Full Length Research Paper

Preliminary study on the establishment of reference intervals for serum prolactin for women in Zaria, Nigeria

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Received 13 March, 2015; Accepted 30 November, 2015

The interpretation of prolactin results is difficult as race related reference intervals are scarce, especially in Nigerian women. Serum prolactin reference intervals were determined from 120 subjects. Subjects were randomly selected from antenatal clinics and general outpatient clinics in one private hospital and three government hospitals in Zaria, Northern Nigeria. They consisted of six groups made up of women that were A-non pregnant, B-first trimester of pregnancy, C-second trimester of pregnancy, D-third trimester of pregnancy, E-labour, and F-puerperium. Their blood samples were collected via venipuncture between 09.00 and 13.00 h after which they were centrifuged and the sera stored at -8°C for further analysis. The samples were analyzed at the Department of Chemical Pathology, Ahmadu Bello University Teaching Hospital, Shika. Serum prolactin was determined with Prolactin quantitative test kits using the principle of solid phase enzyme-linked immunosorbent assay. The concentration of prolactin is proportional to the color intensity of the samples as measured spectrophotometrically at a wavelength of 450 nm. Informed consent and ethical clearance was obtained before commencement of the study. Results were presented as mean ± standard deviation (SD) and data analyzed using one-way analysis of variance, while post-hoc test was carried out. A level of significance of p<0.05 was considered statistically significant, while the reference range was defined as 95% confidence interval. Serum prolactin results showed a significant increase in pregnant women as compared to the non-pregnant, not lactating subjects (20.24±3.41 ng/ml; 95% Confidence Interval (CI): 18.84-21.65 ng/ml). There were also significant trimester related differences in serum prolactin with prolactin levels increasing during the first trimester though not significantly (29.84±5.08 ng/ml). A sharp increase was observed during the second trimester (96.09±18.82 ng/ml; 95% CI: 89.41 to 102.76 ng/ml). There was a further increase at the third trimester (171.11±32.92 ng/ml; 95% CI: 159.03-183.18 ng/ml), a peaking during labour (198.37±15.81 ng/ml; 95% CI: 186.22-210.52 ng/ml) with a significant decline during the first week of the puerperium (197.19±22.11 ng/ml; 95% CI: 183.83-210.55 ng/ml). Apparent changes in serum prolactin levels were similar to that of previous studies; although the ranges for all groups were observed to be narrower as compared to those of other studies.

Key words: Serum prolactin, reference ranges, enzyme immunoassay, Zaria.

INTRODUCTION

Prolactin is a versatile peptide hormone, best known for its role in lactation. Prolactin also has widespread effects amongst which are its profound effects on angiogenesis, leucopoiesis, hematopoiesis, modulation of T- and B- cell
function and as an anti-apoptotic factor (Reuwer et al., 2012; Terra et al., 2011; Ben-Jonathan et al., 2008; Welniaik et al., 2001; Nagy and Berczi, 1989). Prolactin has been reported to play a role in the aetio-pathogenesis of autoimmune diseases like systemic lupus erythematosis, rheumatoid arthritis and multiple sclerosis (Da Costa et al., 2011; Jara et al., 2011; Orbach and Shoenfeld, 2007; Jara et al., 2001; Neidhart et al., 1999). Altered prolactin levels associated with either T-helper cell type 1 and type 2 dominance often characterize autoimmune diseases (Orbach and Shoenfeld, 2007). Pro-carcinogenic effects of prolactin has also been reported (Alhaj, 2012; Manjer et al., 2003). Causes of hyperprolactinemia, include physiological states such as increased prolactin levels in the morning, after exercise, after meals and following sexual intercourse. Increased prolactin also occurs following epileptic seizures, in stress, in expectant fathers as well as in pregnancy and lactation (Kruger et al., 2012; Rojas et al., 2012; Díaz et al., 2013; Ben-Menachem, 2006; Van Cauter and Spiegel, 1997). A circadian and ultradian rhythm is responsible for these fluctuations (Fujimoto et al., 1990; Van Cauter, 1990). The ubiquitous distribution of prolactin receptors and the extrapituitary synthesis of prolactin is responsible for its numerous roles (Marano and Ben-Jonathan, 2014). Assays of prolactin are carried out as part of investigations of hypogonadism in males, erectile dysfunction in males, infertility in women and some metabolic diseases in children (Catti et al., 2012; Seaborg, 2012; Zeitlin and Raifer, 2000).

