Effects of adrenal androgens during the prenatal period on the second to fourth digit ratio in school-aged children

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Abstract

Objectives: We investigated the relationship between the levels of adrenal steroid hormones in cord blood and the second to fourth digit ratio (2D/4D), which is regarded as an indirect method to investigate the putative effects of prenatal exposure to androgens, in school-aged children.

Materials and Methods: Of the 514 mother-child pairs who participated in the prospective cohort study of birth in Sapporo between 2002 and 2005, the following adrenal steroid hormone levels in 294 stored cord blood samples (135 males and 159 females) were measured; cortisol, cortisone, androstenedione and dehydroepiandrosterone (DHEA). A total of 190 out of 350 children who were currently school-aged and contactable for this survey sent back photocopies of their palms for 2D/4D measurements.

Results: 2D/4D in all right hands, left hands, and mean values was significantly lower in males than in females ($p<0.01$). DHEA levels were significantly higher in females. A multivariate regression model showed that 2D/4D negatively correlated with DHEA in males only ($p<0.01$). No correlations were observed in the other adrenal steroid hormones tested in males or in any adrenal steroid hormones in females.

Conclusion: DHEA is mainly secreted in large amounts by the adrenal gland and is transformed into active sex-steroid hormones in peripheral tissues. The present study demonstrated that sex differences in digits were influenced by adrenal androgens during the prenatal period, possibly through intracrinological processes for androgen receptors located in fetal cartilaginous tissues.

Key words

2D/4D; DHEA; prenatal; adrenal; androgen; sex differentiation

Highlights

2D/4D was significantly lower in boys than in girls in school-aged children.

Adrenal steroid hormones levels in cord blood related to sex differences in digits.

2D/4D negatively correlated with level of DHEA in cord blood in males.

No correlations with 2D/4D were observed in the other adrenal steroid hormones.

Sex differences in digits may be influenced by intracrinological processes.

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Abbreviations

CAH: congenital adrenal hyperplasia; DHEA: dehydroepiandrosterone; E: estradiol; INSL3: insulin-like factor 3; T: testosterone; 2D/4D: the second to fourth digit ratio

Introduction

Exposure to sex hormones during the prenatal period is known to have an impact on sexual dimorphism. The extent of prenatal androgen exposure has been shown to affect differentiation to male-typical external and internal genitalia. Regarding digits, since androgen receptors are located in fetal cartilaginous tissues,[1] the second to fourth digit ratio (2D/4D) is affected by the prenatal hormonal environment, such as exposure to higher levels of androgens and other gonad-specific hormones.[2] In humans, 2D/4D was reported to be smaller in males than in females,[3] and is regarded as an indirect method to investigate the putative effects of prenatal exposure to androgens. We previously demonstrated that 2D/4D in school-aged children was affected by prenatal Leydig cell function in males.[4] Furthermore, this hypothesis as the underlying mechanism for differences in digits is supported by the following findings; lower 2D/4D in females with congenital adrenal hyperplasia (CAH),[5-7] higher 2D/4D in males with complete androgen insensitivity syndrome,[8] and higher 2D/4D in men with Klinefelter's syndrome. [9, 10]

Androgens are mainly produced by Leydig cells in the testes of males, and small amounts are secreted by the adrenal glands. The ovaries of females also produce androgens, but to a lesser extent. By focusing on the adrenal glands, Labrie advocated the term ‘intracrinology’ in the field of endocrinology, which describes the local formation of active sex-steroid hormones.[11] In peripheral tissues, dehydroepiandrosterone (DHEA), as an inactive adrenal steroid precursor, is transferred into active androgens and estrogens by enzymes and then exert the important local effects of sex-steroid hormones.[12, 13] Thus, in addition to the gonads, androgens derived from the adrenal

glands also have the potential to affect sexual dimorphism during the prenatal period.

In order to elucidate the mechanisms underlying sexual differences in 2D/4D, hormone exposure needs to be measured during gestation, particularly in the earlier period of pregnancy. However, there is currently no established approach for measuring the hormonal environment earlier in pregnancy because of the ethical issues associated with normal pregnancy. Hormone levels in umbilical cord blood, which is obtained immediately after delivery, reflects a part of the hormonal environment of the fetus at late gestation.[14, 15] Previous studies identified a relationship between fetal hormonal exposure and human development using cord blood.[16-18]

Our previous study showed that no significant relation was identified between 2D/4D in school-aged children and testosterone, estradiol or progesterone in cord blood.[4] In the present study, we focused on androgens derived from the adrenal glands. Therefore, we investigated the relationship between 2D/4D in school-aged children and the levels of adrenal steroid hormones in cord blood.

