Pituitary–ovarian axis during lactational amenorrhoea. I. Longitudinal assessment of follicular growth, gonadotrophins, sex steroids and inhibin levels before and after recovery of menstrual cyclicity

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BACKGROUND: Comparisons of follicular development and hormonal profile in the same women during and after lactational amenorrhoea (LA) are scarce. We report follicular growth, pituitary and ovarian hormone serum levels in the same women during LA and in follicular phases after resumption of menstrual cyclicity. METHODS: Serum samples were obtained from 10 women during LA between days 60 and 89 post-partum and between days 1 and 4 (early follicular phase; EFP) and 7 and 10 (mid-follicular phase; MFP) of the second and third cycles after LA. RESULTS: The number of follicles >3 mm and diameter of the largest follicle were significantly higher during LA when compared to EFP and MFP. Serum levels of inhibin B were similar in LA and EFP and increased significantly in MFP. Pro-αC was significantly higher in EFP than in LA and MFP. Estradiol was similar during all stages. In comparison with EFP and MFP, LA is associated with higher prolactin levels, normal or slightly elevated gonadotrophins and increased number and size of follicles without a parallel increase in estradiol, inhibin B and Pro-αC. CONCLUSIONS: During LA, there is a profound dissociation between follicular growth and follicular endocrine activity, which suggests an alteration in the stimulus–response relationship at the follicular level.

Key words: follicular growth/gonadotrophins/inhibins/lactational amenorrhoea/recovered ovarian cyclicity

Introduction

Lactational amenorrhoea (LA), and its associated anovulation and infertility, is often described as a period of ovarian quiescence determined by signals arising from breast suckling during nursing. This period is characterized by high serum levels of prolactin, low levels of estradiol and progesterone, and gonadotrophin levels which are similar to those observed in the early follicular phase (McNeilly, 1993).

The neuroendocrine mechanisms that determine LA have not been completely understood yet. It has been proposed that elevated prolactin levels as well as decreased LH biopotency contribute to low ovarian activity observed during lactation (Howie et al., 1982; Gross and Eastman, 1985; Diaz et al., 1989; Seron-Ferre et al., 1995). However, there is considerable variability among nursing women, and changes in prolactin and LH are not strictly associated with the maintenance of amenorrhoea. Consequently, they may be contributory factors but not critical ones. Enhanced sensitivity of the hypothalamic–pituitary axis to negative feedback of estradiol (Baird et al., 1979; Perheentupa et al., 2000) and alteration in the pattern of circulating FSH isoforms (Velasquez et al., 2006) may be additional mechanisms involved in the maintenance of anovulation during lactation.

Based on the low levels of estradiol observed in this period, it has been generally accepted that follicular activity is low in lactating women in spite of the fact that their FSH levels are in the normal range of the follicular phase (Kremer et al., 1994). However, serial ultrasonographic examination of the ovaries in lactating women showed that various patterns of follicular growth occur before normal cyclicity is re-established (Flynn et al., 1991). These findings challenge the belief that follicle growth is suppressed during lactational amenorrhoea. At variance with the follicular phase of menstrual cycles, during LA, growing follicles synthesize reduced amounts of sex steroids and it has been proposed that elevated prolactin per se may be directly responsible for this. Elevated levels of prolactin may make follicles less responsive to the steroidogenic action of LH and FSH, without suppressing follicular growth (McNeilly et al., 1982). In vivo and in vitro experimental studies have shown that prolactin, by decreasing P450 aromatase mRNA and protein, may inhibit granulosa cell aromatase activity (Tsai-Morris et al., 1983; Krasnow et al., 1990). These effects
may be exerted through a specific receptor for prolactin present in the human ovary (Schwarzler et al., 1997; Vlahos et al., 2001). Whether or not high prolactin levels also affect peptide hormone secretion from follicles has not been determined yet.

