; Ganz, 2007; Weiss and Goodnough, 2005) [9, 25]

Table 3 showed significant decrease (P<0.05) in Erythropoietin and PCV levels of predialysis subjects (23.3±3.2 iu/l, 16.0±1.4%) compared to dialysis subjects (71.4±14.7 iu/l, 27.0±7.0%) respectively.This could be as a result of improvement in the fibroblasts of the kidneys.This showed that dialysis has a good effect on the repair of the damaged renal tissues by increasing the level of erythropoietin which prevents apoptosis of the cells.

Table 4 showed the correlation of erythropoietin and PCV values in the renal diseases.The table showed that there was a perfect relationship between Epo and PCV of chronic kidney disease subjects on predialysis (PD) and positive relationship in dialysis subjects. This showed that the kidneys might have been damaged to affect the level of erythropoietin which affects the PCV level in predialysis subjects. The feedback is affected in patients with pathological conditions involving the kidneys and the developing anaemia is not adequately compensated by a sufficient increase in the erythropoietin production (Nangaku and Eckardt, 2006) [19].

Conclusion

The study showed that there was decrease in Epo and PCV levels in the renal diseases studied. The depression of erythropoietin level were more pronounced in chronic kidney disease subjects before dialysis (predialysis) and improved on dialysis and chronic kidney disease subjects on predialysis and dailysis showed perfect and positive relationship respectively showing that they do not follow the established inverse relationship in pathological conditions.These subjects need urgent attention to restore the function of the kidneys.

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