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Original Article

Testicular function during adolescence in boys with type 1 diabetes mellitus (T1D): absence of hypogonadism and differences in endocrine profile at the beginning and end of puberty

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Aim: Conflicting results regarding testicular function in adults with type 1 diabetes (T1D) have been reported, but little is known about Leydig and Sertoli cell function during puberty in boys treated with multiple daily insulin doses. Our aim was to assess testicular function in boys with T1D.
Methods: Pubertal boys with T1D (n = 71) and healthy control boys (Control group; n = 104) who were 10–18 years were studied. Both groups were matched by pubertal stage, age, and BMI. Total testosterone (TT), calculated free testosterone (cfT), SHBG, inhibin B, AMH, and gonadotropin levels were determined.
Results: At the beginning of puberty, the T1D group had higher levels of SHBG ($p = 0.003$) and similar androgen levels than the Control group. At the end of puberty, higher TT, and cfT were observed in T1D compared to the Control group ($p < 0.01$ and $p < 0.001$, respectively). Gonadotropins and AMH were similar in both groups. Regression analysis showed that T1D was a significant factor, even after adjusting for Tanner stage and BMI-SDS, affecting TT, cFT, and SHBG levels. BMI-SDS was a significant factor affecting TT and SHBG levels. Higher HbA1c had a negative effect on total testosterone and cFT and a positive effect on SHBG levels in T1D boys.
Conclusion: Adolescents with T1D do not exhibit hypogonadism, as shown by normal gonadotropin, testosterone, inhibin B, and AMH levels. However, in T1D boys, HbA1c and BMI-SDS had a negative association with testosterone levels. Elevated testosterone levels are observed during late puberty, which were not present earlier.

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Background

Abnormalities in growth and pubertal development have been described in patients with type 1 diabetes (T1D), especially in those with insufficient metabolic control (1, 2). The improvement in the treatment of T1D has led to dramatic changes in the care of children and adolescents with this condition. However, this issue has been studied mostly in females (3–5), and it is largely unknown if reproductive function is affected in boys with T1D treated with modern insulin regimens.

Previous studies performed in young males treated with conventional insulin therapy have shown that the

hypothalamic-pituitary-testicular axis may be affected by T1D. Hypogonadotropic hypogonadism associated with insufficient metabolic control has been reported (6), but recent studies in adult men with T1D treated with modern insulin therapy have shown conflicting results regarding testosterone levels (7–11). Moreover, no studies have evaluated testicular function in young boys with T1D receiving multiple daily doses. Previous studies performed in boys with T1D have several limitations including a small number of studied subjects (12, 13), a wide age range with inclusion of adults in the studied group (12, 14), a small number of boys in each pubertal stage (12, 13, 15) and a large proportion of adolescents treated with conventional therapy (12–14).

Adult men with long-standing T1D may exhibit abnormalities in spermatogenesis (16, 17), suggesting that Sertoli cell function may be affected. Inhibin B and anti-Müllerian hormone (AMH) have arisen as useful markers for the assessment of seminiferous tubule function in prepubertal and pubertal males. AMH is a major product of the fetal and prepubertal Sertoli cell; during these periods, its secretion is stimulated by FSH (18, 19). Subsequently, as Sertoli cells mature during puberty, AMH levels decline in response to the increase of intratesticular testosterone, a potent inhibitor of AMH production (19, 20). Inhibin B is produced by Sertoli cells in the prepubertal male gonad (21), where it also reflects the response to FSH stimulation (19). During puberty, inhibin B secretion increases as a consequence of the combined effect of FSH, testosterone, and spermatogenic activity. In the pubertal and adult male, inhibin B is the major component in the negative feedback mechanisms that regulate pituitary FSH secretion (21). Both AMH and inhibin B are low in boys and adolescents with hypogonadotropic hypogonadism (19, 22).

It is unknown whether Sertoli cell function is abnormal during puberty in adolescents with T1D. In addition, a thorough evaluation of testosterone levels in boys treated with multiple daily insulin doses and matched by Tanner stage and body mass index (BMI) has not been performed. Thus, the aim of this study was to assess testicular function, including testosterone and SHBG levels and Sertoli cell function, during puberty in males with T1D receiving multiple daily dose insulin therapy compared to a carefully matched group of healthy children.

