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Gonadotropin Levels in Urine during Early Postnatal Period in Small-for-Gestational Age Preterm Male Infants with Fetal Growth Restriction

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Running title: Gonadotropin in Small-for-Gestational Age Infants

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Abstract

Objectives: The objective of this study was to estimate gonadotropin concentrations in small-for-gestational age (SGA) male infants with the reactivation of the hypothalamic-pituitary-gonadal axis during the first few months of life, which is important for genital development.

Study Design: We prospectively examined fifteen SGA and fifteen appropriate-for-gestational age (AGA) preterm male infants between 2013 and 2014 at Kyoto University Hospital. Gonadotropin concentrations (luteinizing hormone (LH) and follicle-stimulating hormone (FSH)) were measured in serial urine samples from the 7th to 168th postnatal days and compared between SGA and AGA infants using the Mann-Whitney test.

Results: A longitudinal analysis showed that SGA infants had higher LH and lower FSH concentrations ($p=0.004$, $p=0.006$, respectively) than AGA infants.

Conclusions: Male infants that are SGA at birth due to fetal growth restriction (FGR) have gonadotropin secretion abnormalities in the first few months of life.

Introduction

Fetal growth restriction (FGR) may be caused by various factors, including maternal, placental, and fetal factors, and results in infants being small-for-gestational age (SGA) at birth ¹. Based on the Barker hypothesis ², abnormal intrauterine and early postnatal environments are considered to influence later development, with SGA infants being at a high risk of developing various disorders such as endocrinological and metabolic disorders, including abnormal gonadal functions, later in life ³. Although it is still a matter of debate ⁴, males who are SGA at birth may develop testicular dysgenesis syndrome (cryptorchidism, hypospadias, poor semen quality, and testicular cancer) ^{5,6}. A reduced testicular size, lower testosterone (T) concentrations, higher luteinizing hormone (LH) concentrations, elevated serum inhibin B concentrations, and infertility have also been reported in men that are SGA at birth ⁷.

HPG axis development from the fetal phase to the early postnatal period in males occurs as follows. In the male fetus, testosterone is secreted by the testes under the stimulus of human chorionic gonadotropin (hCG) from the placenta, with serum testosterone concentrations increasing between 10 and 20 weeks and decreasing towards term. Large concentrations of testosterone and anti-Müllerian hormone (AMH) play roles in the differentiation of internal and external genitalia ⁹. Testosterone also functions in brain masculinization ¹⁰. LH and FSH are secreted by the anterior pituitary and their

concentrations decrease towards term. LH stimulates the testes to secrete testosterone at a low concentration in order to develop external genitalia after midterm ¹¹. In the early postnatal period, the HPG axis is reactivated and gonadotropin levels increase. This reactivation of the HPG axis occurs in the first few months after separation from the placenta and is called “mini-puberty”. This period is suggested to be important for development of the genitalia ^{12, 13}.

Infants born SGA at birth may exhibit some programming in the HPG axis in these important antenatal and perinatal periods. The increased secretion of FSH ¹⁴ and higher estrogen concentrations have already been reported in girls in a GnRH analog test ¹⁵; however, few studies have investigated the precise time courses of reproductive hormone concentrations in mini-puberty ¹⁶. In the present study, we assessed serial changes in urinary hormone concentrations in SGA males using a non-invasive procedure in the first few months of life, a key phase between the fetal period and adolescence, because mini-puberty is only prominent in males ¹⁷.

Materials and methods

Subjects and study design

We conducted a prospective observational study. Between January 2013 and November 2014, consecutive preterm male infants who were born at Kyoto University Hospital and cared for in the neonatal intensive care unit (NICU) were recruited. This study was performed according to the

Declaration of Helsinki and was approved by the Institutional Ethical Review Committee. Written informed parental consent was obtained for each subject.

Inclusion and exclusion criteria are shown in Figure 1. We classified recruited infants by birth weights: infants who were SGA at birth and those that were appropriate-for-gestational age (AGA) at birth. SGA infants were defined as infants with birth weights that fell within the 1st birth weight decile, while AGA was equal to or greater than the 1st birth weight decile.

Clinical measurements

Data regarding patient characteristics were obtained from medical records (Table 1). We used Japanese standard data that had been stratified according to birth size to calculate standard deviation scores (SDS) and percentiles for each gestational age. Maternal BMI was calculated just after delivery. Penile length at birth was measured three times from the pubic bone to the tip of the penis with a ruler and the average was calculated.