Guidelines determining prolactin reference range for men and women have been defined (Seaborg, 2014; Katayev et al., 2010; Jeffcoate et al., 1986). However, different assays and methods for measuring prolactin are employed by different laboratories. As such, the serum reference range for prolactin is often determined by the laboratory performing the measurement. Prolactin levels also vary with several variables, such as age, sex, menstrual cycle stage, lactation, pregnancy as well as racial and ethnic variability has been demonstrated (Kim et al., 2012; Tanner et al., 2011; Sachidanandam et al., 2009; Jeffcoate et al., 1986).

Thus, accurate interpretation of the measurement is dependent on the circumstances around the given measurement.

Reference intervals estimate the expected values that would contain the 95% of the subjects of the considered population (Baron et al., 1989). Reference ranges utilized in Nigerian laboratories are as specified in the kits without further development of local laboratory reference range. However, there is a need for developing one due to the need to compensate for calibration errors in equipment, compensating for errors in the laboratory methodology, errors in the statistical method used and other variability as suggested by Katayev et al. (2010), Nakayama (1992) and Gaines Das and Cotes (1979).

A previous study on the establishment of reference ranges for some reproductive hormones in Nigerian women including prolactin assays (Amballi et al., 2007). Their study using enzyme immune assay excluded pregnant women, women in labour and the puerperium. This study thus aimed at determining possible difference in the reference ranges for serum prolactin among Nigerian women who were non-pregnant, pregnant, in labour, and the puerperium in Zaria, Northern Nigeria.

**MATERIALS AND METHODS**

**Study site**

The study was conducted among women in Zaria, Northern Nigeria. Zaria is a town located within latitude 11°3’N and longitude 7° 42’E. It is comprised of 2 local government areas with an altitude of 610 m, an annual rainfall of 1056.6 mm and a mean annual temperature of 27°C (Mortimore, 1970).

**Study design**

This study is a cross-sectional, multicenter study. Women were recruited from antenatal clinics, delivery rooms and lying-in wards of five hospitals in Zaria. They consisted of Primary Health Centre, Samaru; Salama Hospital and Maternity, Kwangila; Sabon Gari Comprehensive Health Centre; St Luke’s Hospital, Wusasa and Ahmadu Bello University Health Centre, Samaru, Zaria. While the control subjects consisted of individuals from Ahmadu Bello University (students and staff). Approval for the study was obtained from the Ethical Committee on Human Study of the Kaduna State, Ministry of Health. All participants provided informed consent.

**Study subjects**

A total of one hundred and twenty healthy female subjects participated in the study. Twenty-five subjects served as control for the study. They were non-pregnant, non-lactating women in their reproductive age who were not on hormonal contraceptives. A total of ninety-five women were either pregnant, in labour or in the puerperium. All pregnancies were dated to the last menstrual period. Nine women were in their first trimester, 33 women were in their second trimester while thirty-one women were in their third trimester. Nine women were in labour and 13 were women in puerperium; eight of which had spontaneous vaginal delivery (SVD) and five had a caesarean section (CS). Subjects who were diabetic or hypertensive (and thus on medication) were excluded from the study. A questionnaire was administered to all participants to obtain bio-socio-demographic data while weight, height, blood pressure and blood samples were collected.

**Weight, height and body mass index assessment**

The weights of the subjects were measured while wearing light
clothing to the nearest 0.2 kg with a calibrated weighing scale. Height (without shoes and head attire) was measured to the nearest 0.5 cm with a stadiometer. The body mass index was calculated as weight (kg)/height (m²) (Guyton and Hall, 2006)

Blood pressure measurement
The arterial blood pressure in the brachial artery was measured after seating the subjects for 5 min with legs uncrossed. A mercury sphygmomanometer (Acosson, A. C. Cossor & Son (surigcal) LTD, London) cuff was wrapped round the arm of the subjects and inflated while a 3M Littmann Classic II S.E. Stethoscope (3M Health Care, U.S.A) was used to auscultate. Auscultatory blood pressure measurements were determined using the onset of the first and the disappearance of the fifth Korotkoff sounds denotes the systolic and diastolic blood pressure, respectively (Pickering et al., 2005).