Participants and Methods

Participants

This prospective birth cohort study was based on the Sapporo Cohort, Hokkaido Study on Environment and Children's Health.[19, 20] Study details regarding the population, data collection, sampling of biological specimens, and contents of the questionnaire have been described previously.[19, 20] Briefly, native Japanese women living in Sapporo city or its surrounding areas were enrolled in the study at 23-35 weeks of gestation at Sapporo Toho Hospital between July 2002 and October 2005. Of the 1796 women approached, 25% were excluded because they decided to enroll in the Japanese cord blood bank or deliver the baby at another hospital; therefore, 514 pregnant women were enrolled in this cohort study (participation rate of 28.6%).

This study was approved by the Institutional Ethical Board for Epidemiological Studies at Hokkaido University Graduate School of Medicine and Hokkaido University Center for Environmental and Health Sciences. All participants provided written informed consent. Informed consent on behalf of the children enrolled was provided by their parents.

Measurement of 2D/4D

Ten out of 514 participants were excluded from the study due to miscarriage, stillbirth, relocation, or voluntary withdrawal from the study before delivery. Since 7 sets of twins were born, a total of 511 children (246 males and 265 females) were finally included in the Sapporo Cohort study. Of these, 350 children (68.1%), who are currently school-aged and contactable for this survey, were requested via mail to send

black-and-white photocopies of the palms of both the left and right hands. Measurements of digits were made from photocopies of the ventral surface of the right and left hands. Participants were instructed to straighten their fingers and lightly place their hands palm down on the photocopy machine. Measurements were made to the nearest 0.5 mm from the mid-point of the finger crease proximal to the palm to the tip of the finger using steel Vernier calipers. 2D/4D was calculated by dividing the length of the second digit by that of the fourth.[3] All measurements were taken twice by two observers blinded to participant information in order to confirm the measurements obtained as described previously.[4]

Adrenal steroid hormone measurements in cord blood samples

At the time of delivery, a 10-30-mL blood sample was collected from the umbilical cord and stored at -80°C for later analysis.

The following hormone levels in 294 stored cord blood samples (135 males and 159 females) were measured. Cortisol, cortisone, androstenedione, and DHEA levels were measured using LC-MS/MS.[21, 22] All hormone measurements were performed by Aska Pharma Medical Co., Ltd. (Kanagawa, Japan).

Statistical analyses

Data on the characteristics of participants, 2D/4D, and sex hormone levels were presented as a group mean ± standard deviation and were analyzed between groups using a one-way ANOVA. Sex hormones were converted to a log10 scale as these data did not fall into a normal distribution. The relationship between 2D/4D and sex hormone levels in cord blood samples was calculated using a multiple linear regression analysis.

The inclusion of covariates was based on biological considerations and adjustments were made for maternal age (continuous), birth weight (continuous), maternal smoking during pregnancy (yes or no), and maternal alcohol consumption during pregnancy (yes or no). All statistical analyses were performed using JMP pro 10 (SAS institute Inc., NC, USA), except for the intra-class correlation coefficient for right and left 2D/4D measurements, which was calculated using SPSS statistics version 19 (IBM, IL, USA). Significance levels were set to 0.05 for all comparisons.

Results

1) 2D/4D

A total of 190 children, including 88 males and 102 females, sent back photocopies of their palms. In all right hands, left hands, and mean values, 2D/4D was significantly higher in females than in males: 94.9 +/- 0.3 vs. 93.2 +/- 0.4 in right hands (p=0.0006), 94.9 +/- 0.3 vs. 93.5 +/- 0.4 in left hands (p=0.0082) and 94.9 +/- 0.3 vs. 93.3 +/- 0.4 in mean values (p=0.0006), as described previously.[4] 2D/4D fell into a normal distribution in all right hands, left hands, and mean values. The mean 2D/4D value in both hands was used to determine its relationship with sex hormones as a representative value of each participant.

2) Adrenal steroid hormones in cord blood samples

The detection percentages of cortisol in males and females were 98.5% and 96.8%, while those of cortisone in males and females were 97.0% and 93.0%, respectively. The other adrenal steroid hormones were detected in all samples (Table 1). In samples with non-detected cortisol or cortisone level, a half of detection limit (DL) was used as a value of cortisol or cortisone level in data analysis. The intra-assay and inter-assay coefficients of variations in terms of adrenal steroid hormone measurements were as follows; cortisol: 3,9%-10.9%, Cortisone: 1.3%-9.9%, androstenedione: 4.8%-6.5%, and DHEA: 2.3%-3.7% in the intra-assay coefficients of variations, and cortisol: 7.6%-11.3%, Cortisone:7.8%-11.3%, androstenedione: < 5.6%, and DHEA: 6%-15.0% in the inter-assay coefficients of variations.

