World Journal of Pharmaceutical and Life Sciences
WJPLS
www.wjpls.org
SJIF Impact Factor: 3.347

CORRELATION OF VALUES OF HAEMOGLOBIN AND ERYTHROPOIETIN IN DIFFERENT TRIMESTERS OF PREGNANCY IN FEDERAL MEDICAL CENTRE OWERRI

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Article Received on 27/01/2016 Article Revised on 17/02/2016 Article Accepted on 09/03/2016

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ABSTRACT

The correlation values of haemoglobin (HB) and erythropoietin (Epo) in the different trimesters of pregnancy was conducted in Federal Medical Center Owerri, with the aim of determining the HB and EPO levels among pregnant women in different trimester of pregnancy in order to detect anaemia. A total of 200 women chosen randomly, prospectively and consecutively were used for the study: 50 pregnant women in their first trimester, 50 pregnant women in their second trimester and 50 pregnant women in their third trimester served as the test groups, while 50 non-pregnant women served as the control. The mean age of the subjects was 34 years. Laboratory investigation for PCV and HB were done using the methods described by Cheesbrough (2006), miccrohaemaricit and Cyanmethaemoglobin; and erythropoietin level estimation was done using ElISA. Results of the laboratory investigation were analysed statistically using Pearson product moment of correlation using statistical package for social packages (SPSS). Laboratory result showed that pregnant women in the first trimester were mildly anaemic, with HB and erythropoietin level of 10.5g/dl and 75.77pg/mL respectively; while those in second and the third trimesters where not anaemic, with HB and EPO levels of 10.99g/dl and 99.88pg/ml in the second trimester and 11.67g/dl, 34.62 and 72.84pg/ml in the third trimester. The Correlation of erythropoietin and HB

between first and second trimester were 0.041 and -0.282 showing a very weak correlation which were not statistically significant since P>0.05, also for the second and third trimester the correlation values were -0.282 and 0.0000 showing also a very weak correlation which was not statistically significant with P>0.005.

KEYWORDS: Haemoglobin, Erythropoietin, Pregnancy, Anaemia.

INTRODUCTION

Anaemia which is common in pregnancy can lead to serious complications of pregnancy such as preterm delivery which have serious consequences for both mother and foetus. Most anaemia in pregnancy results from iron-deficiency. It is estimated that about 2,150 million people are iron deficient (WHO, 1999). In developing countries, nearly half of the population is iron-deficient. Roughly 47% of non-pregnant women and 60% of pregnant women have anaemia worldwide. It has been clearly demonstrated that anaemic pregnant women are at greater risk of death during perinatal period. Close to 500, 000 maternal deaths have been ascribed to childbirth or early post –partum, with anaemia contributing 20-40% of such deaths (WHO, 1962). Anaemia poses a 5-fold increase in the overall risk of maternal death related to pregnancy and delivery. In Zaria, Nigeria Harrison (Harrison, 1982) reported that mortality for women during delivery or shortly after was 20%. Severe anaemia is however associated with very poor overall socioeconomic and health conditions in certain countries and regions of developing world.

Anaemia has different diagnostic markers such as low haemoglobin concentration, packed cell volume; red cell indices such as mean cell volume (MCV), mean corpuscular haemoglobin concentration (MCHC) etc. Erythropoietin, a precursor of red blood cell, theoretically is expected to increase in anaemic states. Several researchers have studied the levels of haemoglobin, packed cell volume and erythropoietin in pregnant women. This study is aimed at correlating the different diagnostic indices of anaemia- haemoglobin concentration and erythropoietin in different trimesters of pregnancy for better management of anaemia in pregnant women.

Pregnancy is the fertilization and development of one or more offspring, known as embryo or foetus, in a woman's uterus. It is the common name for gestation in humans. Child-birth usually occurs about weeks after conception in women who have normal menstrual cycle of

four weeks; this is approximately 40weeks from the start of the last normal menstrual period (LMP).

Pregnancy is typically divided into three periods or trimesters, each about three months (Collins English Dictionary, 2012 and American Heritage Dictionary, 2001).

Obstericians define each trimester as lasting for 14 weeks, resulting in a total duration of 42 weeks, although the actual duration is about 40 weeks.

