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Orıgınal Article

Salivary Sex Steroid Levels in Infants and Their Relation with Infantile Colic

Kalaycı FM et al. Minipuberty and Infantile Colic

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What is already known on this topic?
To timings of minipuberty and infantile colic coincide. There exists no data regarding the relationship between them.

What this study adds?
Sex steroid production might be altered during minipuberty in subjects with infantile colic.

Abstract
Background: Hypothalamic-pituitary-gonadal axis is active during minipuberty, timing of which coincides with infantile colic. To the best of our knowledge, the relationship between these entities has not been investigated yet.
Methods: Saliva samples were collected from 15- to 60-day-old term infants (n=139) between 9 am and 5 pm. Group 1 included infants with infantile colic (n=68, 54.4% female). Remaining healthy infants constituted Group 2 (n=71, 47.9% female). The salivary levels of estradiol (E_{sal}) in females and testosterone (T_{sal}) in males were studied in duplicate by using the ELISA method.
Results: The median (25^{th}-75^{th} centile) age and birth week for all infants were 33 (29-43) days and 39 (38.1-40) weeks, respectively. Levels of T_{sal} in males [Group 1, 73.35 (59.94-117.82) pg/mL vs Group 2, 77.66 (56.49-110.08) pg/mL, p=0.956] and E_{sal} in females [Group 1, 3.91 (2.76-5.31) pg/mL vs Group 2, 4.03 (1.63-12.1) pg/mL, p=0.683] were similar among the groups. However, only in subjects with infantile colic (Group 1), E_{sal} and body mass index (BMI) standard deviation scores of females were slightly correlated (Group 1, r_s= 0.393, p=0.016 vs. Group 2, r_s= 0.308, p=0.076) and there was a significant correlation between the sampling time and T_{sal} in males (Group 1, r_s= 0.469, p=0.009 vs. Group 2, r_s= -0.005, p=0.976).
Conclusions: Random salivary sex steroid levels were similar among groups. However, only in subjects with infantile colic, salivary estradiol levels in females were positively correlated with BMI and salivary testosterone levels were higher later in the day among males. Thus, sex steroid production might be altered during minipuberty in subjects with infantile colic.
Keywords: Gonadal activity, newborn, puberty, fussing

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Introduction

Classical puberty begins in adolescence; however, the hypothalamic-pituitary-gonadal (HPG) axis is also active during the first months of life resulting in an increase in gonadotropins and sex steroids (minipuberty) (1-4). In boys, the increase in follicle stimulating hormone (FSH) and luteinizing hormone (LH), primarily LH, reaches its highest value in 4-10 weeks after birth and they both decrease to prepubertal levels around six months. Serum testosterone level, which increases with LH secretion, peaks between 1-3 months and decreases to prepubertal levels between 6-9 months (2, 5). Similarly in girls, serum estradiol levels peak between 30-60 days of life, then fall below the prepubertal level towards one year of age (5). In the medical literature, there exist data regarding salivary testosterone levels in male infants, but data on salivary estradiol levels in female infants are limited (6,7,8,9). The importance of minipuberty is not fully understood; until now, minipuberty is found to affect genital organ development, body composition, and cognitive functions (3).

Infantile colic was defined by Wessel in 1954 as excessive irritability and crying in the evening for no apparent reason, starting in the first weeks of life (10). It is seen in 10-40% of otherwise healthy infants aged one to five months. While various hypotheses including gastrointestinal, neurodevelopmental, and psychosocial causes have been proposed, its etiology is currently unknown (11, 12).

Among the few and heterogeneous data regarding the impact of minipuberty on behavior, there are some findings indicating that sex steroid levels might be associated with behavioral patterns in infants as well as in adolescents (3,9,13,14). Since sex steroid levels are elevated during a period when infantile colic is common, we aimed to evaluate the relationship between minipuberty and infantile colic by measuring testosterone and estradiol levels in saliva of infants. To the best of our knowledge, there is no similar study in the literature.