The median concentration of DHEA was significantly higher in females. No

significant differences were observed in the other hormones between males and females (Table 1).

A focus on the presence or absence of 2D/4D data revealed no significant differences in adrenal steroid hormone levels in children who sent back photocopies for 2D/4D and those who did not (Table 2).

3) Relationship between 2D/4D and adrenal steroid hormones

Combined with the data on 2D/4D and sex-steroid hormones levels in cord blood, a total of 117 children, including 45 males and 72 females, were available for an analysis of the relationship between sexual dimorphism of the digits and the hormonal environment during gestation.

The characteristics of mothers for the analysis were as follows; older mothers, mothers with a lower body mass index, a higher annual household income, higher educational level, and fewer smokers during pregnancy. The characteristics of infants for the analysis were more males, heavier birth weight, and older gestational age at birth (Table 3).

A multivariate regression model showed that 2D/4D negatively correlated with DHEA in males only. No correlations were observed in any of the other adrenal steroid hormones with 2D/4D in males or females (Table 4). This result indicated that 2D/4D was affected by adrenal androgens.

Discussion

The extent of prenatal androgen exposure is known to have an impact on sexual dimorphism. Androgen receptors located in fetal cartilaginous tissues have been implicated as an underlying mechanism for sex differences in digits.[1] Therefore, 2D/4D has been used as an indirect method to investigate the putative effects of prenatal exposure to androgens. In the present study, 2D/4D was smaller in males than in females, which was consistent with previous findings.[3, 4, 23] The present study focused on the relationship between the extent of fetal exposure of adrenal steroid hormones using cord blood and sex difference in digits, since our previous study showed that no significant relation was identified between 2D/4D in school-aged children and sex-steroid hormones in cord blood, such as testosterone (T), estradiol (E) and progesterone[4]. Sex differences in digits revealed that 2D/4D in school-aged children negatively correlated with DHEA in males only. No correlation was observed between 2D/4D and the other adrenal steroid hormones in males or females. Thus, the present study revealed that exposure to adrenal androgens during the prenatal period also affects sexual dimorphism in the digits.

Although the concentration of hormones in cord blood did not entirely reflect the hormonal environment during pregnancy, we used cord blood to measure adrenal steroid hormone levels due to the ethical issues associated with normal pregnancy. Our previous results revealed that T and T/E levels in cord blood were significantly higher in males than in females[4], which was consistent with findings of other groups.[24, 25] These results indicate that testosterone is predominantly produced by Leydig cells in the fetal testes of males and the concentration of hormones in cord blood reflect as a

part of hormonal environment during pregnancy.

In the present study, we measured adrenal steroid hormone levels and found that the median concentration of DHEA was significantly higher in females. DHEA secreted by the adrenal glands is an inactive precursor steroid that is converted into sulphated DHEA (DHEA-S) in the adrenal glands and small intestine.[26] Gender differences in DHEA and DHEA-S are controversial, with some studies reporting significantly higher concentrations in the cord blood of females,[24] whereas others report no significant differences between males and females.[27] Keen at al. found that maternal, fetal, and obstetric factors may influence androgen levels in cord blood.[15] However, we did not detect any relationship between the concentration of DHEA in cord blood and maternal, fetal, and obstetric factors, such as maternal age, maternal body mass index, maternal smoking during pregnancy, maternal alcohol consumption during pregnancy, birth weight, or gestational age in the present study. Thus, the measurement of adrenal steroid hormones in cord blood has not yet been established in detail.[14]

CAH has been used as one of the representative models to investigate the effects of prenatal androgen exposure. Most cases of CAH are caused by a steroid 21-hydroxylase deficiency, which results in elevated concentrations of adrenal androgens, including DHEA, beginning at the 8^{th} week of gestation.[28] Females with CAH, in particular, show genital ambiguity, such as clitoral enlargement, labial fusion, and interference in urogenital sinus separation. In sexual dimorphism of the digit ratio, 2D/4D was previously reported to be lower in CAH patients than in healthy controls, particularly in females.[5-7] Albeit the special condition of CAH, these findings demonstrate that prenatal exposure to adrenal androgens may affect sexual dimorphism in the digit ratio.