Normal pregnancy is characterized by profound changes in almost every organ system to accommodate the demands of the fetoplacental unit. The haematologic system must adapt in a number of ways such as provision of vitamins and minerals for foetal hamatopoiesis (iron, maternal anaemia and preparation for bleeding at delivery) which requires enhanced haemostatic function. Haematological changes as well as physiological changes are influenced by maternal hormonal changes.

During pregnancy, the total blood volume increases by about 1.5 litres, mainly to support the demands of the new vascular bed and to compensate for blood loss occurring at delivery (Surabhi *et al*., 2012). Increase in blood volume is more marked in multiple pregnancies and in iron-deficiency states. Expansion of plasma volume occurs by 10-15% at 612 weeks of gestation (Haroon *et al*., 2003) ie within the first trimester. Red cell mass, driven by an increase in maternal erythropoietin production) also increases but relatively less, compared with the increase in plasma volume; the net result being a dip in haemoglobin concentration. Thus there is dilutional anaemia.

By the late second trimester, the drop in haemoglobin is typically by 1-2g/dl and stabilizes thereafter in the third trimester when there is reduction in maternal plasma volume (owing to an increase in atrial natriuretic peptide). Women who take iron supplements have less pronounced changes in haemoglobin as they increase their red cell mass in a more proportionate manner than those not on haematinic supplements.

The red cell indices change little in pregnancy. However, there is a small increase in mean corpuscular volume (MCV) of an average of 4fl in an iron-replete woman. Post pregnancy, plasma volume decreases as a result of diueresis, and the blood volume returns to non-pregnant values. Haemoglobin increases consequently.

Anaemia during pregnancy is defined as less than fifth percentile of the distribution of haemoglobin(Hb) (CDC, 2011). The cut- off values vary by trimester for pregnant women. In the first trimester, Hb level for pregnant women should not be less than 11.0g/dl. In the second trimester Hb level should not be less than 10.5g/dl. In the third trimester, Hb level should not be less than 11.0g/dl(CDC, 2011).

Pregnant women are at a higher risk of iron-deficiency anaemia because of the increased iron requirements of pregnancy. In pregnant women, Hb levels drop during the first and second trimester because of blood volume expansion. Among pregnant women who do not take iron supplements, haemoglobin and haematocrit remain low during the third trimester (CDC, 2011).

Erythropoietin is gaining popularity as a therapeutic option during pregnancy and postpartum period for treatment of anaemia (Sienas *et al*., 2013). According to the research conducted by Sienas *et al*. (2013) further investigation is needed to establish a standard dosage and dosing interval.

Erythropoietin (EPO) is a glycoprotein hormone that controls erythropoiesis or red blood cell production. It is a cytokine (protein signaling molecule) for erythrocyte precursors in the bone marrow (Siren *et al*., 2001).

In addition to erythropoiesis, erythropoietin also has other functions. For example, it plays an important role in the brain's response to neuronal injury (Siren *et al*., 2001).

Exogenous erythropoietin is produced by recombinant DNA technology in cell culture. It has been used illicitly as a performance –enhancing drug. It can often be detected in blood due to slight differences from the endogenous erythropoietin eg in features such of post-translational modification (FDA Safety Information).

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

Erythropoietin (EPO) concentration has been proved to increase 2-4 folds in the course of pregnancy (Conrad *et al*., 1996; Nangaku & Eckardt, 2007; McMullin *et al*., 2003) and plateau is achieved after 20 weeks ie second trimester of gestation(Rikonen *et al*., 1994). The

reason for this phenomenon is hazy (Kowalska-kanka & Maciejewski, 2013). It is however believed that physiological blood dilution in pregnancy, increase of renal oxygen consumption due to intensified glomerular filtration as well as paracrine and autocrine mechnanisms are likely to be responsible for increased EPO renal secretion in pregnancy (Conrad *et al*., 1996).

Pregnancy anaemia, most frequently caused by iron –deficiency, can have serious consequences for both mother and child. Maternal haemoglobin concentration < 10.5g/dl increases the risk of prematurity and low birth weight of the newborn (McMullin *et al*., 2003).