Materials and Methods

a. Subjects

This study included term infants aged 15-60 days old without any additional disease who applied to outpatient clinics in a single center between March and October 2021. Infants who had any disorder of sex development or a systemic pathology such as gastrointestinal malformation and gastroesophageal reflux or who had used antibiotics in the last week were not included in the study. Infantile colic was diagnosed according to official Rome IV criteria (episodes of crying and irritability lasted longer than one week, at least three hours a day, and at least three days in the same week) (11, 15). Careful physical examinations of these infants were performed to exclude other systematic

causes of irritability. All of the infants were prepubertal and none had an abnormal external genital structure. The parents of the subjects with infantile colic were contacted by phone when they were 6 months old and it was confirmed that they did not have any other diseases.

b. Data Collection

All relevant data including demographic features, family history, anthropometric measurements, physical examination findings were recorded. Standard deviation scores (SDS) for weight, length, head circumference, and BMI were calculated according to the Turkish child population using Child Metrics (15, 16). Weight for length SDS was calculated according to World Health Organization (WHO) data (16,17).

Saliva samples were collected using Salimetrics® SalivaBio Oral Swab and stored at -80°C. Measurement was made with Salimetrics® 17 β-estradiol ELISA kit for estradiol (1-3701) in girls and Salimetrics® Testosterone ELISA kit for testosterone (1-2402) in boys in duplicate. When the first and second results were statistically compared for testosterone and estradiol, p values were 0.922 and 0.347, respectively. Average of two measurements were used in the study. Both kits work with the Sandwich ELISA method and can read at a wavelength of 450 nm. The sensitivity of the estradiol kit was 0.1 pg/mL, and the testosterone kit was 1 pg/mL. The measurement ranges were 1-32 pg/mL for estradiol and 6.1-600 pg/mL for testosterone.

c. Statistical Analysis

In order to be able to find a significant difference of 5 pg/mL between the mean of sex steroids of the groups in a situation where the standard deviation values of the groups were 4 and 8 pg/mL, based on a type 1 error of 0.05 and a power of 0.80, the minimum number of subjects for each gender among the groups was determined as 27. Taking unexpected errors into account, we planned to include 20% more cases. Statistical analysis was performed using the IBM SPSS Statistics 24 program. The distribution of the data was evaluated using the Kolmogorov–Smirnov test. Descriptive statistics were given as numbers and percentages for categorical variables and median value (25^{th}-75^{th} centile) for numerical variables. Mann-Whitney U test was used to compare numeric variables and chi-square test was used for categorical data. The correlation of the parameters was tested with Spearman correlation analysis. A p value <0.05 was considered significant.

d. Ethics

This study was conducted with the approval of Local Ethical Committee (2019/22-22). Financial support was provided by the Department of Scientific Research Projects of our University (2020.KB.SAG.40) and Turkish Society for Pediatric Endocrinology and Diabetes (TSPED-2020-04). An informed written consent form was obtained from parents before participating the study and it was performed in accordance with the principles of the Declaration of Helsinki.

Results

A total of 139 infants (48.9% males) were included in the study. The median age was 33 (29-43) days, and the median gestational age was 39 (38.1-40) weeks. Majority of the newborns (n=88, 63.3%) were born by caesarean section (C/S).

A hundred of infants (71.9%) were fed only with breast milk, 35 (25.2%) with both breast milk and formula, and four (2.9%) only with formula. The median value of daily stool counts was calculated as 3 (2-4; 1-9). Only three of them (2.2%) were not using any medication, 120 (86.3%) were using vitamin D, 15 (10.8%) were using vitamin D and probiotics and one of them (0.7%) were using only probiotics.

The subjects were divided into two groups as those with infantile colic (Group 1, n=68) and those without (Group 2, n=71). The infants among the groups had similar demographic and anthropometric features except gestational age and weight-for-length SDS (Table 1). In addition, the ages, education, health status, drug use, smoking rates of parents were similar among the groups (data not shown).