Dimeric inhibins are glycoproteins predominantly produced in the gonads (Burger, 1992) and their primary role is the regulation of hypophysial FSH biosynthesis and secretion (de Jong, 1988; de Kretser and McFarlane, 1996; Burger and Robertson, 1997). They are composed of α and β subunits. Heterodimerization of α subunit with either form of the β subunit, βα and ββ, generates dimeric inhibin A and inhibin B respectively. In women, the ovary is the main source of circulating inhibin A, B and Pro-αC (Groome et al., 1994, 1995, 1996; Sehested et al., 2000). Inhibin B is produced at early stages of follicular development, whereas inhibin A is preferentially secreted by more differentiated follicular and luteinized cells (Welt et al., 1997; Smitz and Cortvrindt, 1998; Sehested et al., 2000). Inhibin A is present in peripheral blood throughout gestation at concentrations 50-fold higher than those observed in the normal menstrual cycle (Muttukrishna et al., 1995). In the early post-partum period, immunoreactive inhibin and inhibin A levels fall markedly and a subsequent rise in FSH is associated with an increase in inhibin B levels (Burger et al., 1994, 2000). To our knowledge, the relationship between follicular activity and inhibin levels during LA has not been reported.

In the present study we assessed longitudinal changes in follicular growth and inhibin serum profile in women during lactational amenorrhoea and after recovery of menstrual cyclicity, and we examined their relationships with pituitary and other ovarian hormones. The main objective was to find differences between the two physiological stages that would guide further research to understand the mechanisms involved in anovulation associated with breastfeeding.

Materials and methods

Subjects

Twenty-two healthy nursing women interviewed at the maternity ward of Hospital San Borja–Arriarán in the central area of Santiago, Chile, were invited to participate in the study. Women were selected among those who intended to exclusively breastfeed their child for ≥6 months and met the following criteria: age 18–33 years, parity 1–3, normal pregnancy and vaginal term delivery, exclusively breastfeeding on demand, body mass index 20–30 kg/m² and haemoglobin concentration of ≥12 g/dl. Women with chronic diseases, requiring chronic drug therapy or who planned to use a hormonal contraceptive post-partum were excluded. Within the first 2 days after delivery, a member of the research team instructed each mother on how to breastfeed on demand.

Women were instructed not to give their baby any liquid or solid food or water and to use their breast as the only source of water and nutrients, except for the administration of vitamin drops, for 6 months. All babies were singletons, had normal weight at birth and normal post-partum evolution. Normality of weight and height for the age of the babies was determined following the Boston percentile weight curve for male infants (Vaughan, 1964).

Study protocol

Women were followed at the Family Planning Clinic of Instituto Chileno de Medicina Reproductiva (ICMER). They were given appointments to return on days 7, 20, 30, 40 and 55 post-partum and monthly thereafter for reinforcement of breastfeeding instructions and to assess the rate of infant growth and maternal and infant health. Each mother kept a daily record of the number of suckling episodes and episodes of vaginal bleeding. If formula milk feeds or solid feeds were used they were also recorded in the diary. Full or exclusive breastfeeding was defined as the condition in which breast milk was the only source of nutrients and water for the infant up to 6 months of age and the only source of milk, thereafter. Partial lactation was defined as the condition in which the infant received breast milk and other nutrient supplement before the age of 6 months and milk supplement thereafter. First post-partum menses was defined as the first bleeding episode occurring after puerperal bleeding consisting of ≥3 consecutive days followed by a second bleeding within 60 days. A general physical examination, a Papanicolaou smear and haemoglobin measurement were performed on day 30. On the 40th day visit, the procedure to be followed during the study was explained to each participant. Ten women mentally and physically healthy, still amenorrhoeic and fully breastfeeding at 60 days post-partum were enrolled in the ultrasonographic and endocrine study.