Placenta

Placental weight SDS were calculated using reference data. Pathological findings were obtained from medical records. The following results were considered to be related to FGR: a small placenta, marginal cord insertion, hemangioma, infarctions, and symptoms of pregnancy-induced hypertension

(PIH) (decidual vasculopathy, accelerated maturation of the placental villi, infarctions, and increased fibrin deposition).

Urine samples and biochemical assays.

Spot urinary samples were collected from the 7th to 168th postnatal days at intervals of less than four weeks with cotton during delivery and at every outpatient visit after discharge.

LH and FSH concentrations were quantified in duplicate using the dissociation-enhanced lanthanide fluorescent immunoassay (DELFIA, Wallac Oy, Turku, Finland) ¹⁸. The relationships between the urinary levels of gonadotropins and serum levels of the same hormones were described previously ¹⁸.

Testosterone concentrations were measured in triplicate using enzyme-linked immunosorbent assays (ELISA); i.e., the testosterone EIA kit 582701 (Cayman; detection limit: 6 pg/ml, concentration range: 3.9-500 pg/ml, intra-assay coefficient of variation (CV): 4.4-19.1%; inter-assay CV: 14.2-7.7%).

Urinary parameters were corrected according to creatinine levels in order to adjust for changes in urinary concentrations. Urinary creatinine concentrations were analyzed using an enzymatic method.

Statistical analyses

Regarding patient profiles, categorical variables are presented as numbers, and comparisons involving these variables were performed with Fisher's exact test. Continuous variables are presented as the mean $\pm$ SD values, and comparisons involving these variables were performed with the

Student's *t*-test. Since data on gonadotropin and testosterone concentrations in our subjects were not normally distributed, they were shown as median and interquartile range (IQR) values. Hormonal concentration data included outlying observations and were derived from small sample sizes; therefore, we used the Friedman and Mann-Whitney tests during comparisons of these variables. Gonadotropin concentrations of 0 mIU/ml obtained using DELFIA were defined as 0.1 mIU/ml because the minimum detectable value of this assay is approximately 0.1 mIU/ml. LH/T ratios were compared using the Mann-Whitney *U* test. Results were regarded as significant if the *P* value was <0.05. All statistical analyses were performed using the statistical package SPSS (version 21.0; SPSS, Inc., Chicago, IL).

Results

Patient profiles

During the study period, 195 infants (104 male infants) were delivered in the NICU at Kyoto University Hospital. Thirty of these infants met the inclusion criteria. They were divided into 15 infants that were SGA at birth and 15 that were AGA at birth (Figure 1).

The background characteristics of all infants were as follows: mean± standard deviation (SD) gestational age: 33.2± 2.5 weeks, mean birth weight: 1626.6± 522.5 g, mean birth weight SDS: -1.3± 1.5, mean birth height 40.4± 4.6 cm, and mean birth height SDS -1.1± 1.3. The characteristics of the SGA and AGA groups are shown in Table 1. The gestational ages of both groups did not differ

significantly ($p=0.6$). However, SGA infants were significantly smaller than AGA infants (mean birth weight, mean birth weight SDS, mean birth height, and mean birth height SDS: $p=0.003$, $p<0.001$, $p=0.1$, and $p=0.006$, respectively). Penile length at birth was shorter in the SGA group than in the AGA group ($p=0.009$). None of the maternal factors differed significantly between the groups. The placenta was significantly lighter in the SGA group than in the AGA group ($p=0.03$). Placental blood flow obstructions were significantly more common in the SGA group (14 infant, $p=0.007$) than in the AGA group (2 infants). All infants in the SGA group involved a clinical diagnosis of maternal PIH or more than one placental pathological finding that was indicative of PIH or a placental blood flow obstruction (hemangioma or marginal cord insertion).

Endocrinological functions

A total of 117 and 94 samples were successfully collected from the SGA and AGA groups, respectively. Table 2 shows the concentrations of LH, FSH, and testosterone (median (IQR) values). Figure 2A-C shows postnatal changes in urinary gonadotropin and testosterone concentrations in the SGA and AGA groups (median (IQR) values).

Comparisons within each group

The concentrations of gonadotropins and testosterone increased in the 1st-2nd month and decreased by the 6th month in both groups. LH concentrations in the SGA group and testosterone concentrations in the SGA group and AGA group significantly changed throughout the entire study period (Friedman

Test; SGA: LH: $p < 0.001$, FSH: $p = 0.1$, and testosterone: $p < 0.001$; AGA: LH: $p = 0.2$, FSH: $p = 0.2$, and testosterone: $p = 0.001$) (Table 2).