Subjects and methods

Pubertal boys (n = 71) with T1D and healthy control boys (n = 104) who were 10–18 yr were studied. All patients treated in Hospital San Borja Arriarán during 2009–2010 were invited to participate. This diabetes unit provides care to all the patients with T1D treated in the Public Health System who lives in central Santiago, Chile. Inclusion criteria were a diagnosis of T1D, the presence of severe insulin deficient diabetes treated with multiple daily insulin doses from the time of diagnosis, diabetes duration longer than 1 yr in males younger than 18 yr and a testicular volume of 4 mL or more. To evaluate the effect of metabolic control on gonadal function, the hemoglobin A1c (HbA1c) level was not an exclusion criteria. The exclusion criteria were type 2 or other type of diabetes; the presence of honeymoon, as previously described (23); precocious or delayed puberty, abnormal thyroid function; sex steroid use; and the presence of chronic comorbidities such as genetic diseases, malnutrition, obesity [defined by a BMI higher than the 95th percentile), celiac disease, or kidney, liver or heart disease. Daily insulin dose was determined from the average daily dose used in the past 15 d obtained from diabetes logs.

Healthy boys (Control group), were matched by age, pubertal stage, and BMI, and recruited from two schools located near our institute. The inclusion criteria were normal fasting glucose, absence of chronic diseases, and no use of medications. Boys with precocious or delayed puberty according to Marshall and Tanner were excluded (24).

Study protocol

Pubertal development was assessed by one of the authors (A. R. or X. G.) according to Marshall and Tanner (24). Testicular volume was determined by a Prader orchidometer. Pubertal stage was considered to be early puberty in boys if the genitalia were Tanner stage 2, as intermediate puberty if the genitalia were Tanner stages 3 and 4, and late puberty if pubertal development was complete (Tanner stage 5). Pubic hair development was not used as a proxy of pubertal development because the appearance of sexual hair has an ethnic component and the Chilean population is known to have a delay in pubic hair development and to be non-hirsute (25).

Weight was measured using a conventional Seca scale with a precision of 100 g, and height was measured with a Harpenden stadiometer. Standard deviation scores (SDS) were calculated for height, weight, and BMI using current NCHS standard curves (26).

The protocol was approved by the institutional review board of the San Borja Arriarán Hospital. All parents signed informed consents, the patients verbally assented to participate in the study and those older than 15 yr old signed an assent form.

Hormone assays

Blood samples were obtained between 8:00 and 9:30 hours after an overnight fast. After centrifugation, serum was separated and stored at $-20^{\circ}C$ until

hormone determination. Total testosterone (TT), SHBG, inhibin B, AMH, and gonadotropin levels were measured. Calculated free testosterone (cfT) was estimated from testosterone, SHBG, and albumin, estimated as 4 g/L, as previously described (27). Free androgen index (FAI) was calculated as previously reported (27).

To correct for the variability reported for the testosterone assay, serum testosterone levels was measured with two different assays. The average of both assays is reported and the results obtained individually with each assay are shown in the Supporting Information Table S1. The correlation of both assays was $r = 0.93$ ($p < 0.0001$). One of the assays was a competitive-specific binding RIA for testosterone (Diasource, Nivelles, Belgium) that has a sensitivity of 10 ng/dL (0.347 nmol/L) and intra- and interassay coefficients of variation (CVs) of 5.1 and 6.4%, respectively. The second testosterone assay was an electrochemilumeniscent immunoassay (ECLIA, Testosterone II immunoassay ref. 05200067, Roche Diagnostics GmbH, Mannheim, Germany) that uses a Cobas e411 analyzer, as previously described (28). The limit of detection of the assay was 10 ng/dL. Intra- and interassay CVs were 2.4 and 2.6%, respectively.

LH and FSH were determined in serum by ECLIA (Roche Diagnostics GmbH and Mannheim), as previously described (28). HbA1c levels were measured using a commercially available automatic system (DCA 2000, Bayer Diagnostics, Tarrytown, NY, USA). SHBG levels were measured by immunoradiometric assays from Diasource; intra- and interassay CVs were 3.9 and 6.9%, respectively.

AMH was determined using an ultrasensitive enzyme-linked immunoassay (ELISA) specific for human AMH (EIA AMH/MIS®, Immunotech, Beckman-Coulter Co., Marseilles, France, ref. A11893), as previously described (29). Intra- and interassay CVs were 10.5 and 9.4%, respectively. Inhibin B was measured by ELISA (Inhibin B Gen II ELISA Beckman Coulter®, Inc., Brea, CA, USA). Assay sensitivity was 10 pg/mL, and the intra- and interassay CVs were 4.0 and 5.6%, respectively.