Characteristics of the subjects were further analyzed according to gender. Although it was observed that salivary estradiol levels decreased with increasing age in female babies (n=71), this situation was not at the level of statistical significance (r_s=-0.224, p=0.061) (Figure 1a). Females with infantile colic (Group 1-F) were born slightly earlier compared to healthy control female subjects (Group 2-F), while remaining features including salivary estradiol levels were similar (Table 2). Correlation analyses in females revealed that saliva estradiol levels showed significant correlation with BMI SDS only in Group 1 (Table 3).

In male infants, salivary testosterone levels decreased with increasing age (Figure 1b). BMI SDS and weight-for-length SDS were found to be significantly higher in the males with infantile colic (Group 1-M); although salivary testosterone levels were similar between the groups (Table 4). Correlation analyses were also done for male babies (Table 5). There was a moderate negative correlation between testosterone and BMI SDS only in the infantile colic group. When the correlation analysis of the sample collection time and testosterone level was examined, it was found that the subjects in the infantile colic group whose samples were taken later in the day had higher salivary testosterone levels (Figure 2). There was no similar association in the control group. In addition, when examined with partial correlation by controlling age, the relationship between testosterone and sample collection time was found to be stronger (r_s=0.469, p=0.009).

Discussion

Many factors were investigated in studies examining the etiology of infantile colic. Infantile colic is more common in preterm babies (10,18). While term babies were already included in our study, the median gestational age of the infantile colic group was slightly lower than that of the control group. When the girls and boys were examined separately, it was seen that this difference was caused by the girls. According to these data, it can be said that earlier birth, even at term, might be associated with infantile colic in girls.

In our study, there was not a significant association between salivary estradiol levels and age in the female infants. This situation might be explained by the findings of Kuiri-Hänninen *et al*. They found fluctuating urinary estradiol levels in subjects aged between one week and six months (19). We observed a positive correlation between salivary estradiol levels and BMI SDS both in all cases and in the infantile colic group. This situation might be associated with extraglandular estrogen production in the increased adipose tissue (20,21). However, we did not observe a significant difference between random salivary estradiol levels of girls with and without infantile colic. Alexander et al. also found no association of salivary estradiol levels (4.73±0.86 pg/mL) of 3-4 months old female infants with their preferences for various stimuli (9).

Decreasing salivary testosterone levels with age in male infants in our study are in line with the existing knowledge. Testosterone levels in the saliva of male infants aged one to three months, which were measured in duplicate with ELISA method, were found as 79.09±22.75 pg/mL, levels of which were like our study (6). In older infants aged between 2.7 and 4.8 months, the mean testosterone level was 40.39±13.39 pg/mL (7). In another study conducted with the same method and studied in duplicate, the salivary testosterone levels of 3–6-month-old boys were between 27.51 and 58.13 pg/mL (8). In our study, random salivary testosterone levels were not different between males with and without infantile colic, but, in the infantile colic group, there was a significant positive correlation between sample collection time and testosterone level. According to this, higher testosterone levels later in the day may be related with the onset of colic attacks in the evening. Also, we observed that boys with infantile colic have higher BMI SDS and weight for length SDS at the time of examination, which may be related to the fact that parents try to feed restless babies more. Significant relationship between salivary testosterone levels (40.68±10.69 pg/mL) in 3–4-month-old male infants and their behavior was also observed by Alexander et al. They reported that higher salivary testosterone levels were associated with stronger preferences for male-typical stimuli in videos (9).

Study Limitations

Biochemical demonstration of puberty with measuring serum levels of FSH and LH would be beneficial, however, measuring sex steroids in otherwise healthy infants is similarly informative and we successfully provided relevant data by using a noninvasive method. Collecting

saliva samples from infants during their symptoms can be suggested. On the other hand, this is not practical in an irritable infant, and we could have missed the temporal relationship of testosterone in boys if we have done so.