Some authors found no correlation between EPO and haemoglobin concentration in women with normal pregnancy in the first or second trimester. This phenomenon is explained by weakened EPO response to anaemia in early pregnancy and its intensification in the final stages (Rikonen *et al*., 1994; Erdem *et al*., 2002).

Erdem *et al*. (2002) noticed that pregnant women with anaemia have significantly lower haemoglobin and ferritin concentration, and higher blood serum erythropoietin level, compared to healthy pregnant patients. It was also found that EPO concentration in cord blood of newborn babies born by mothers with anaemia is significantly higher than in healthy women's babies.

It has finally been acknowledged that anaemia in pregnancy causes EPO secretion as a response to low haemoglobin concentration and ferrum deficiency.

Aims and objectives of the study

Having elucidated the haematologic physiologic changes in pregnancy, this study is aimed at correlating Haemoglobin concentration erythropoietin levels to detect anaemia in pregnant women in Federal Medical centre Owerri through.

- Determination of their haemoglobin level in different trimesters of pregnancy.
- Determination of erythropoietin levels in different trimesters of pregnancy.
- Correlation of the obtained haemoglobin and erythropoietin values for the different trimesters of pregnancy.

MATERIALS AND METHODS

Study Area

This study was conducted at the antenatal clinic of Federal Medical Centre Owerri with co-ordinate location of latitude $5^0$28'59"N and longitude $7^0$01'49'E and 159 metres above sea level. Owerri is made up of mainly Igbo ethnic group and few other nationalities with high rate of literacy while civil service is their major occupation. There were four groups A, B, C and D. Groups A, B, C were the test group while group D was the control group. Random sampling technique was employed. Laboratory analysis was done in the haematology and chemistry sections of the laboratory in Federal Medical Centre Owerri.

Study Population and Sample Size-

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 of anaemia in Nigeria according to Olatunbosun (2014) is 60% (0.60)

$$n = \frac{(1.96)^2 \times 0.60 \times 0.40}{(0.05)^2}$$

n = 221, approximately 200.

The sample size will be 200 subjects.

A total of 200 women were used for the study; 150 pregnant women and 50 non-pregnant women. One hundred and fifty pregnant women aged 22-45 years attending antenatal clinic of Federal Medical Centre Owerri between February and May 2015, fifty subjects for each trimester, and fifty non-pregnant women aged 22-45 years was used as controls.

Sampling- the women for the test groups were selected at random for the three trimesters of pregnancy and also for the control group- 50 women for each group. Group A represents pregnant women in the first trimester. Group B represents pregnant women in the second trimester and group c represents pregnant women in the third trimester. Group D were non-pregnant women who visited the clinic within the period of study.

Ethical Clearance

The study was approved by the federal medical centre research and ethical committee and ethical clearance was obtained before the study commenced. Participant information sheet (PIS) was given to the prospective participants. After reading and understanding the PIS, questions were asked and proper explanations given. They consented to participate in the study by signing the informed consent form.

Subjects

Clinical Criteria for Diagnosis of Pregnancy

Foetal heart-beat identification

The demonstration of foetal heartbeat is made possible by auscultation, Doppler technology or sonography. This can be performed from about 19 weeks of pregnancy (Bastian & Piscitelli, 1997).

Maternal perception of foetal movement

The mother can feel the movement of the foetus as early as the six-weeks of pregnancy. This can also be detected by ultrasound (De Vries, 1984).

Ultrasonographic demonstration of pregnancy

Ultrasonogrphic evidence of pregnancy can be seen as early as 4-5 weeks' gestation. Modern ultrasound techniques have made possible and have advanced the diagnosis and prognosis of early pregnancy. It has not however replaced biochemical testing for the diagnosis of early pregnancy but has greatly improved the differentiation of normal versus abnormal uterine pregnancies and the determination of extrauterine pregnancies (Bastian & Piscitelli, 1997).