Beginning on day 60 post-partum, vaginal ultrasound examination of the ovaries and concomitant blood sampling were performed twice a week, for 4 consecutive weeks in order to assess follicular growth and hormonal profile. At this time all women were on exclusive breastfeeding and lactational amenorrhoea. When menstrual cycles resumed, vaginal ultrasound examination and blood sampling were performed on day 1–4 (early follicular phase, EFP) and 7–10 (mid-follicular phase, MFP) after the onset of menses on the 2nd and 3rd post-partum menstrual cycles. All samples were collected ≥2 h after the preceding breastfeeding episode. Blood was allowed to clot and then centrifuged to harvest the serum, which was stored frozen at −20°C until assayed. Serum levels of FSH, LH, prolactin, estradiol, progesterone, inhibin A, inhibin B and Pro-αC were determined during lactational amenorrhoea between days 60 and 69 (LAIII), 70 and 79 (LAII), 80 and 89 (LAIII) post-partum and during EFP and MFP of the 2nd and 3rd post-partum menstrual cycles. The study protocol was approved by the Ethics Review Committee of ICMER and all women enrolled gave informed written consent.

Ultrasonographic examination

Follicular development was assessed by the same observer randomly using Corometrics Aloka 620 ultrasonographic equipment with 5 MHz transvaginal transducer (Medical Systems, Inc., Yokogawa, Japan) or SA 600C with 7.5 MHz transvaginal transducer (Medison Co. Ltd, Seoul, Korea) according to availability. The diameters of all follicles ≥5 mm were recorded. Depending on the shape of each follicle, measurements were done in two or three planes and their mean value was considered the diameter of the follicle.

Hormone measurements

All samples from an individual woman were measured in duplicate in the same assay run. FSH and LH levels in serum samples were determined by enzyme immunoassay (EIA) using FSH and LH EIA kits (Immunometrics Ltd, London, UK). Prolactin, estradiol and progesterone levels were determined by radioimmunoassay (RIA) using the reagents and methodologies of the WHO Special Programme of Research, Development and Training in Human Reproduction (1987). The WHO 2nd IRP 78/549 and WHO 1st IRP 68/40 were used as FSH and LH standards respectively. Assay sensitivities and coefficients of variation for FSH, LH, prolactin, estradiol and progesterone are shown in Table I.

Serum inhibin A, B and Pro-αC were measured using a two-site enzyme-linked immunosorbent assay (ELISA) (Oxford Bio-Innovation...
Sensitivity and coefficients of variation (CV) of hormone assays

Characteristics of the subjects and number of breastfeeding episodes at the two study periods

Results

Breastfeeding characteristics of the participants

Out of 22 women screened, 10 entered and completed the study. Table II shows their breastfeeding condition at various study periods. Breastfeeding episodes were significantly more frequent during amenorrhoea than at the time of follicular phase sampling, when they had decreased to almost 50% (P < 0.01). Three women (subjects 6, 7 and 10) had all or some samples of the follicular phase taken after weaning (Figure 1).

Although eight women were on exclusive breastfeeding at the time of first bleeding, the number of breastfeeding episodes per 24 h had decreased by almost 40% (12 ± 1 versus 7 ± 1, P < 0.01). Figure 1 shows individual breastfeeding conditions, the first three consecutive menstrual episodes and the timing of sampling.

Statistical analysis

Blood samples taken during lactational amenorrhoea were included in the analysis only up to 14 days before the first vaginal bleeding. Samples having estradiol or progesterone concentration below the detection limit of the assay were assigned the value of the lower limit of detection.

In total, 10 women were included in the study. Since each woman provided multiple measures during each period of study, an average of individual values was calculated for each woman within each period. Therefore, every woman contributed with a single datum for each period, which was used to calculate the mean ± SEM during LAI, LAII, LAIII, EFP and MFP. Comparisons between two groups were assessed using paired Student’s t-test. Comparisons between more than two groups were carried out using repeated measures one-factor analysis of variance or its equivalent non-parametric statistical method as appropriate. Each ANOVA was followed by multiple comparisons test. Spearman’s method was applied to assess the correlation between variables. All statistical calculations were performed using GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego (CA, USA), www.graphpad.com. P < 0.05 was considered statistically significant.