Conclusion

In conclusion, random levels of sex steroids in the saliva of subjects with infantile colic were not different from those of the control infants. On the other hand, significant correlation of salivary estradiol levels and BMI of females and higher salivary testosterone levels later in the day among the subjects with infantile colic indicate an alteration of sex steroid production in subjects with infantile colic.

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Author Contributions

Dr. Fulya Mete Kalaycı designed the study, collected data and drafted the initial manuscript. Dr. Özlem Gürsoy Doruk designed the data collection instruments and carried out the initial analyses. Drs. İbrahim Mert Erbaş, Osman Tolga İnce, Makbule Neslişah Tan, Adem Aydın, Ayhan Abacı, Ece Böber contributed to data collection, performed the literature search and critically reviewed and revised the manuscript. Dr. Korcan Demir conceptualized and designed the study, coordinated and supervised data collection, and critically reviewed and revised the manuscript for important intellectual content. All authors approved the final manuscript as submitted and agree to be accountable for all aspects of the work.

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Table 1. The demographic and anthropometric characteristics regarding presence of infantile colic. Group 1: Subjects with infantile colic, Group 2: Subjects without infantile colic

	Group 1 (n=68)	**Group 2 (n=71)**	***p***
Age (in days)	34.5 (29-43.8)	33 (29-43)	0.439
Gender			
Female	37 (%54.4)	34 (%47.9)	0.442
Male	31 (%45.6)	37 (%52.1)	
Mode of delivery			
SVD	21 (%30.9)	30 (%42.3)	0.164
C/S	47 (%69.1)	41 (%57.7)	
Gestational age	38.5 (38-39.6)	39.2 (38.5-40.2)	<0.001
Birth weight SDS	-0.03 [(-0.43)-0.61]	0.01 [(-0.72)-0.52]	0.903
Birth length SDS	0.00 [(-0.38)-0.75]	-0.19 [(-0.66)-0.45]	0.089
Birth head circumference SDS	0.07 [(-0.36)-0.43]	0.07 [(-0.64)-0.79]	0.899
Weight SDS	0.4 [(-0.2)-0.84]	0.15 [(-0.32)-0.62]	0.168
Length SDS	0.16 [(-0.48)-0.77]	0.23 [(-0.34)-0.91]	0.634
BMI SDS	0.13 [(-0.11)-0.75]	0.03 [(-0.67)-0.52]	0.056
Weight for length SDS	0.3 [(-0.16)-1.11]	-0.04 [(-0.87)-0.79]	0.021
Head circumference SDS	0.04 [(-0.52)-0.68]	-0.06 [(-0.71)-0.60]	0.607

Data are presented as median (25p-75p). SVD: Spontaneous vaginal delivery, C/S: cesarean section, SDS: standard deviation score, BMI: Body mass index

Table 2. The demographic and anthropometric characteristics regarding presence of infantile colic in females. Group 1-F: Female subjects with infantile colic, Group 2-F: Females without infantile colic