Biochemical Criteria for Diagnosis of Pregnancy

Human chorionic gonadotropin hormone synthesis and secretion in normal and aberrant pregnancies

Human chorionic gonadotopin is a glycoprotein hormone secreted by the syncytiotrophoblast with a molecular weight of 36, 700Da. The hCG molecule contains 30% carbohydrate (Dart *et al.*, 1999). hCG is the hormone that classically has been measured to diagnose pregnancy. It is composed of α and β-subunits which are non-covalently linked. The β-subunit confers specific activity to the hormone and is the subunit most commonly measured in most pregnancy assays. Detection of hCG in plasma is not possible until implantation has

occurred. The level of hCG peaks (100, 000mIU/ml) at about 10weeks' gestation (6) after which it decreases by 20, 000mIU/ml in midpregnancy and remains at that level till term.

Abnormal elevations, plateaus or decreasing titres of β-hCG, raises the possibility of ectopic pregnancy. Therefore β-hCG is diagnostic of both normal and abnormal pregnancies.

Enzyme-linked immunosorbent assay (ELISA) is a quick, easy method of hCG determination. This assay uses highly specific monoclonal antibodies for hCG. It is very sensitive; the sensitivity is as low as 5mIU/ml (Bandi *et al.*, 1987).

Urine and serum follicle- stimulating hormone

Total and serum follicle stimulating hormone (FSH) β- subunit levels in the post –ovulatory period are lower in conception cycles than in non-conception cycles. Qui et al.(1997) showed that mean serum and urinary FSH levels rise significantly above the post ovulatory base-line by day 10-12, following LH surge in non-pregnant conception cycles but did not rise at any time following ovulation in conception(Qui et al., 1997). They reported that the sensitivity and specificity of urinary FSH to detect pregnancy were 88.9% and 89.3% respectively.

Early pregnancy factor

This has been studied as an alternative to β-hCG determination, because it can be detected in the blood prior to hCG (Sinosich et al., 1985). EPF is a placental protein and one of the first pregnancy markers known to appear in the blood.

Exclusion Criteria

Pregnant women who attend the antennal clinic, but refused to disclose their gestational period and those that refused to give their consent were excluded.

Inclusion Criteria

Pregnant women at different trimesters and control women (non-pregnant women) within the ages to 22-45 years who gave their consent were included.

Sample Collection and Methodology

The blood was collected from the ante-cubital vein close to the skin, the median cubital vein in the inside of the elbow. The skin over the blood vessel was cleaned with 70% alcohol.

A tourniquet was wrapped around the upper arm to increase the pressure of the blood in the arm veins and speed up the process. The blood was collected into EDTA containers for

haemoglobin, PCV estimation and Plain containers for erythropoietin determination (Cheesbrough, 2006; ALPCO, 2014).

Determination of Haemoglobin Level

Method: Haemoglobincyanide technique

Principle: Whole blood is diluted 1 in 201 in a modified Drabkin's solution which contains potassium ferricyanide and potassium cyanide. The red cells are haemolyzed and the haemoglobin is oxidized by the ferricyanide to methaemoglobin. This is converted by the cyanide to stable haemoglobincyanide (HiCN). Absorbance of the HiCN solution is read in a spectrophotometer at wavelength 540nm or in a filter colorimeter using a yellow-green filter. The absorbance obtained is compared with that of a reference HiCN standard solution. Haemoglobin values are obtained from tables prepared from a calibration graph or if using a direct read-out haemoglobin metre, from the digital display (Cheesbrough, 2006).

Procedure

- 20µl (0.02ml) of well-mixed venous blood was carefully measured and dispensed into 4ml Drabkin's neutral diluting fluid.
- It was stoppered and left at room temperature, protected from sunlight for 4-5minutes.
- The colorimeter was set at 540nm wavelength.
- The colorimeter was zeroed with Drabkin's fluid and the absorbance of the samples was read.
- Using the table prepared from the calibration graph, the haemoglobin values were obtained.