Statistical analysis

Sample size was calculated based on the testosterone levels observed in young adults with T1D that showed higher testosterone levels (14), which show that in order to reach a significance level of 5% and a power of 90%, the smallest appropriate sample size was 16 subjects in each group. Normal distributions were evaluated using the Shapiro–Wilk test. Only inhibin B was normally distributed. The distribution of Tanner stages between the two groups was assessed using Pearson X^2. Continuous variables were compared using Mann–Whitney's U test, except for inhibin B, which was analyzed using Student's t-test. Differences in the prevalence of hypogonadism were assessed with the X^2 test.

An analysis of covariance (ANCOVA) was used to determine the effect of T1D on androgens and SHBG levels adjusted by pubertal stage (model 1). Regression analysis was used to evaluate the effect of T1D, adjusted by age, and BMI-SDS (model 2), on hormonal levels. Pubertal stage and age cannot be assessed in the same model because they have colinearity. β and standard error of β determined from the regression analysis is reported. The evaluation of normality of the residuals performed using a q–q plot showed that the residuals were normally distributed. The same regression analyses were performed in adolescents who had finished their pubertal development (late puberty) to eliminate pubertal development as a variable. Regression analysis was performed in T1D boys to evaluate the effect of HbA1c level, BMI-SDS, and insulin dose on androgens and SHBG levels.

Results are expressed as mean ± SEM. All statistic calculations were run on SPSS for Windows (version 19.0, SPSS, Inc., Chicago, IL, USA). A significance level of 5% was employed.

Results

The clinical and anthropometric characteristics of T1D ($n = 71$) and Control group ($n = 104$) boys and the degree of metabolic control and daily insulin dose of the patients with T1D are shown for the whole group in Table 1 and according to pubertal development in Table 2. Age, pubertal stage, and BMI-SDS were similar in both groups; the only significant difference was a younger age in T1D than in Control group during intermediate puberty. One T1D and one healthy boy were excluded from the study due to the presence of precocious puberty. No case of delayed puberty was detected.

Table 1. Anthropometrics, pubertal development, and metabolic control of boys with T1D

	T1D N = 71	C N = 104
Age (yr)	13.9 ± 0.2	14.0 ± 0.1
Pubertal development		
Early puberty (Tanner 2)	N = 16	N = 16
Intermediate puberty (Tanner 3–4)	N = 25	N = 58
Late puberty (Tanner 5)	N = 30	N = 30
Height (SDS)	−0.1 ± 0.1	0.1 ± 0.1
BMI-SDS	0.4 ± 0.1	0.5 ± 0.1
T1D duration (yr)	4.9 ± 0.3	
Age of onset of T1D (yr)	9.3 ± 0.3	
Insulin dose (U/Kg/d)	1.1 ± 0.04	
HbA1c (%)	8.3 ± 0.1	

Table 2. Testicular volume and hormonal profile in boys with T1D and a healthy control group

	Early puberty		Intermediate puberty		Late puberty	
	T1D (n = 16)	C (n = 16)	T1D (n = 25)	C (n = 58)	T1D (n = 30)	C (n = 30)
Age (yr)	11.7 ± 0.3	11.9 ± 0.23	13.4 ± 0.33	14.0 ± 0.16*	15.7 ± 0.2	15.2 ± 0.28
Testicular volume (mL) [Mode (range)]	5 (4–8)	5 (4–8)	15 (10–18)	15 (10–18)	25 (20–25)	20 (20–25)
HbA1c (%)	8.4 ± 0.3		8.2 ± 0.3		8.3 ± 0.2	
TT (ng/dL)	67.7 ± 16	53.6 ± 20	400 ± 31	339 ± 23	**565 ± 28**†	**439 ± 25**
cfT (pmol/L)	22 ± 4.9	25.6 ± 10	262 ± 30	232 ± 17	**454 ± 25**‡	**340 ± 18**
FAI	2.8 ± 0.6	3.7 ± 1.4	38.7 ± 5.2	34 ± 2.4	**69.1 ± 3.9**§	**53.1 ± 2.7**
SHBG (nmol/L)	**89.9 ± 9.7**¶	**53.2 ± 6.4**	45.9 ± 4.4	32.6 ± 1.5	30.7 ± 2.1	29.4 ± 1.5
AMH (pmol/L)	284.1 ± 41.3	335.9 ± 71.1	80.2 ± 9.3	96.3 ± 12.0	103.5 ± 9.5	93.2 ± 10.3
Inhibin B (ng/L)	**197 ± 38**‖	**258 ± 28**	212 ± 11	202 ± 7.7	256 ± 11	237 ± 12
LH (IU/L)	1.3 ± 0.3	1.0 ± 0.2	3.3 ± 0.5	2.6 ± 0.1	4.5 ± 0.3	3.9 ± 0.3
FSH (IU/L)	2.1 ± 0.2	2.2 ± 0.2	2.9 ± 0.2	3.4 ± 0.2	3.1 ± 0.3	3.0 ± 0.3