	Group 1-F (n=37)	**Group 2-F (n=34)**	***p***
Age (in days)	32 (29-43)	34 (29-47)	0.522
Mode of delivery **NSVD** **C/S**	 11 (%29.7) 26 (%70.3)	 14 (%41.2) 20 (%58.8)	0.313
Gestational age	38.28 (37.71-39.21)	39 (38.42-39.88)	<0.001
Birth weight SDS	-0.14 [(-0.62)-0.46]	-0.11 [(-0.72)-0.52]	0.773
Birth length SDS	-0.19 [(-0.42)-0.75]	-0.19 [(-0.66)-0.75]	0.619
Birth head circumference SDS	0.36 [(-0.36)-0.36]	-0.18 [(-0.36)-0.36]	0.743
Crying frequency (day/week)	5 (4-6; 0-7)	0 (0-5; 0-7)	<0.001
Last feeding time (min)	30 (17.5-60; 10-120)	30 (20-60; 15-180)	0.902
Sampling time **9:00-12:00** **13:00-17:00**	 21 (%56.8) 16 (%43.2)	 19 (%55.9) 15 (%44.1)	0.941
Weight SDS	0.41 [(-0.23)-0.82]	0.12 [(-0.29)-0.77]	0.542
Length SDS	0.02 [(-0.5)-0.76]	0.26 [(-0.71)-0.91]	0.936
BMI SDS	0.13 [(-0.09)-0.77]	0.36 [(-0.55)-0.70]	0.881
Weight for length SDS	0.32 [(-0.15)-1.18]	0.06 [(-0.82)-1.02]	0.208
Head circumference SDS	-0.08 [(-0.78)-0.69]	-0.19 [(-0.72)-0.62]	0.995
Weight gain (g/day)	33.75 (27.64-38.44)	32.61 (23.75-40.13)	0.782
Salivary estradiol (pg/mL)	3.91 (2.76-5.31)	4.03 (3.22-5.40)	0.683

Data are presented as median (25p-75p). SVD: Spontaneous vaginal delivery, C/S: cesarean section, SDS: Standard deviation score, BMI: Body mass index

Table 3: Correlation of salivary estradiol level with demographic and clinical parameters in females, Group 1-F: Females with infantile colic, Group 2-F: Females without infantile colic.

	All females (n=71)	**Group 1-F (n=37)**	**Group 2-F (n=34)**
Age (in days)	r_s=-0.224 (p=0.061)	r_s= -0.142 (p=0.4)	r_s= -0.268 (p=0.125)
Birth length SDS	r_s=-0.11 (p=0.361)	r_s= -0.007 (p=0.965)	r_s= -0.211 (p=0.231)
Sampling time	r_s=0.017 (p=0.887)	r_s= -0.003 (p=0.987)	r_s= 0.042 (p=0.811)
Weight SDS	r_s=0.132 (p=0.274)	r_s= 0.109 (p=0.521)	r_s= 0.181 (p=0.305)
BMI SDS	r_s=0.346 (p=0.003)	r_s=0.393 (p=0.016)	r_s=0.308 (p=0.076)
Weight for length SDS	r_s=0.241 (p=0.043)	r_s=0.287 (p=0.085)	r_s=0.215 (p=0.221)
Crying frequency (day/week)	r_s=-0.078 (p=0.518)	r_s= -0.216 (p=0.2)	r_s= -0.009 (p=0.962)

SDS: Standard deviation score, BMI: Body mass index

Table 4. The demographic and anthropometric characteristics regarding presence of infantile colic in males. Group 1-M: Male subjects with infantile colic, Group 2-M: Males without infantile colic.

	Group 1-M (n=31)	**Group 2-M (n=37)**	***p***
Age (in days)	37 (31-49)	31 (29-39.5)	0.086
Mode of delivery			
SVD	10 (%32.3)	16 (%43.2)	0.353
C/S	21 (%67.7)	21 (%56.8)	
Gestational age	39 (38.28-40)	39.71 (38.7-40.4)	0.139
Birth weight SDS	0.13 [(-0.25)-0.76]	0.09 [(-0.66)-0.50]	0.542
Birth length SDS	0.00 [(-0.45)-0.91]	0.00 [(-0.91)-0.45]	0.072
Birth head circumference SDS	0.07 [(-0.29)-0.79]	0.07 [(-0.64)-0.79]	0.895
Crying frequency (day/week)	5 (4-6)	0 (0-4.5)	<0.001
Last feeding time (mn)	60 (30-90)	60 (30-105)	0.369
Sampling time			
9:00-12:00	16 (%51.6)	26 (%70.3)	0.115
13:00-17:00	15 (%48.4)	11 (%29.7)	
Weight SDS	0.38 [(-0.10)-0.86]	0.16 [(-0.36)-0.55]	0.196
Length SDS	0.23 [(-0.49)-0.87]	0.22 [(-0.27)-0.99]	0.649
BMI SDS	0.12 [(-0.15)-0.76]	-0.13 [(-0.68)-0.27]	0.018
Weight for length SDS	0.25 [(-0.29)-1.06]	-0.17 [(-0.95)-0.60]	0.044
Head circumference SDS	0.22 [(-0.38)-0.68]	0.10 [(-0.67)-0.60]	0.483
Weight gain (g/day)	39.25 (28.52-49.88)	34.13 (26.58-44.44)	0.203
Salivary testosterone (pg/mL)	73.35 (59.94-117.82)	77.66 (56.49-110.08)	0.956