Determination of Erythropoietin Level

Method: EPO Immunoasay

Principle: The EPO Immunoassay is a two-site ELISA (Enzyme linked Immunosorbent Assay) for the measurement of the biologically active 165 amino-acids chain of EPO. It utilizes two different mouse monoclonal antibody to human EPO specific for well-defined regions of the EPO molecule. One mouse monoclonal antibody to human EPO is biotinylated and other mouse monoclonal antibody to human EPO is labeled with horseradish preoxidase (HRP0 for detection). In this assay calibrators, controls or patient samples are simultaneously incubated with the enzyme-labelled antibody and a biotin coupled antibody in a streptavidin-coated microplate well. At the end of the assay incubation, the microwell is washed to

remove unbound components and the enzyme bound to the solid phase is incubated with the substrate, tetramethylbenzidine (TMB). An acidic stop solution is then added to the reaction and converts the colour to yellow. The intensity of the yellow colour is directly proportional to the concentration of EPO in the sample. A dose-response curve is generated using results obtained from the calibrators. Concentration of EPO present in the patients' samples is determined directly from this curve (ALPCO, 2014).

Sample Collection and Storage

The determination of EPO was performed on human serum. The samples were collected between 7:30am and 12:00pm because diurnal variation has been reported in literature (Cahan *et al.*, 1992).

Whole blood was collected without anticoagulant and allowed to clot between 2-8^{0}C .It has been reported that serum samples clotted at room temperature (22^{0}C to 28^{0}C) caused a decrease in EPO value as assessed by radioimmunoassay of about 30% over clotting on ice or lower temperature.

The sera were separated after centrifuging and stored at 2^0 C.

Assay procedure

- The streptavidin coated plates were placed in a holder to run all six calibrators, A-F of the EPO calibrators, controls and patient samples.
- 200µl of calibrators and controls and samples were pipette into designated or mapped well.
- 25µl of reagent 2 (enzyme labeled antibody) was dispensed into each of the wells, which already contain the calibrators, controls and samples).
- 25 µl of reagent 2(Enzyme labeled antibody was dispensed into each of the sample wells). Then the microplate was tapped firmly against the bench to achieve thorough mixing of the sample reagents. The tapping was done five times to achieve complete mixing.
- The microplate was covered with aluminum foil to avoid exposure to light, and then placed on a rotator at 170 rpm for 2hours at room temperature.
- The fluid in the microplate was aspirated completely from each well and then the wells were washed five times with the working solution using automatic microplate washer.
- 150µl of the ELISA reagent B(TMB substrate) was added into each of the wells. The microplate was tapped again for 5 times.

- The microplate was covered with a foil to avoid exposure to light and then placed on a rotator set at 170 rpm for 30mins at room temperature.
- 100µl of the Stop solution was added into each of the wells. Then the microplate was tapped again for five times.
- Prior to reading the blank wells were filled with distilled water.
- The absorbance of the solution in the wells was read within 10 minutes, using a microplate reader set to 450nm.
- A second reading of the plate was done against the blank wells with the reader set at 405nm.
- Using the final absorbance values obtained in the previous step, the calibration curves were constructed using 405nm reading and 450nm reading, through cubic spline, point-to-point interpolation the concentration of EPO is quantified.

  Reference range: 3.22-31.9mIU/mL 0r 31.25-2000pg/mL.

RESULTS

Table 1: Mean and standard deviation of Haemoglobin concentration in different trimesters of pregnancy.

Subjects	Mean	SD	Significance
First trimester	10.51	0.8793	0.033
Second trimester	10.99	1.0683	0.000
Third trimester	11.67	1.3729	0.000
Control	11.97	1.1011	0.191

*P < 0.05

Table 2: Mean and standard deviation of Erythropoietin Values in different trimesters of pregnancy.

Subjects	Mean	SD	Significance
First trimester	75.77	43.09036	0.087
Second trimester	91.88	35.1929	0.754
Third trimester	72.84	23.0075	0.000
Control	122.82	71.7944	0.001

Table 3: Correlation Analysis of the Haemoglobin level and erythropoietin levels in the subjects.

Hb/Epo	1st trimester	2nd trimester	3rd trimester
Pearson Correlation	0.041	-0.282	0.000
Sig(2-tailed)	0.778	0.047	0.995
N	50	50	50

Table 4: Correlation Analysis of the erythropoietin levels and HB Levels in the subjects

EPO/HB	1st trimester	2nd trimester	3rd trimester
Pearson Correlation	0.041	-0.282	0.000
Sig(2-tailed)	0.778	0.047	0.995
N	50	50	50

DISCUSSION

Looking at the laboratory results of the study, the statistical analysis of the results and comparing them with previous related studies conducted by other researchers, it is found that most are in line with the results of the previous related studies.