Serum levels of gonadotrophins and prolactin

Table III shows the hormonal profile during LA and in the follicular phase. No significant difference was found in FSH serum levels between the three LA periods or when they were compared to MFP. However, FSH levels were significantly lower in EFP than in all other periods studied (P < 0.05). LH levels followed a similar pattern, although significantly lower LH serum levels were observed in EFP only when compared to MFP (P < 0.05).

A progressive decrease in the level of prolactin was observed from LAIII onwards. Significantly lower levels of prolactin were found in EFP and MFP when compared to LAI, LAII and LAIII (P < 0.05).

Ultrasonographic examination

No significant difference was found in the number of follicles ≥3 mm and in the diameter of the largest follicle present during LAI, LAII and LAIII. However, both parameters were significantly higher in LA than in EFP and MFP (P < 0.05) (Figure 2A and B respectively).

Serum steroid and inhibin profile

No significant differences were found in estradiol and progesterone serum levels between any studied periods, although estradiol results may be biased by the proportion of samples obtained during LA (46%) that had estradiol values below the detection limit of the assay.

No difference was found in Pro-αC levels between the three LA periods or when they were compared to MFP; however, Pro-αC levels were significantly increased in EFP (Table III, P < 0.05).

Inhibin A serum levels were below the detection limit of the assay in all samples analysed. Serum inhibin B levels were low.
throughout the three LA periods and in EFP; whereas a significant increment occurred in MFP \( (P < 0.05) \) (Table III).

In order to relate estradiol, Pro-\( \alpha \)C and inhibin B levels to the total cohort of follicles present in the ovary, the ratio of serum concentrations to total follicular diameter—which integrates size and number of antral follicles—was calculated. The total follicular diameter corresponds to the sum of the mean follicular diameter of follicles >3 mm. A significantly higher inhibin B

### Table III. Serum levels of gonadotrophins, prolactin and ovarian hormones during three lactational amenorrhoea periods and at early and mid-follicular phase of the 2nd and 3rd recovered cycles

<table>
<thead>
<tr>
<th></th>
<th>LA\textsubscript{I}</th>
<th>LA\textsubscript{II}</th>
<th>LA\textsubscript{III}</th>
<th>EFP</th>
<th>MFP</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>FSH (IU/l)</strong></td>
<td>8.5 ± 1.0</td>
<td>7.8 ± 0.5</td>
<td>6.8 ± 0.5</td>
<td>4.5 ± 0.4\textsuperscript{a}</td>
<td>6.8 ± 0.4</td>
</tr>
<tr>
<td><strong>LH (IU/l)</strong></td>
<td>4.4 ± 1.2</td>
<td>4.1 ± 0.7</td>
<td>3.3 ± 0.7</td>
<td>2.4 ± 0.4\textsuperscript{b}</td>
<td>4.8 ± 0.6</td>
</tr>
<tr>
<td><strong>Prolactin (mIU/l)</strong></td>
<td>963 ± 134</td>
<td>1070 ± 140</td>
<td>856 ± 125</td>
<td>560 ± 98\textsuperscript{c}</td>
<td>448 ± 87\textsuperscript{d}</td>
</tr>
<tr>
<td><strong>Estradiol (pmol/l)</strong></td>
<td>118.1 ± 9.5\textsuperscript{*}</td>
<td>112.6 ± 5.9\textsuperscript{*}</td>
<td>128.5 ± 14.2\textsuperscript{*}</td>
<td>136.7 ± 7.5</td>
<td>132.9 ± 10.1</td>
</tr>
<tr>
<td><strong>Pro-( \alpha )C (pg/ml)</strong></td>
<td>2.3 ± 0.3</td>
<td>2.1 ± 0.2</td>
<td>2.0 ± 0.1</td>
<td>3.1 ± 0.6</td>
<td>2.1 ± 0.1</td>
</tr>
<tr>
<td><strong>Inhibin B (pg/ml)</strong></td>
<td>122.2 ± 20.4</td>
<td>130.8 ± 17.6</td>
<td>130.2 ± 19.6</td>
<td>204.7 ± 23.2\textsuperscript{c}</td>
<td>177.9 ± 24.6</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM.

