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Gonadotropin Levels in Mothers Who Have Had Two Sets of DZ Twins

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Abstract. Serum gonadotropin, estradiol, prolactin and alpha-1-antitrypsin levels were measured in the first four days of the menstrual cycle in 14 women who were cycling normally. FSH, and to a lesser extent LH levels, were significantly higher in a group of 8 women who had at least one set of DZ twins (6 of whom had 2 sets) than in a control group of 6 women with no DZ twins. Estradiol levels also tended to be higher in mothers of twins but there were no significant differences in prolactin or alpha-1-antitrypsin concentrations. There were no differences between the means of the two groups in age, height, weight or number of pregnancies.

Key words: Dizygotic twinning, Gonadotropins, Mothers of twins

INTRODUCTION

While much effort has been expended in understanding the causes of multiple births in sheep and cattle, little work has been done on the etiology of dizygotic (DZ) twinning in humans. Bulmer [1] summarised the evidence that DZ twinning tends to aggregate in families and suggested that this tendency is genetic in origin. Except for a few unusual case reports [18, 19] monozygotic (MZ) twins do not appear to aggregate in families: their occurrence is apparently random and it seems that their etiology is quite distinct from that of DZ twins.

It has been assumed, largely by inference from animal evidence, that the immediate physiological cause of the tendency to have multiple births is unusual hormone levels in the mother which produce multiple ovulation. This assumption was bolstered by the large number of multiple pregnancies resulting from early trials of gonadotropin treatment for

infertility [5, 6]. Milham [12] hypothesised that "mothers of dizygotic twins should have higher gonadotropin levels than mothers of single births" but, to our knowledge the only direct evidence of unusual hormone levels in women who give birth to DZ twins under natural circumstances comes from the work of Nylander [13, 14].

He measured follicle stimulating hormone (FSH) and luteinising hormone (LH) levels daily for ten days from the seventh day of the menstrual cycle in 15 Yoruba women in western Nigeria, 6 of whom had had only singleton infants, 7 who had one set of twins and 2 who had two sets of twins. Mean FSH levels were higher at the peak, and for four days before and after the peak, in women who had twins compared with those who had singletons. The levels were higher still in the two mothers with two sets of twins. However, none of the differences reported was statistically significant. Mean LH levels were less clearly related to twinning history. Nylander [14] followed this up with a similar study of serum gonadotropin levels in mothers of singletons, MZ twins and DZ twins in Aberdeen, Scotland. Although the peak FSH level was higher in DZ mothers than in mothers of MZ twins or singletons, the difference was smaller than in the Nigerian sample and was not significant. The fact that gonadotropin levels were higher in Nigerian mothers of twins and singletons than in their Aberdeen counterparts led Nylander to speculate that the diet of African women may be responsible.

Since it is generally accepted that follicles are recruited for growth early in the menstrual cycle [9, 10] it is unlikely that a higher mid-cycle FSH peak, even if real, is directly relevant to the cause of multiple ovulation. We hypothesise that the tendency to DZ twinning will be associated with higher than normal FSH levels in the early follicular phase. In order to test this hypothesis we have chosen an extreme group, women who have had two sets of DZ twins under natural circumstances, and compared their gonadotropin levels in the early follicular phase of the menstrual cycle with those of control mothers of singletons. Furthermore, to investigate whether this hypothesised elevation of FSH is the result of reduced negative feedback of lower estradiol levels, we also measured estradiol concomitantly with serum FSH.

A third hypothesis is that women with a tendency to DZ twinning have an increased frequency of the S allele of the Protease inhibitor polymorphism (Pi) which in turn is associated with decreased alpha-1-antitrypsin levels (AAT) [2]. Accordingly, Pi was typed and AAT levels were measured in mothers of twins and controls.

SUBJECTS AND METHODS

Subjects

Mothers with two sets of twins were ascertained by scanning the Australian NH&MRC Twin Registry for cases of two sets of twins registered at the same address. From more than 15,000 pairs nationwide, 180 such cases were found. Letters were sent to the mothers of these pairs explaining the aims of the research project and seeking volunteers who were: (a) aged 18-40 (ie, not menopausal); (b) not using oral contraceptives or other hormone preparations: (c) not pregnant or lactating; and (d) who had not had a hysterectomy. Appeals were also made through mothers-of-twins clubs affiliated with the Australian Multiple Birth Association.