Urinalysis
A fresh mid-stream urine sample was collected from the participating subjects into sterile, clean and dry bottles. The urine samples were immediately tested by inserting a multistrip (3) urinalysis strip affixed with chemically specific reagent pads. Detectable levels of protein and glucose and the urine pH was determined by visually comparing the color reaction with the included color chart to determine the level of each chemical factor.

Sample collection
Participants were instructed about the procedure on arrival at the health facility. They rested quietly for a minimum duration of 30 min; other parameters were also obtained on the same day (for some participants they proceeded for their antenatal visits thereafter). The blood samples were taken between 09.00 and 12.00 h. Five milliliters of venous blood was collected at the cubital fossa using a 5 ml syringe and a 23G needle. The sample was then transferred into a clean, plain and dry bottle which was centrifuged using a bench centrifuge at 3000 rpm for 5 min. The sera were transferred into plain bottles and frozen at <8°C till assay was carried out.

Prolactin assay
Serum prolactin concentration was determined for the samples using Prolactin Quantitative Test Kits. The principle of the test was based on solid phase enzyme linked immunosorbent assay. The level of prolactin was determined by measuring the detectable color change using a Microwell reader at 450 nm (Batzer, 1980). The assay was carried out at the Department of Chemical Pathology, Ahmadu Bello University Teaching Hospital, Shika, Zaria.

Data analysis
Results were presented as frequencies, percentages and mean ± standard deviation (SD), while data was analyzed using one-way analysis of variance using SPSS version 22, followed by a Tukey’s Post-hoc test, while the reference range (defined as 95% confidence limits) was determined as arithmetic mean ± 2 SD. Results were considered statistically significant with p<0.05.

RESULTS
The result is for 120 subjects comprising non-pregnant controls, women in their first, second, and third trimesters, women in labour and the puerperium. The data presented includes the mean ± SD and the central 95 percentile of the results of the apparently healthy Nigerian women in Zaria (Tables 1, 2 and 3).
Women below 25 years constituted the highest proportion of the study subjects (52%). While a large proportion of the subjects were Hausa women (49%). All the women had some form of education with the tertiary education group constituting the highest proportion (38%). Most of the subjects were housewives (35%) while the least were artisans (5%).

The mean age of the women in labour and the puerperium was significantly higher than that of the control (p<0.05). There was also a significant increase in weight in women in labour as compared to the non-pregnant control, pregnant women and women in puerperium (p<0.05). There was no significant difference in the height and BMI of the women across all groups (p>0.05). Mean systolic blood pressure significantly increased among women in labour and the puerperium as compared to the non-pregnant control (p<0.05). A significant decrease in the mean arterial pressure and diastolic blood pressure was observed in pregnant women and while women in labour had an observed significant increase in the mean arterial pressure and diastolic blood pressure as compared to the control non-pregnant women (p<0.05).

There was a significant increase in mean serum prolactin concentration in women in the 2nd trimester, 3rd trimester, labour and puerperium as compared to the control non-pregnant group (p<0.05). However, there was no significant difference in mean serum prolactin concentration between the non-pregnant control women and the first trimester pregnant women nor between the women in labour and the puerperium (p>0.05).

DISCUSSION
Pregnancy, labour and the puerperium induces a number of physiological variations in most body systems. Some of these changes are marked and hence a need for reference ranges for women across these transitional phases of their reproductive life. Prolactin hormone plays a role in establishing and maintaining lactation amongst its several functions (Freeman et al., 2000). A preliminary cross-sectional study to establish reference ranges of serum prolactin was undertaken in this study.

A large proportion of the women in the study were below 25 years. This is as a result of the region of the study area (Northern Nigeria) which is reputed for high rates of teenage marriages and child bearing which has resulted in a high total fertility rate of over 7 births per woman (National Population Commission, 2009; Wolf et al., 2008).