*These assay means have a slight positive bias as results below the detection limit of the assay were expressed as the assay detection limit.

\textsuperscript{a}P < 0.05 when compared to LA\textsubscript{I}, LA\textsubscript{II}, LA\textsubscript{III} and MFP.

\textsuperscript{b}P < 0.05 when compared to MFP.

\textsuperscript{c}P < 0.05 when compared to LA\textsubscript{I}, LA\textsubscript{II} and LA\textsubscript{III}.

\textsuperscript{d}P < 0.05 when compared to LA\textsubscript{I}, LA\textsubscript{II}, LA\textsubscript{III} and EFP.

LA\textsubscript{I} = day 60–69; LA\textsubscript{II} = day 70–79; LA\textsubscript{III} = day 80–89 post-partum; EFP = early follicular phase; MFP = mid-follicular phase.
Inhibins during lactational amenorrhoea and follicular phase

A significant inverse correlation was found between prolactin and inhibin B serum levels during lactational amenorrhoea and in the follicular phase ($r = -0.482$, $P < 0.001$, LA$_{I}$ + LA$_{II}$ + LA$_{III}$; $r = -0.377$, $P < 0.05$, EFP + MFP). No significant correlation was found between ultrasonographic findings and hormonal parameters.

Discussion

Several physiological parameters were compared between lactational amenorrhoea—in which ovulation is impeded—and follicular phases of ensuing menstrual cycles attempting to identify clues to understand the mechanisms involved in LA. All volunteers participating in the study were fully nursing and in amenorrhoea at the time of the first run of examinations, and all of them were sampled again during the follicular phase of the second and third cycles after resumption of menstrual cyclicity. They were all lactating when first menses occurred and eight out of 10 women were exclusively breastfeeding. However, the number of breastfeeding episodes per 24 h had diminished by nearly half when compared to day 60 post-partum. The decrease in the number of breastfeeding episodes over time is dependent on multiple environmental and intrinsic biological factors, which to some extent were controlled by inclusion/exclusion criteria applied in the selection of participants. This was further strengthened by applying the same management of post-partum care to all volunteers, which included continued support by the same nurse to the mother’s intention to practice full breastfeeding on demand as long as possible. To the best of our knowledge, the decline in breastfeeding frequency that eventually led to the end of LA in this study was a process dictated by nature rather than extraneous influences. Accordingly, prolactin levels were higher during LA than in the follicular phase. This pattern is in keeping with the higher absolute concentration (pg/ml) and concentration per follicle >3 mm tended to be higher during the follicular phase than in LA, reaching a 2–3-fold increase in MFP.

Correlation between inhibins and other hormonal and ovarian ultrasound parameters

A significant inverse correlation was found between estradiol and Pro-αC serum levels during lactational amenorrhoea and in the follicular phase ($r = -0.377$, $P < 0.05$, EFP + MFP; §$P < 0.05$ when compared to LA$_{I}$, LA$_{II}$, LA$_{III}$). Therefore, absolute concentration (pg/ml) and concentration per follicle >3 mm tended to be higher during the follicular phase than in LA, reaching a 2–3-fold increase in MFP.

Significantly higher estradiol and Pro-αC ratios were observed in EFP and MFP when compared to LA$_{I}$, LA$_{II}$, LA$_{III}$ ($P < 0.01$) (Figure 2D).