Seven mothers who satisfied these criteria were tested. A further mother of a single set of DZ twins (Table 1, mother T7) was included in the sample because she had two sets of DZ twin siblings and was judged to be from a high-risk family. If twin pairs were not of opposite sex, then mothers answered questions on their physical characteristics including eye colour, hair colour, and height, or produced photographs to satisfy the investigators that their twins were in fact DZ. One mother (T8) of an opposite-sex pair produced photographs of her same-sex girls which strongly suggested they were

MZ but she has nevertheless been included in the experimental group. Practical considerations, including the young age of many of the twins, prevented blood typing of the same sex pairs to confirm zygosity diagnoses.

An attempt was made to obtain a control for each mother of twins who was matched for age, parity, and weight. Initially we asked each mother of twins to find a friend willing to participate who satisfied all the screening criteria and was similar to themselves in age, number of pregnancies and weight, but not twinning. This was partly successful, but it proved more difficult to obtain control volunteers than mothers of twins. Six controls were tested successfully. One of these (C5) had a single pair of MZ twins but no other twins in her family. We thus had to abandon our ideal of a matched pair control study and its attraction of greater statistical power.

Protocol

Subjects were asked to telephone their hospital contact on the first day of menstrual bleeding (Day 1). Blood collection took place in several centres. Samples were then collected on cycle days 1, 2, and 3, or 2, 3, and 4: one subject (C6) was sampled on days 1, 4, and 5. Further appointments were then made for either day 8 or day 9 and then for approximately day 21 (19-23). Blood samples were thus collected on five days of the cycle.

On each of the first four sampling days, 4 X 10 ml intravenous blood samples were drawn at 20 min intervals (ie, at 0, 20, 40, and 60 min). On the fifth sampling day (cycle day 19-23) only a single blood sample was drawn which was assayed for progesterone to check that the cycle had been ovulatory. Since abnormally high prolactin levels can influence ovarian steroidogenesis and produce infertility, prolactin was assayed in the first four sampling days to check that all our subjects were in the normal range. All sampling took place in the morning. Samples were placed in 10 ml clotting tubes and allowed to stand at room temperature until the clot had retracted. Then 2 X 2 ml aliquots of serum from each sample were frozen at -20 until assay.

Assays

Samples were transported in a frozen condition from the several centres where they were collected, to the Endocrine Laboratory at the Prince of Wales Hospital, Sydney, for assay for follicle stimulating hormone (FSH), luteinising hormone (LH), estradiol, prolactin and progesterone. Hormone levels were estimated by radioimmunoassay using kits from Diagnostic Products Corporation (Los Angeles) for LH and FSH, from Radio Isotopen Service (Wurlingen, Switzerland) for estradiol and Amersham International (UK) for prolactin; the progesterone assay (nmol/l) was based on the method of Garza et al [4]. Peptide assay standards were M.R.C. 68/40 (LH), 69/104 (FSH) and 75/504 (prolactin: 19 units/mg). Coefficients of variation were less than 6.4% within and 13% between batch for all hormones. Alpha-1-antitrypsin was measured by immunoturbidometry and Pi phenotyping was carried out using the Immobiline method (LKB) with a pH range of 3.8-4.8.

Replicate gonadotropin assays on each day were made in order to minimise any variability in estimation due to the pulsatility of gonadotropin release from the anterior pituitary gland [21]. Because the replicate samples were available, replicate assays were also obtained for estradiol, prolactin and alpha-1-antitrypsin.

To ensure that batch effects are not confounded with group differences, all samples should be individually randomised and then assayed in the assigned random sequence. This was not possible with our samples but efforts were made to reduce the risk of confounding.

RESULTS

The mean of multiple samples in each subject for each day was computed. These daily means for LH and FSH and other information for each subject are given in Table 1.

The mothers of twins and control group are remarkably well matched for age, height, weight, and number of pregnancies (Table 2). Because our prior hypothesis is that mothers of twins will have greater FSH and LH concentrations than controls, we may perform a one-tailed t-test on the difference between their means (Table 3). The prior expectation is confirmed, directionally at least, for both hormones on all sampling days.