Data are presented as median (25p-75p). SDS: Standard deviation score, BMI: Body mass index

Table 5. Correlation of salivary testosterone levels with demographic and clinical parameters in males Group 1-M: Male subjects with infantile colic, Group 2-M: Males without infantile colic

	All males (n=68)	Group 1-M (n=31)	Group 2-M (n=37)
Age (in days)	r_s=-0.622 (p<0.001)	r_s= -0.695 (p<0.001)	r_s= -0.594 (p<0.001)
Birth length SDS	r_s=-0.288 (p=0.017)	r_s= -0.424 (p=0.018)	r_s= -0.190 (p=0.260)
Sampling time	r_s=0.148 (p=0.229)	r_s= 0.369 (p=0.041)	r_s= -0.005 (p=0.976)
Weight SDS	r_s=-0.126 (p=0.307)	r_s= -0.424 (p=0.017)	r_s= 0.132 (p=0.437)
BMI SDS	r_s=-0.167 (p=0.172)	r_s=-0.528 (p=0.002)	r_s=0.057 (p=0.738)
Weight for length SDS	r_s=0.043 (p=0.729)	r_s=-0.200 (p=0.280)	r_s=0.168 (p=0.320)
Crying frequency (day/week)	r_s=0.175 (p=0.153)	r_s=0.120 (p=0.520)	r_s= 0.256 (p=0.127)

SDS: Standard deviation score, BMI: Body mass index

Figure 1. Correlation between salivary sex steroid levels and age in (a) females and (b) males with and without colic

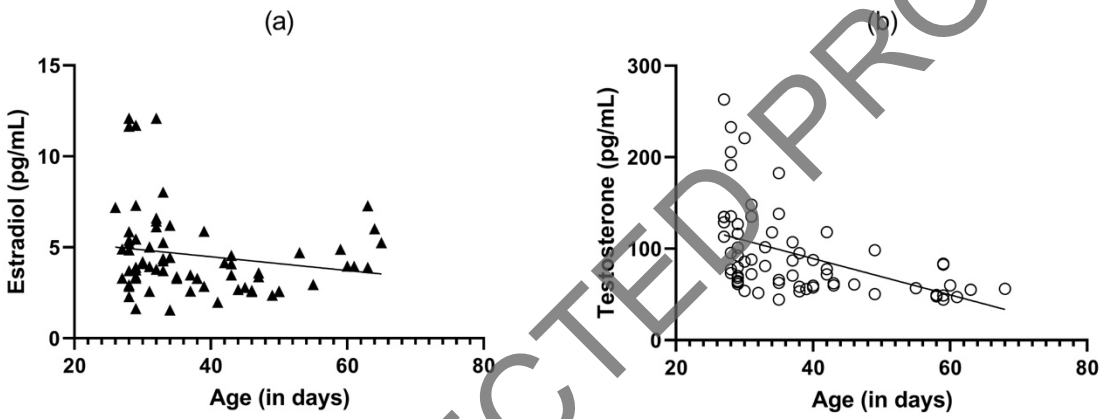

Figure 2. Correlation between salivary sex steroid levels and sampling time in the infantile colic group (a, c) and control group (b, d)