Data are shown as the mean ± SEM. Bold values highlight that a significant difference exists.
*p = 0.042 T1D vs. C.
†p = 0.004 T1D vs. C.
‡p = 0.001 T1D vs. C.
§p = 0.002 T1D vs. C.
¶p = 0.003 T1D vs. C.
‖p = 0.02 T1D vs. C.

Similar testicular volume was observed in the T1D and Control group (Table 2). Metabolic control was similar in T1D boys in different pubertal stages. Testicular function during pubertal development showed a different hormonal profile in boys with T1D at the beginning and at the end of puberty. Boys with T1D in early puberty had higher levels of SHBG ($p = 0.003$), lower inhibin B levels ($p = 0.02$) and similar androgen levels compared to Control group in the same stage of pubertal development. During intermediate puberty, similar hormonal levels were observed in T1D and Control group. At the end of puberty, higher TT, FAI, and cfT levels were observed in the boys with T1D compared to Control group in the same pubertal stages ($p = 0.004$, $p < 0.002$, and $p < 0.001$, respectively). Similar results were observed with both testosterone assay (Table S1), except for elevated testosterone levels in T1D boys compared with Control group in intermediate puberty was observed with assay 2 (ECLIA), which was not observed with the average testosterone level or assay 1 (Table S1). Gonadotropins and AMH were similar in both groups for all the stages of puberty.

The results of the regression analysis are shown in Table 3. T1D was a significant factor, even after adjusting for Tanner stage and BMI-DS, affecting TT, cfT, and SHBG levels (models 1 and 2). A significant interaction between T1D and pubertal stage was observed for testosterone, cfT, and SHBG levels. Age, BMI-SDS, and pubertal stage were significant factors for predicting androgens and SHBG levels. In boys at the end of pubertal development, T1D was a significant factor for TT and cfT ($p = 0.005$ and $p = 0.001$, respectively) but not SHBG levels.

The regression analysis of the factors affecting androgen and SHBG levels in T1D boys showed that higher HbA1c had a negative effect on TT and cfT and a positive effect on SHBG levels in T1D boys (model 3). Insulin dose did not exhibit any association with hormonal levels. In T1D boys, BMI-SDS had a negative association with testosterone and SHBG levels.

Discussion

We report the first study of testicular, including Leydig and Sertoli cell function in a rather large group of boys with T1D compared to a carefully matched control group. We observed no evidence of hypogonadism during puberty in this study of tubular and interstitial testicular function in 71 pubertal boys with T1D treated with multiple daily insulin doses compared with a control group matched by Tanner stage and BMI-SDS. Subtle differences were observed in the hormonal profile in T1D boys at the beginning and at the end of puberty. In addition, we found that androgen levels in T1D are negatively affected by worse metabolic control and BMI during pubertal development.

The finding of normal gonadotropin, testosterone, AMH, and inhibin B levels during pubertal development in T1D patients suggests that in boys receiving multiple daily insulin doses, hypogonadism does not occur frequently. These data differ from data of men treated with conventional treatment (6) or in cases of poor control (30). Similarly, in women, hypogonadism has also become less prevalent than was reported in the 1980s (3).

We observed that at the beginning of puberty, T1D boys had elevated SHBG levels in the presence of

Table 3. Regression analysis

	Total testosterone			Calculated free testosterone			SHBG		
	β	SEβ	p	β	SEβ	p	β	SEβ	p
Model 1: All children									
T1D	57.8	22.9	0.013	46.4	17.7	**0.009**	13.4	2.9	**<0.0001**
Pubertal stage	139	10.1	**<0.0001**	122.3	7.8	**<0.0001**	−12.2	1.3	**<0.0001**
Interaction T1D/Tanner			**<0.0001**			**0.008**			**<0.0001**
Model 2: All children									
T1D	64.3	23.0	0.006	55.2	17.9	**0.002**	11.2	2.9	**<0.0001**
Age	77.1	6.0	**<0.0001**	78.1	5.5	**<0.0001**	−6.9	0.8	**<0.0001**
BMI-SDS	−40.8	12.6	0.002			ns	−8.3	1.6	**<0.0001**
Model 2: Tanner 5 boys only									
T1D	92.9	31.7	**0.005**	88.5	26.4	**0.001**			ns
Age	50.4	10.3	**<0.0001**	46.5	8.6	**<0.0001**			ns
BMI-SDS			ns			ns	−6.8	1.2	**<0.0001**
Model 3: T1D boys									
Pubertal development	158.1	13.8	**<0.0001**	141.2	11.8	**<0.0001**	−17.8	2.2	**<0.0001**
HbA1c	−24.7	10.4	**0.02**	−26.8	8.9	**0.004**	4.5	1.7	**0.01**
Insulin dose			ns			ns			ns
BMI-SDS	−56.8	18.1	0.003			ns	−6.9	2.9	**0.02**