TABLE 1 - Mean LH and FSH Levels (mIU/ml) for Four Sampling Days of Cycle as Shown
Mothers of Twins Have Two Sets of DZ Twins Except Where Indicated. Progesterone Level (nmol/l) on or about Day 21 of Cycle is Also Shown

		Age	Height (cm)	Weight (kg)	No. of pregnancies	Progesterone (day 21)	1st sampling day			2nd sampling day			3rd sampling day			4th sampling day		
							Day	LH	FSH	Day	LH	FSH	Day	LH	FSH	Day	LH	FSH
Mothers of twins	T1	36	167	60	3	27.1	2	11.3	11.7	3	10.3	11.2	4	9.4	8.7	9	5.6	5.5
	T2	40	167	95	3	11.6	1	1.9	5.3	2	2.0	5.7	3	2.1	5.1	8	3.2	4.0
	T3	39	175	64	4	18.5	1	1.4	6.1	2	2.6	5.8	3	1.0	5.5	8	56.8	10.8
	T4	38	172	79	2	25.2	2	4.9	8.6	3	2.3	8.2	4	3.3	7.8	9	3.4	4.8
	T5	36	170	62	6	23.1	2	5.4	8.3	3	4.8	7.4	4	6.0	7.3	8	5.3	6.6
	T6	40	167	65	3	43.1	2	2.5	8.2	3	1.6	8.3	4	2.0	7.1	8	1.9	4.5
	T7[a]	32	172	61	2	64.0	1	5.9	6.5	2	4.0	7.5	3	3.9	6.9	8	3.2	5.4
	T8[b]	37	165	57	4	17.6	2	0.6	7.6	3	1.9	7.5	4	2.8	7.7	9	2.2	6.6
Controls	C1	35	170	76	4	6.5	2	0.2	6.8	3	0.3	6.8	4	0.8	7.0	9	1.9	6.0
	C2	36	180	79	2	24.5	1	0.1	6.2	2	0.1	6.9	3	1.5	6.8	8	2.4	5.8
	C3	33	170	86	2	27.8	1	2.8	5.9	2	3.9	5.6	3	4.3	6.1	8	6.3	6.3
	C4	40	162	64	6	38.3	1	0.4	2.3	2	2.5	3.6	3	1.5	3.7	8	4.6	8.1
	C5[c]	34	172	53	1	61.8	2	0.2	6.3	3	1.2	7.7	4	2.0	7.5	9	0.5	6.8
	C6	40	162	53	3	53.0	1	1.7	6.2	4	1.9	4.8	5	2.3	4.3	8	1.8	2.9

a Only one set of DZ twins but has two sets of DZ siblings.

b One set of DZ and one set of probable MZ twins.

c One set of MZ twins.

TABLE 2 - Comparability of Mothers of Twins and Controls for Age, Height, Weight, and Number of Pregnancies

	Mothers of twins (N = 8)		Controls (N = 6)		t	P
	Mean	SD	Mean	SD		
Age (yrs)	37.3	2.7	36.3	3.0	0.60	0.56
Height (cm)	169.6	3.5	169.5	6.8	0.04	0.97
Weight (kg)	68.0	12.9	68.5	13.9	0.06	0.95
Pregnancies	3.38	1.30	3.00	1.79	0.46	0.66

TABLE 3 - Hormone and α_1-Antitrypsin (α_1 AT) Levels in Mothers of Twins and Controls. T-Tests Are One Tailed Except Where Indicated

	Sampling day	Mothers of twins (N = 8)		Controls (N = 6)		t	P
		Mean	SD	Mean	SD		
LH	1	4.24	3.47	0.90	1.11[b]	2.55	0.015
LH	2	3.69	2.89	1.65	1.43	1.58	0.070
LH	3	3.81	2.71	2.07	1.21	1.46	0.085
LH	4	10.20	18.88	2.92	2.13[b]	1.08	0.16
FSH	1	7.79	1.97	5.62	1.65	2.18	0.025
FSH	2	7.70	1.72	5.90	1.53	2.03	0.032
FSH	3	7.01	1.20	5.90	1.55	1.52	0.077
FSH	4	6.03	2.14	5.98	1.72	0.04	0.48
Estradiol	1	177	75	161	53	0.42	0.68[a]
	2	204	80	154	44	1.39	0.19[a]
	3	222	81	152	62	1.76	1.04[a]
	4	574	330	292	190	1.86	0.088[a]
Prolactin	1	4.99	3.36	3.48	1.78	0.99	0.34[a]
	2	5.29	2.78	4.38	3.47	0.54	0.60[a]
	3	5.40	3.94	5.35	4.75	0.02	0.98[a]
	4	4.71	2.42	4.47	4.17	0.14	0.89[a]
α_1 AT	1	2.58	0.35	2.58	0.26	0.05	0.48
	2	2.54	0.26	2.60	0.32	0.41	0.34
	3	2.59	0.24	2.60	0.28	0.09	0.46
	4	2.69	0.51	2.60	0.26	−0.38	–

a Two-tailed probabilities.

b Group variances different at 5% level, so degrees of freedom < 12.

The difference is significant at the 10% level for both hormones on the first three sampling days and at the 5% level for LH on day 1 and for FSH on days 1 and 2.