Most of the women were of Hausa ethnicity (49.2%). The
Table 1. The socio-demographic details of the participants; non-pregnant, pregnant, in labour and in the puerperium women.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Group</th>
<th>Non-pregnant (n=25)</th>
<th>Pregnant (n=73)</th>
<th>Labour (n=9)</th>
<th>Puerperium (n=13)</th>
<th>Totals</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Frequency</td>
<td>Percentage (%)</td>
<td>Frequency</td>
<td>Percentage (%)</td>
<td>Frequency</td>
<td>Percentage (%)</td>
</tr>
<tr>
<td>Age group (years)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;20</td>
<td>5</td>
<td>20</td>
<td>19</td>
<td>76</td>
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<td>0</td>
</tr>
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<td>21-25</td>
<td>10</td>
<td>27</td>
<td>22</td>
<td>59.5</td>
<td>1</td>
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<tr>
<td>26-30</td>
<td>4</td>
<td>13.8</td>
<td>22</td>
<td>79.9</td>
<td>2</td>
<td>6.9</td>
</tr>
<tr>
<td>&gt;31</td>
<td>6</td>
<td>20.7</td>
<td>10</td>
<td>34.5</td>
<td>6</td>
<td>20.7</td>
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<tr>
<td>Hausa</td>
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<td>3.4</td>
<td>43</td>
<td>72.9</td>
<td>5</td>
<td>8.5</td>
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<tr>
<td>Igbo</td>
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<td>30</td>
<td>5</td>
<td>50</td>
<td>2</td>
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<td>Yoruba</td>
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<td>52.9</td>
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<td>41.2</td>
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<tr>
<td>Others</td>
<td>11</td>
<td>32.4</td>
<td>18</td>
<td>52.9</td>
<td>2</td>
<td>5.9</td>
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<tr>
<td>Educational status</td>
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<td>0</td>
<td>19</td>
<td>76.0</td>
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<td>12</td>
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<td>Primary</td>
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<td>3</td>
<td>37.5</td>
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<tr>
<td>Secondary</td>
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<td>70</td>
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<td>47.8</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Occupation</td>
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<td>Civil Servant</td>
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<td>53.3</td>
<td>7</td>
<td>46.7</td>
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<tr>
<td>Trader</td>
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<td>0</td>
<td>17</td>
<td>73.9</td>
<td>2</td>
<td>8.7</td>
</tr>
<tr>
<td>Student</td>
<td>17</td>
<td>50</td>
<td>16</td>
<td>47.1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>House Wife</td>
<td>0</td>
<td>0</td>
<td>30</td>
<td>71.4</td>
<td>5</td>
<td>11.9</td>
</tr>
<tr>
<td>Artisan</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>50</td>
<td>2</td>
<td>33.3</td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>358.8</td>
<td>292</td>
<td>988.5</td>
<td>36</td>
<td>165.6</td>
</tr>
</tbody>
</table>

reason being that Zaria is located in Northern Nigeria, where one of the major ethnic groups is Hausa, a language used as a lingua franca in most of the region. An appreciable number of the women (38.3%) had attained tertiary education (Table 1). This is a marked improvement from the study of Okereke et al. (2013), where less than 5% of the female respondents participating in their study (site also in Northern Nigeria) had attained tertiary education. Other researchers have observed a lower educational status for females in Northern Nigeria as compared to other regions of the country (Korode, 2008; African Girl-Child Education Initiative, 2001; Umar, 1996). Their study was carried out in a rural setting. The findings of a high percentage of the women in our study area attaining a tertiary education (Table 1), is due to the fact that Zaria is an urban
Table 2. The age, anthropometric measurement and blood pressure of the subjects.