Comparisons between SGA and AGA groups

Figure 2 shows changes in median LH, FSH, and testosterone concentrations estimated from the 7th to 168th postnatal days in the SGA and AGA groups. In comparisons of median gonadotropin and testosterone concentrations in the SGA and AGA groups over the entire period (Figure 3), the SGA group had significantly lower FSH concentrations (Mann-Whitney test; SGA: median: 4.5 mIU/mg creatinine, IQR: 2.4-9.5; AGA: median: 7.2 mIU/mg creatinine, IQR: 4.8-10.7; $p = 0.006$), significantly higher LH concentrations (SGA: median: 8.6 mIU/mg creatinine, IQR: 4.5-23.0; AGA: median: 6.3 mIU/mg creatinine, IQR: 3.4-11.5; $p = 0.004$), and significantly lower testosterone concentrations (SGA: median: 37.4 ng/mg creatinine, IQR: 19.9-54.5; AGA: median: 49.5 ng/mg creatinine, IQR: 28.0-73.6; $p = 0.003$) than those in the AGA group.

Discussion

In the present study, we evaluated longitudinal and serial urinary gonadotropin and testosterone concentrations in SGA and AGA preterm male infants during the first few months of life; i.e., “mini-puberty”. The secretion of each hormone was detected during the entire study period; however, not all hormonal concentrations changed significantly. As a result, we found that SGA infants had lower FSH and higher LH concentrations than AGA infants during mini-puberty. We also evaluated

testosterone concentrations in urine using ELISA in the same samples ¹⁹ and found that testosterone concentrations were significantly lower in SGA infants than in AGA infants. However, for specificity, agreements between different immunoassays, and measurement ranges, data obtained using immunoassays were considered to be less accurate than those obtained using mass spectrometric methods (MS). We speculate that high LH secretion is caused by low testosterone secretion through the negative feedback acting on the HPG axis, which suggests that SGA male infants develop primary hypogonadism during mini-puberty. These results are consistent with previous findings showing that FGR is involved in male reproductive functions ²⁰.

In contrast with the strong secretion of LH, FSH concentrations were significantly lower in SGA infants than in AGA infants, which is in contrast to the findings of a previous study ¹⁴. In infancy, the androgen receptor is not expressed in Sertoli cells ²¹; therefore, the effects of negative feedback from Sertoli cells on FSH secretion may be weak. However, FSH concentrations are also affected by inhibin ^{22,23}. Thus, the mechanisms responsible for modulating FSH secretion may be complex.

Difficulties are associated with definitively evaluating the clinical characteristics of SGA infants because these infants do not constitute a homogeneous population. Maternal, placental, and fetal factors all cause FGR ¹. In the present study, we excluded infants suspected of being SGA at birth because of fetal factors, e.g., genetic/chromosomal factors or malformations, and multiplets were also excluded from this study. In addition, infants with cryptorchidism or hypospadias, which are frequently observed in male SGA infants with FGR ²⁴, were also excluded because these conditions

may be caused by genetic factors²⁵. In order to avoid the influence of ethnic factors, only Asian infants were included in the present study. Thus, this study examined infants that were SGA at birth only due to FGR, and FGR in these infants was considered to have been caused by an abnormal intrauterine environment; i.e., maternal and placental factors. Furthermore, PIH and a small placenta were significantly more common among the SGA group. All SGA cases in this study involved a small placenta and/or pathological placental findings.

In preterm infants, the concentrations of reproductive hormones and timings of the peaks of each hormone in mini-puberty differ from those of full-term infants, as described previously¹². We excluded extremely preterm infants who were born before 28 weeks of gestation, and the gestational ages of the SGA and AGA groups did not differ significantly ($p=0.32$). Thus, the influence of preterm birth on hormone concentrations in SGA and AGA infants was eliminated from this study.

This study had several limitations. The number of infants was small because we had to exclude those who were SGA at birth due to fetal factors. Although infants were born preterm, we were unable to compare their data with those of full-term infants because of the difficulties associated with following up full-term infants as frequently as preterm infants, who repeatedly visit medical institutions.

Another limitation is the other medical issues that occur in preterm infants, e.g., respiratory, circulatory, and nutritional issues, which may have affected their hormone concentrations. Although we did not assess these factors, all infants were discharged from hospital around full term.

The hypersecretion of LH and low secretion of FSH during the early postnatal period in infants that were SGA at birth due to FGR may have been caused by antenatal programming. More longitudinal follow-up studies are needed in order to establish whether gonadal functional disturbances during mini-puberty in male infants that are SGA at birth influence gonadal functions in adolescence/adulthood.

In conclusion, male infants that are SGA at birth due to FGR have higher LH and lower FSH secretion than AGA male infants in the first few months of life.