Our previous study showed that no significant relation was identified between 2D/4D in school-aged children and testosterone level in cord blood.[4] This result indicates that testosterone levels in cord blood at birth do not reflect fetal exposure in the critical period of digit development at approximately 14 weeks of gestation. On the other hand, our previous study also revealed 2D/4D was negatively correlated with insulin-like factor 3 (INSL3) in males. Since INSL3, a gender-specific fetal hormone, is constitutively produced by Leydig cells in the fetal testis after sex determination[29], we considered that INSL3 in cord blood reflect androgen exposure during the important developmental window of earlier pregnancy for the digits as well as male reproductive development. Namely, 2D/4D was affected by prenatal Leydig cell function in males in our previous study.[4]

In addition to the previous finding, the present study revealed that DHEA in cord blood negatively correlated with 2D/4D in males. The adrenal cortex in the fetus morphologically consists of a fetal zone, transitional zone, and definitive zone. The fetal zone, resembling the zona reticulosa in adults for steroidogenesis, occupies the central region of the fetal adrenal cortex and expresses P450c17 in order to produce DHEA from approximately the 10^{th} week of gestation.[30] DEHA is transformed into androgens and estrogens in peripheral target tissues by enzymes. The intracellular levels of sex steroids produced from DHEA are locally controlled in androgen-sensitive or estrogen-sensitive tissues. This is referred to as intracrinology, a new field of endocrinology.[11-13] This concept has mainly been applied to women after menopause and prostate cancer in men. After menopause in women, the secretion of estrogens from the ovaries ceases, and estrogens as well as androgens are produced in peripheral tissues from DHEA, which may affect bone physiology, libido, muscle mass,

fat tissue, and the vaginal epithelium. Since adrenal androgens are transformed into active androgens such as T and dihydrotestosterone with prostate cancer, anti-androgen drugs are used to block the androgen receptor in combination with medication to inhibit androgen secretion from the testes. The results of the present study indicate that adrenal androgens during the prenatal period also play an important role in the development of sexual dimorphism in the digits, possibly through intracrinological processes for androgen receptors located in the fetal cartilaginous tissues of digits, in addition to androgens produced by Leydig cells in the testes. However, the mechanisms responsible remain unclear and intracrine androgen-estrogen regulation of digital cartilage is still based on our speculation. Further studies are needed in order to confirm the effects of adrenal androgens on sexual dimorphism during the prenatal period.

Prenatal exposure to androgens may affect social behavior. By focusing on the relationship between personality and prenatal environment, our study group previously reported that 2D/4D negatively correlated with the masculine score in males and females, while no correlation was observed between 2D/4D and the feminine score in the Pre-school Activities Inventory. This study concluded that the prenatal hormonal environment, such as androgen exposure during early gestation, may be one of the important factors influencing masculine-typical dimorphic brain development and behavior in school-aged children.[23] Furthermore, previous studies reported that individuals with a lower 2D/4D, who may have been exposed to high levels of androgens prenatally, were more likely to exhibit aggressive behaviors.[31-33] On the other hand, adrenal androgens have been associated with conduct disorders in children. A study conducted by van Goozen et al. revealed that the level of DHEA-S positively

correlated with conduct disorders, such as aggression and delinquency, in boys.[34] Barzman et al. also found that a higher level of DHEA correlated with aggression in children.[35] Taken together with these findings, prenatal exposure to DHEA has the potential to affect sexual dimorphism in the digits of infants as well as social behaviors, and we consider the results of the present study to be the first evidence to confirm this phenomenon.

However, there were still some limitations in the present study. First, compared between participant and non-participant characteristics in the current analysis of Sapporo cohort, there were some characteristic differences in terms of maternal age at delivery, maternal body mass index before pregnancy, annual income, maternal educational level, maternal smoking during pregnancy, ratio of male/female infants, body weight at birth and gestational age at birth. Therefore, it may suggest selection bias. Second, since we analyzed data on hormonal exposure using cord blood, hormone exposure during the earlier period of gestation still remains unknown. Third, the number of children who are currently school-aged and for whom we had data on 2D/4D and sex hormones was small because only 190 out of 350 children (54.3%) sent back photocopies of their hand palms to measure 2D/4D. Therefore, larger studies are needed in order to reveal the effects of sex hormone levels *in utero*, particularly during the earlier period of gestation, on physical changes in children.

Keen at al. found that maternal, fetal, and obstetric factors may influence androgen levels in cord blood.[15]

Conclusions

DHEA is mainly secreted in large amounts by the adrenal gland and is transformed into active androgens and estrogens in sex-steroid hormone-sensitive tissues, which is referred to as intracrinology. The present study demonstrated that sex differences in digits was influenced by adrenal androgens during the prenatal period, possibly through intracrinological processes for androgen receptors located in fetal cartilaginous tissues.