<table>
<thead>
<tr>
<th>Parameter (mean±SD)</th>
<th>Non-pregnant women (n=19)</th>
<th>Pregnant women (n=46)</th>
<th>Women in labour (n=9)</th>
<th>Women in puerperium (n=12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>26.05±5.44</td>
<td>24.87±5.01</td>
<td>32.78±5.12*</td>
<td>30.75±6.34*</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>163.73±8.47</td>
<td>161.76±11.35</td>
<td>165.50±1.41</td>
<td>161.13±2.43</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>58.84±11.12</td>
<td>63.39±13.22</td>
<td>78.38±3.11*</td>
<td>65.60±5.52</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>21.93±4.92</td>
<td>24.36±5.60</td>
<td>28.25±0.07</td>
<td>25.33±2.42</td>
</tr>
<tr>
<td>Systolic B.P (mmHg)</td>
<td>112.63±12.40</td>
<td>109.33±11.16</td>
<td>135.71±19.88*</td>
<td>133.00±20.03*</td>
</tr>
<tr>
<td>Diastolic B.P (mmHg)</td>
<td>77.89±9.18</td>
<td>70.11±9.80*</td>
<td>87.14±11.13*</td>
<td>91.00±13.70</td>
</tr>
<tr>
<td>Mean Arterial B.P (mmHg)</td>
<td>89.47±9.18</td>
<td>83.19±9.64*</td>
<td>103.33±13.19*</td>
<td>105.00±15.65</td>
</tr>
</tbody>
</table>

*P<0.05.

Table 3. Mean serum level of prolactin for all subjects; non-pregnant, pregnant, in labour and in puerperium women.

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean ± SD of serum prolactin concentration (ng/ml)</th>
<th>95% confidence interval for mean reference range (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-pregnant (n=25)</td>
<td>20.24±3.41</td>
<td>18.84 – 21.65</td>
</tr>
<tr>
<td>1st Trimester (n=9)</td>
<td>29.84±5.08</td>
<td>25.94 – 33.75</td>
</tr>
<tr>
<td>2nd Trimester (n=33)</td>
<td>96.09±18.82</td>
<td>89.41 – 102.76</td>
</tr>
<tr>
<td>3rd Trimester (n=31)</td>
<td>171.11±32.92</td>
<td>159.03 – 183.18</td>
</tr>
<tr>
<td>Labour (n=9)</td>
<td>198.37±15.81</td>
<td>186.22 – 210.52</td>
</tr>
<tr>
<td>Puerperium (n=13)</td>
<td>197.19±22.11</td>
<td>183.83 – 210.55</td>
</tr>
</tbody>
</table>

A significant decrease in diastolic blood pressure and mean arterial blood pressure during pregnancy was observed (Table 2). These findings are physiological variations associated with pregnancy. The decrease is associated with a decrease in systemic vascular resistance. Pregnancy hormones (estradiol-17b, progesterone and relaxin) have been implicated in the vascular change (Conrad, 2011; Kristiansson and Wang, 2001). Relaxin especially, is said to cause a induce a rapid and sustained vasodilation by activation of endothelial nitric oxide synthase and increases in arterial gelatinase (s) activity; which still subsequently activates the endothelial (ET(B) receptor/nitric oxide vasodilatory pathway.

Blood pressure (systolic and diastolic) was understandably significantly higher in women during labour (Table 2). It has longed been recognized that blood pressure rises during labour by 15 to 50 mmHg (Radcliffe, 1944). The rise occurs during uterine contractions and aids in maintaining a satisfactory placental circulation (Cooks and Briggs, 1903; Edwards, 1958).

In Nigeria, hyperprolactinemia is quite common in infertile patients (Meraiyebu et al., 2012; Olootu et al., 2012). It presents with galactorrhea, amenorrhea and sterility. Attempts have been made at establishing normal reference ranges for the Nigerian female population (Amballi et al., 2007; Iwamoto et al., 2004; Dada et al., 1981). These studies excluded women in other transitional stages of their reproductive cycle (pregnancy, labour, and the puerperium). Their studies entailed using radioimmunoassay method, comparing that with an enzyme immunoassay method, determining prolactin levels in men and women at various phases of their menstrual cycles. Pregnancy is associated with profound changes in laboratory markers amongst which are the reproductive hormones. A local reference range that describes the change across the non-pregnant, pregnant, labour and puerperium stages was the interest of this study.

In this study, serum prolactin levels in non-pregnant women were significantly lower than in other groups (Table 3).