Inspection of the results in Table 1 suggests that the significant differences in gonadotropin levels may be due to the particularly high levels of one mother of twins (T1). However, repeating the t-test with this case omitted makes little difference to the results and their significance. Similarly, omitting in turn the control mother (C1) who may have had an anovulatory cycle (day 23 progesterone only 6.5 nmol/1), or the control mother with MZ twins (C5), or the control mother sampled on days 1, 4, and 5 (C6), does not substantially alter the results nor the significance of the differences reported here. To further emphasise the main findings, LH and FSH means and standard errors for the two groups are shown graphically in Fig. 1 and 2.

Our subsidiary hypothesis, if FSH levels were indeed higher in mothers of DZ twins, was that this would be due to lower estradiol levels causing less feedback inhibition of pituitary gonadotropin production. Contrary to our hypothesis, estradiol levels are actually *higher* in DZ mothers than in controls on all four sampling days (Table 3). The difference increases with sampling day and is actually significant at the 10% level (2-tail test) on day 8/9. This is largely due to a fourfold increase over day 3/4 levels in two mothers of twins (T1, T8) which may have been due to the imminence of multiple ovulation. Estradiol means and their standard errors are shown graphically in Fig. 3.

Our third hypothesis, that alpha-1-antitrypsin levels would be lower in mothers of twins than in controls, is directionally true for the first three sampling days but is false for the fourth sampling day (Table 3). None of the differences is significant. All subjects were PiM phenotype, there being 4 M1M1 and 2 M1M2 in the controls and 3 M1M1, 1 M1M2, 1 M1M3 and 2 M2M3 types in the mothers of twins.

Prolactin levels were not significantly different in the two groups of women and all subjects were within the normal range.

DISCUSSION

In order to understand the etiology of DZ twinning, one must first investigate the endocrine factors which affect the recruitment, maturation and ovulation of at least two follicles. The process of follicular maturation and ovulation has been the focus of intense study over the past decade [9-11, 16]. It appears that the early stages of folliculogenesis are primarily dependent on gonadotropin (especially FSH) but that in the later stages of recruitment of the dominant follicle, both gonadotropins and follicular estrogen biosynthesis are important [3, 7].

The hypothesis that mothers of DZ twins have higher serum gonadotropin levels in the early follicular phase of the menstrual cycle is strongly supported by our data, despite the small numbers in this study. Furthermore, we have demonstrated that these higher gonadotropin levels are not a consequence of reduced negative feedback by estradiol in this group. It is unlikely that these results are artefacts produced by differences in prolactin levels, age, weight or parity in the two groups.

In selecting for study women who had had two sets of DZ twins, we have given ourselves the optimum chance of detecting hormonal differences, although we do not have data to confirm that dual ovulations occurred in the cycles we observed. We believe that this is the first report of gonadotropin levels measured in the very early part of the menstrual cycle of DZ twin mothers. The only other reports regarding the measurement of