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Table 1 Sex hormone levels in cord blood of males and females

DL: detection limit

		Males					Females				
	DL	n	50th	25th-75th	>DL (%)		n	50th	25th-75th	>DL (%)	p-value
Cortisol (ng/mL)	0.250	135	38.3	22.5-65.3	98.5		159	39.3	22.8-62.8	96.8	0.3671
Cortisone (ng/mL)	0.100	135	95.3	70.5-123.4	97.0		159	93.2	69.9-123.0	93.0	0.1382
androstenedione (ng/mL)	0.010	135	0.47	0.38-0.61	100		159	0.44	0.35-0.57	100	0.8782
DHEA (ng/mL)	0.010	135	2.08	1.59-2.76	100		159	2.32	1.91-3.22	100	0.0018

DHEA: dehydroepiandrosterone

Table 2 Sex hormones in cord blood and 2D/4D

	Males					Females				
	2D/4D (+)		2D/4D (-)			2D/4D (+)		2D/4D (-)		
	n	50th Min Max	n	50th Min Max	p-value	n	50th Min Max	n	50th Min Max	p-value
Cortisol (ng/mL)	45	36.7 0.125 188.5	89	41.5 0.125 179.9	0772	65	40.39 0.125 173.1	93	36.4 0.125 242.7	0.892
Cortisone (ng/mL)	45	95.0 0.05 253.7	89	99.8 0.05 299.7	0.307	65	85.3 0.05 190.2	93	95.4 0.05 181.2	0.098
androstenedione (ng/mL)	46	0.47 0.10 3.21	89	0.47 0.10 5.93	0.459	71	0.44 0.18 7.46	88	0.45 0.15 1.06	0.149
DHEA (ng/mL)	46	2.08 0.67 27.95	89	2.08 0.93 19.2	0.522	71	2.32 0.95 54.28	88	2.40 1.11 114.2	0.732

DHEA: dehydroepiandrosterone

Table 3 Characteristics of participants

The values in brackets represent percentages. *: p<0.05, **: p<0.01

		2D/4D (+) and Sex hormones (+)		2D/4D (-) and/or Sex hormones (-)		
		n	Mean ± SD	n	Mean ± SD	
Maternal characteristics						
Age at delivery (years old)		117	31.8 ± 4.1	387	30.4 ± 5.0	**
Pre-pregnancy BMI (m^2/kg)		117	20.7 ± 2.7	384	21.4 ± 3.4	*
Parity	Primiparous	61 (52.1)		179 (46.3)		
	Multiparous	56 (47.9)		208 (53.7)		
Annual household income (million yen per year)	<5	64 (55.2)		279 (72.5)		**
	≥5	52 (44.8)		106 (27.5)		
Educational level (years)	≤12	38 (32.5)		185 (47.8)		**
	≥13	79 (67.5)		202 (52.2)		
Smoking during pregnancy	Non-smoker	106 (90.6)		296 (76.5)		**
	Smoker	11 (9.4)		91 (23.5)		
Alcohol consumption during pregnancy	Non-drinker	74 (63.3)		276 (71.3)		
	Drinker	43 (36.7)		111 (28.7)		
Infant characteristics						
Gender	Males	46 (39.3)		196 (50.7)		*
	Females	71 (60.7)		191 (49.3)		
Birth weight (g)		117	3104.8 ± 304.6	387	3018.6 ± 431.6	*
Gestational age (weeks)		117	39.3 ± 1.6	387	38.7 ± 1.6	**

Table 4 Relationship between 2D/4D and sex hormones in cord blood

Hormone levels	Total			Males			Females		
	n	B (95%CI)	R^2	n	B (95%CI)	R^2	n	B (95%CI)	R^2
Cortisol (ng/mL)	113	0.094 (-0.608, 1.878)	0.115	44	0.1174 (-1.787, 3.744)	0.032	69	0.062 (-1.047, 1.704)	0.170
Cortisone (ng/mL)	112	0.075 (-0.541, 1.288)	0.110	43	0.092 (-1.562, 2.747)	0.026	69	0.083 (-0.623, 1.307)	0.173
androstenedione (pg/mL)	113	-0.076 (-2.870, 1.187)	0.112	44	-0.268 (-9.563, 0.840)	0.088	69	-0.028 (-2.302, 1.811)	0.167
DHEA (pg/mL)	113	-0.136 (-3.573, 0.533)	0.125	44	-0.361* (-8.862, -0.697)	0.141	69	-0.074 (-3.036, 1.594)	0.172

DHEA: dehydroepiandrosterone

Covariates: maternal age, birth weight, maternal smoking during pregnancy, maternal alcohol consumption during pregnancy