Conflict of interest

There is no potential conflict of interest to disclose.

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Table1. Perinatal characteristics of SGA and AGA infants in this study

	SGA	AGA	P-value
	n=15	n=15	
Gestational age (weeks)	33.4±2.8	33.0±2.4	0.6
Very preterm (28-31 weeks)	6/15	5/15	0.5
Birth weight (g)	1354.4±389.3	1899.8±431.4	0.003
Birth weight SD score	-2.5±0.5	-0.1±0.5	<0.001
Birth height (cm)	39.0±3.4	41.8±5.1	0.1
Birth height SD score	-1.7±0.7	-0.4±1.1	0.006
Penile length at birth (cm)	2.2±0.4	2.7±0.5	0.009
Placental weight (g)	261.5±65.5	381.7±123.7	0.03
Placental weight SD score	-1.6±0.5	0.4±0.9	<0.001
Placental blood flow obstruction	11/14	2/10	0.007
Maternal age (years)	34.7±3.5	35.2±3.8	0.7
Maternal height (cm)	156.3±6.0	157.6±6.0	0.6
Maternal body mass index (kg/m ²)	22.8±3.6	23.5±5.3	0.7
Primiparous	11/15	8/15	0.2
Twin	2/15	1/15	0.5
Emergency cesarean section	14/15	11/15	0.2
Assisted reproductive technologies	8/15	4/15	0.1
Pregnancy-induced hypertension	8/15	3/15	0.06
Maternal smoking	6/15	3/15	0.2
Antenatal steroid use	8/15	7/15	0.5

Continuous variables are presented as the mean ± SD values. The Student's *t*-test.

Categorical variables are presented as numbers. Fisher's exact test.

Table 2. Concentrations of LH (mIU/mg creatinine), FSH (mIU/mg creatinine), and testosterone (ng/mg creatinine). Median (IQR). Comparison within each group; the Friedman Test ($p < 0.05$).

Age (days)	LH				FSH				T			
	SGA		AGA		SGA		AGA		SGA		AGA	
7-13	22.7	(12.0-42.8)	6.6	(4.1-9.6)	2.5	(1.0-5.9)	3.4	(2.1 -5.7)	30.9	(20.1-51.0)	54.1	(28.9-75.3)
14-20	34.9	(26.2-69.4)	11.1	(6.2-14.7)	9.0	(5.2-13.4)	20.4	(10.7-23.5)	43.0	(30.1-51.0)	42.8	(36.9-74.8)
21-27	9.3	(7.1-15.1)	10.9	(5.4-17.3)	4.3	(1.8-5.2)	8.3	(5.9-10.7)	24.5	(12.1-46.0)	53.5	(36.8-87.9)
28-34	18.2	(11.1-59.8)	7.3	(5.4-12.8)	7.4	(6.7-14.5)	5.8	(4.8-6.7)	44.7	(38.3-71.1)	60.4	(52.0-79.6)
35-41	10.0	(9.0-18.9)	4.7	(2.3-10.1)	6.0	(3.2-11.3)	7.7	(6.8-13.4)	52.6	(32.4-68.8)	79.3	(77.3-113.3)
42-48	4.8	(3.1-11.0)	8.3	(3.7-12.2)	5.2	(4.8-6.3)	8.8	(7.9-13.2)	45.6	(39.7-60.8)	68.0	(51.1-87.6)
49-55	9.2	(5.0-17.8)	3.0	(1.5-3.3)	5.0	(2.4-9.6)	8.4	(7.6-9.4)	52.8	(48.7-62.5)	42.4	(33.1-64.7)
56-83	8.0	(5.9-17.4)	4.7	(3.5-6.7)	4.1	(2.8-11.6)	7.6	(5.4-9.5)	40.4	(29.4-55.2)	48.2	(28.3-52.7)
84-111	4.8	(4.4-6.7)	3.8	(3.1-9.1)	4.1	(2.2-8.0)	6.1	(5.3-6.9)	28.2	(20.3-38.6)	23.0	(15.5-41.0)
112-139	4.1	(2.9-6.5)	3.7	(1.8-6.7)	2.7	(2.6-3.1)	1.2	(0.9-3.1)	16.1	(8.1-24.3)	12.7	(10.8-16.2)
140-168	2.8	(1.3-4.2)	1.9	(1.4-2.2)	2.4	(2.1-6.0)	1.0	(0.9-2.3)	4.4	(3.2-6.3)	15.5	(9.4-20.5)
P value	<0.001		0.2		0.1		0.2		<0.001		0.001	