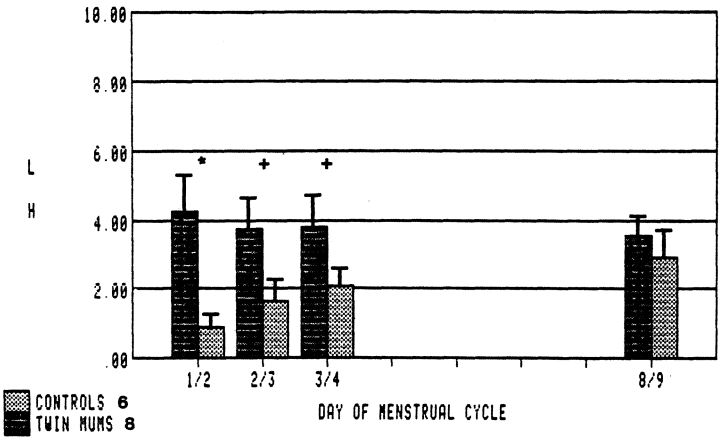

Fig. 1

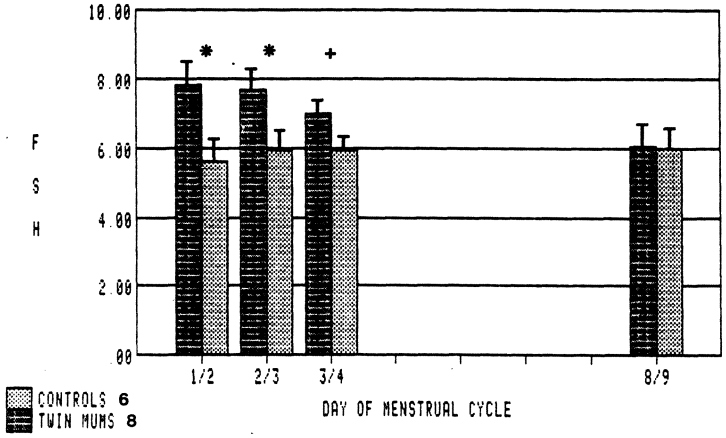

Fig. 2

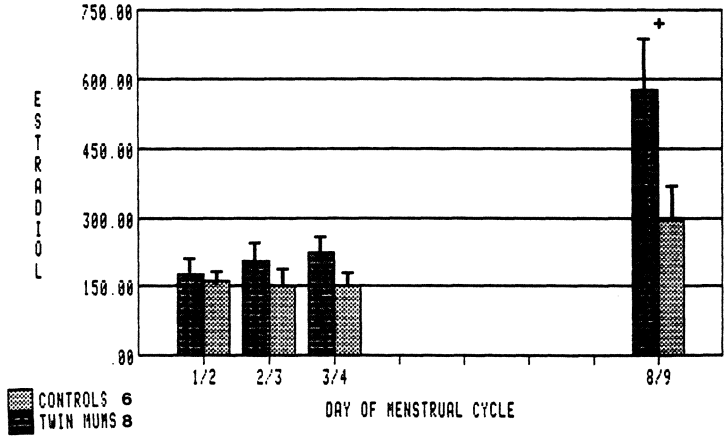

Fig. 3

Figures 1-3. Hormone levels in mothers of DZ twins (N=8) and controls (N=6). Standard error bars are shown. LH and FSH, mIU/ml; estradiol, pmol/l.
* means differ at 5% significance level;
\+ means differ at 10% significance level.

gonadotropin levels in DZ twin mothers are from Nylander [14, 15]. However, his studies measured gonadotropin in the late part of follicular phase and at ovulation and not at the early part of the follicular phase when the number of follicles recruited is determined. Mid-cycle FSH levels were higher in DZ twin mothers than in mothers of singletons in both a Nigerian and a Scottish sample. The difference was particularly evident in the Yoruba sample and greatest for two mothers of two sets of twins, although no differences in either study were statistically significant.

Nylander [15] postulates that diet is responsible for the propensity of some women to multiple ovulation and for the higher gonadotropin levels in Yoruba (who have the highest DZ twinning rate reported) than in Scots. Dietary differences might also account for the very low mid-cycle gonadotropin levels in Japanese [20] who also have the lowest DZ twinning rate reported. However, while it is difficult to disprove this explanation, we find it implausible particularly in view of the comparability of our two groups of mothers in their domestic habits. The tendency for DZ twinning to run in families for many generations prompted Bulmer [1] to interpret the high mother-daughter and sister-sister correlations for DZ twinning (about 0.3 and 0.5 respectively) in whites as evidence for genetic determination. The conflict between Nylander's and Bulmer's hypotheses of the determination of DZ twinning cannot be resolved without a properly designed study.

Our hypothesis that higher gonadotropin levels in the mothers of DZ twins would be a reflection of low estradiol levels causing reduced feedback inhibition of gonadotropin release is not only not supported by our data but is actually contradicted by them: estradiol levels in DZ mothers are higher than in controls although not significantly so. This is consistent with very recent results suggesting that early follicular gonadotropin levels may be predominantly under feedback inhibition by inhibin-F [8]. Estradiol values on day 8/9 are significantly elevated in the DZ mothers. This may be partly explained by the dramatically elevated estradiol levels in 2 of the 8 DZ mothers where the estradiol levels are approximately four times higher than those on day 3/4. These very high levels would be expected if there were two dominant follicles destined to ovulate, but we have no evidence on this point.

A further factor which has been associated with the tendency to DZ twinning is decreased levels of alpha-1-antitrypsin (AAT) as found in women heterozygous for the Pi^S allele [2]. Schumacher & Pearl [17] found that cervical mucus AAT concentrations were particularly low at mid-cycle and it is possible that even lower levels in women carrying this allele may enhance fertility in some way. However, none of the DZ mothers carried the S allele and their AAT levels were no different from those of controls, although this may be because they were measured in serum and in the follicular phase rather than at ovulation. The lack of evidence for any involvement of the Pi polymorphism or AAT levels in the tendency to DZ twinning among this group of women at very high risk, in contrast to the evidence for association with a more moderate tendency in mothers of single DZ sets, may suggest heterogeneity in the etiology of DZ twinning.

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