Pregnant women in all trimesters presented with a higher concentration of serum prolactin as compared to their non-pregnant control group. The increase was with each increasing trimester. Estradiol levels, rising...
throughout pregnancy, act at the hypothalamic level to increase prolactin secretion. The increase is about 10 to 20. During pregnancy, prolactin increases arginine activity, stimulate ornithine decarboxylase activity and enhance the rate of transport of polyamines into the mammary gland. The resultant effect is increased spermine and spermidine synthesis required for milk production (Voogt et al., 2001). From our study, non-pregnant women had a reference range of 18.84 to 21.65 ng/ml, while the pregnant women had a reference range of 25.94 to 33.75, 89.41 to 102.76, and 159.03 to 183.18 ng/ml for the first, second and third trimester, respectively. These pattern of the reference range values are similar to the review of Abbassi-Ghanavati et al. (2009) who obtained a reference range of serum prolactin for non-pregnant women of 0 to 20 ng/ml, and for pregnant women as 36 to 213, 110 to 330, and 137 to 372 ng/ml for first, second, and third trimesters, respectively. Though, the reference ranges for the subjects in our study area yielded lower values and narrower ranges. It was observed that the values were also similar to reference values (in non-pregnant females) provided in a study by Ambali et al. (2007), his study however, excluded pregnant women and women in their puerperium. Reference ranges for serum prolactin determined by Dada et al. (1981), had values that compared reasonably with those in our study.

In our study, there was a significant increase in serum prolactin in labour and puerperium as compared to the non-pregnant women. However, there was a slight decrease in mean serum prolactin among the women in puerperium when compared with those in labour that was however not statistically significant. Prolactin increases during pregnancy and reaches a peak 10 h before delivery (Onur et al., 1989; Biswas and Rodeck, 1976; Fang and Kim, 1975). In the first week postpartum, prolactin levels usually decline 50%. The decline is due to the abrupt drop of estrogen as well as progesterone allowing prolactin to induce lactation (Speroff and Fritz, 2005). Prolactin levels subsequently increase due to suckling which activates mechanoreceptors around the nipple resulting in stimulation of the hypothalamus and consequent increased pituitary secretion of prolactin (Diaz et al., 1989; Andersen et al., 1981). The findings are similar to the study by Godo et al. (1988) and Vemer and Roland (1981). The slight decrease in the puerperium and labour is due to the ineffectiveness of suckling during the early puerperium, the loss of estrogen and progesterone following delivery. The decrease in serum prolactin is more likely in our subjects who were all within the first week of puerperium when suckling is not so established. Subsequently, serum prolactin levels are known to increase as suckling becomes more established and eventually dwindle to near non-pregnant level by the sixth month of lactation (Riordan, 2005; Walker, 2006).

Limitations include inability to carry out a longitudinal study as opposed to the cross-sectional study undertaken; having its flaws. This study was a randomized study, carried out in the Northern region of the country and thus the population might not necessarily be representative of the general population of women in Nigeria. Due to the small sample size and site of the study, we were unable to examine ethnicity differences in serum prolactin levels in major Nigerian tribes.

**Conclusion**

This is a preliminary cross-sectional study of reference intervals of serum prolactin spanning through pregnancy, labour and puerperium in Nigerian women in Zaria. Apparent changes in serum prolactin levels for all groups were observed to be lower and narrower as compared to those of other studies. This suggests that there may be racial differences in serum prolactin levels in women. It is envisaged that these intervals will aid in the monitoring of women in the different transitional phases of their reproductive period.

**RECOMMENDATIONS**

Further studies were recommended utilizing a larger sample size. A multi-centre study recruiting participants across the regions of the country will aid in deriving a reference range for Nigerian women. Studies including menopausal women in the study are also being suggested (due to the implication of serum prolactin women in breast cancer risk especially in menopausal women). More pertinent is the need for a longitudinal study following up the serum changes in prolactin from a non-pregnant state, through pregnancy and the puerperium in Nigerian women. Serum prolactin reference ranging in Nigerian children has also not been previously elucidated and is being suggested as considerations of another study. A study comparing serum prolactin levels in Caucasians, Nigerians and other Africans, utilizing the same laboratory is also being suggested.

**Conflict of Interests**

The authors have not declared any conflicts of interests.

**ACKNOWLEDGEMENTS**

The authors wish to appreciate the participants of the study, the various health professionals at the respective health services and the ethical committee of the Ministry of Health, Kaduna for their co-operation in the study.

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