Studying Table 1 which shows the mean haemoglobin concentration of the subjects in different trimesters of pregnancy, it is seen that the mean haemoglobin concentration in the first trimester subjects is 10.5g/dl, with a standard deviation of 0.9, which is a small value, which implies that the haemoglobin concentrations of pregnant women in this group are all close to the mean value. From the WHO definition of anaemia in pregnancy and from CDC, (2011), the cut-off value for Hb in the first trimester is 11.0g/dl. It can therefore be inferred that subjects in this group are mildly anaemic.

Studying the mean haemoglobin concentration of subjects in the second trimester which are 11.0g/dl with standard deviations of 1.1 a, we see that most pregnant women in this group are healthy that is not anaemic, since the mean haemoglobin concentration was up to the cut-off values given by WHO and CDC, which are 10.5g/dl a pregnant women in their second trimester.

In group C, studying Table 2, we also see that most pregnant women in this group are not anaemic as shown in their mean haemoglobin concentration 11.7g/dl But checking the cut-off values given by CDC (2011), 11.0g/dl for Hb, it could be seen that most of the subjects are not anaemic.

The erythropoietin concentration of the studied groups does not have a specific pattern of increase or decrease. Looking at the subjects in group A that is pregnant women in their first trimester, we see that some have high erythropoietin values while others have low erythropoietin values. This is clearly shown in the mean EPO and standard deviation, which are 75.8 and 42.7 respectively. A standard deviation of 42.7 shows a wide range of variation among the values and when compared with their mean. This finding supports the fact that previous work on erythropoietin secretion in pregnancy is controversial.

Some researchers observed a weakened EPO response to anaemia during the first trimester of pregnancy and its intensification in the final stages of pregnancy (Erdem *et al*., 2002).

This observation of Erdem *et al*. (2002) can be used to explain the low EPO values found in the anaemic pregnant women in the first trimester.

Also, when studying the EPO Values of the pregnant women in the second and third trimesters, it is observed that most of the women have high EPO values. This can be explained by the observations of different authors: the finding of Erdem *et al*., (2002) about the intensification of erythropoietin response in the final stages of pregnancy, the studies conducted by Conrad *et al*.(1996) and Nangaku & Eckardt (2007), who proved EPO concentration increases 2-4 folds in the course of pregnancy and that plateau is achieved after 20 weeks of gestation i.e in the second trimester of gestation.

Moreso, according to Conrad *et al*.(1996), physiological blood dilution in pregnancy, increase in renal oxygen consumption due to intensified glomerular filtration as well as paracrine and autocrine mechanisms are likely to be responsible for increased EPO renal secretion in pregnancy.

The correlation analysis conducted for Haemoglobin and Erythropoietin in the various trimesters indicated a very weak linear correlation of haemoglobin and Erythropoietin in the first trimester and third trimester, and a negative correlation in the second trimester and were also not statistically significant as ($P>0.05$) as seen in table 3. All also indicated a statistical significant difference in the three trimesters ($P<0.05$).

In view of the above findings, we recall that some authors found no correlation between EPO and haemoglobin concentration in women with normal pregnancy in the first or second trimester (Rikonen *et al*., 1994). This is explained by weakened EPO response to the dilutional anaemia observed in these trimesters (Erdem *et al*., 2002).

CONCLUSION

Haemoglobin concentration and packed cell volume of the subjects in different trimesters of pregnancy were, apparently, only affected by the dilutional anaemia inherent in the early and middle stages of pregnancy. We cannot draw absolute conclusion on this because of some of the limitations of this study. We did not exclude pregnant women who are on haematinic supplements from those who are not.

This study shows a strong correlation between haemoglobin concentration in pregnancy. However, the correlation of erythropoietin concentration with either haemoglobin concentration shows a weak negative correlation. Since none of the subjects has a malfunction of the kidney, we conclude that erythropoietin concentration is only affected in pregnancy in the presence of kidney malfunction.

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