**Figure 2.** Ultrasound findings and inhibin profile. (A) Number of follicles >3 mm; (B) diameter of the largest follicle; (C) inhibin B; (D) ratio of Pro-αC or estradiol serum levels/total follicular diameter during lactational amenorrhoea (LA) and in the follicular phase. LA$_{I}$ = day 60–69 post-partum; LA$_{II}$ = day 70–79 post-partum; LA$_{III}$ = day 80–89 post-partum; EFP = early follicular phase; MFP = mid-follicular phase; foll. diam. = follicular diameter; Inh B = inhibin B; E$_{2}$ = estradiol. *$P < 0.05$ when compared to LA$_{I}$, LA$_{II}$, LA$_{III}$; #$P < 0.05$ when compared to LA$_{I}$, LA$_{II}$, LA$_{III}$ and EFP; §$P < 0.05$ when compared to LA$_{I}$. 

ratio was observed in MFP when compared to all other periods and in EFP when compared to LA$_{I}$ ($P < 0.05$) (Figure 2C). Therefore, absolute concentration (pg/ml) and concentration per follicle >3 mm tended to be higher during the follicular phase than in LA, reaching a 2–3-fold increase in MFP.
with previous studies that described a similar decrease in basal prolactin levels and a diminished response to suckling with time after delivery (Howie et al., 1982; Diaz et al., 1989; Nunley et al., 1991). When menstrual cyclicity was recovered, prolactin levels approached the upper limit of the normal range for non-nursing women.

Based upon earlier studies in a similar sample of the same population (Diaz et al., 1988), we assume that the follicular phases studied belong to ovulatory cycles in most if not all cases. The fact that we found no differences between the second and third cycles studied strongly suggests that full recovery of normal ovarian cyclicity had been achieved.

The present study clearly demonstrates that follicular growth to >3 mm is not absent during LA, and that it follows patterns that differ from those observed in the early and mid-follicular phase of the menstrual cycle in the same women. Both the number of follicles >3 mm and the diameter of the largest follicle during LA significantly exceed those observed in EFP and MFP. Our finding of a large number of follicles >3 mm confirms previous reports (Flynn et al., 1991; Perheentupa et al., 2000, 2003). It clearly shows that the degree of follicular growth taking place during LA is much higher than what has been accepted in the past based upon the low levels of estradiol observed in the circulation. In addition, during LA, we found a striking dissociation between follicular growth and follicular endocrine activity. Estradiol and inhibin B levels during LA failed to reflect the extent of follicular growth observed in this condition. When follicular endocrine activity was expressed as serum hormone level/total follicular diameter ratio, values obtained for inhibin B, estradiol or Pro-αC revealed a sharp decrease in the endocrine activity during lactational amenorrhea when compared to the follicular phase.

The increased number and size of follicles during LA may reflect the fact that FSH immunoreactive levels were continuously higher in this period as compared to those in the follicular phase in which comparable levels were attained only in MFP. Strangely enough, these sustained higher FSH levels—that clearly induced abundant follicular growth—were unable to stimulate the endocrine activity of these follicles.

A similar dissociation, i.e. low inhibin B levels despite normal number of 6–9 mm follicles and normal levels of immunoreactive FSH, has been reported recently in patients with functional hypothalamic amenorrhea. This was attributed to partial loss of inhibition(s) that keep inhibin B secretion at a low level, whereas decreased LH bioactivity (Seron-Ferre et al., 1995) is less likely to contribute, since at this stage granulosa cells lack LH receptors.

The absence of circulating inhibin A observed in all women during lactational amenorrhea indicates that although the diameter of follicles was higher than in MFP, none of them was functionally competent or adequately stimulated.

In summary, these results show a major difference in the endocrine activity of antral follicles between LA and the follicular phase after resumption of menstrual cyclicity, in spite of comparable serum gonadotrophin levels. Ongoing follicular growth during LA is more abundant and advanced than in the early follicular phase, produces relatively scanty amounts of steroids, inhibin B and Pro-αC and does not reach the stage of follicular maturation. Follicular response to FSH, the levels of which are steadily as high as the highest basal levels seen in the follicular phase, is clearly altered during lactational amenorrhea.

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