SE β, standard error of β.
Bold values highlight that a significant difference exists. Model 1 shows the effect of T1D and pubertal stage on androgen and SHBG levels. Model 2 shows the effect of T1D, age, and BMI-SDS on androgen and SHBG levels. Model 3 shows the effect of pubertal stage, HbA1c, insulin dose, and BMI-SDS on androgen and SHBG levels. Standardized β is shown.

similar testosterone, AMH, and gonadotropin levels. The higher SHBG levels observed at the beginning of puberty in T1D boys may decrease the exposure of target tissues to androgens by decreasing the available free fraction of steroids (31); furthermore, these levels may have a role in the pathogenesis of the recently reported delay of onset of virilization of genitalia in boys with T1D (1). Elevated SHBG levels at the beginning of puberty have also been previously reported in pubertal boys and girls with T1D (12, 32).

In different studies of adult males with T1D, the results of reported androgen levels are inconsistent, with some showing elevated levels (7, 8, 10, 14) and others showing normal TT with decreased cfT (6, 9–11). A small sample size may explain these inconsistencies. Our present study that included 30 adolescents at late pubertal stages confirms preliminary data obtained in a small sample of adolescents, which indicated that androgen levels are elevated at the end of puberty in boys with T1D (13). The higher TT, FAI, and cfT observed in our T1D boys during late puberty may explain the catch-up of pubertal events reported in boys with T1D (1). Elevated testosterone levels have been associated with retinopathy in adult men with T1D (33) and with nephropathy in adolescents (34), and the elevated androgens that we observed at the end of puberty in boys with T1D occurs at a time when chronic complications are exacerbated.

A role of intensive insulin treatment on the elevated testosterone levels has been postulated. Previously, Christensen et al. (8) showed that after intensive insulin treatment and improved glycemic control, elevations of free testosterone, and SHBG were observed in T1D young men with insufficient metabolic control receiving intensive insulin therapy. In women with T1D, O'Hare et al. (35) also showed that improvement of metabolic control lead to elevations in testosterone levels. Previously, hyperandrogenism in women with T1D has been postulated to be secondary to non-physiologic hyperinsulinemia associated with exogenous insulin treatment, which stimulates androgen secretion by the ovary (36–38). Insulin and IGF-1 receptors are expressed in Leydig cells (39, 40), and the hyperinsulinemia usually observed in T1D (41) could lead to an elevated secretion of testosterone by Leydig cells, similar to what has been reported in women. Insulin has been shown to have a stimulatory effect on testosterone secretion by the testis in adult men (42), and decreasing insulin levels with diazoxide showed an opposite effect (43).

Other possible mechanisms of hyperandrogenism in T1D males can also be postulated. Decreased aromatization is an alternative hypothesis to explain the higher testosterone levels that were observed in T1D patients, but this mechanism has not been studied in T1D. Hypothalamic and pituitary function may be affected by T1D (3). LH dynamics has been shown to be

affected in hyperinsulinemic women who have insulin resistance, and they are hypergonadotropic and have a relative resistance of their pituitary responsiveness to insulin (44). However, the design of the study did not allow the assessment of overnight gonadotropins, which would have allowed more convincing statements on neuroendocrine secretory dynamics.

We observed a negative association of BMI, HbA1c, and testosterone/cFT levels, which demonstrated that androgen levels decrease when metabolic control deteriorates and increasing BMI. The hyperglycemia and insulin deficiency that occurs in patients with insufficient metabolic control have a negative effect on gonadotropin secretion (3, 45). Insulin deficiency leads to diminished expression of kisspeptin1, a neuropeptide that has a key role in stimulating the secretion of hypothalamic GNRH (45–48).

The negative association of BMI with testosterone levels is similar to what has been reported in adults with T1D, with T2D, or obesity (9, 49–52). We observed a lack of association of BMI with androgen levels in the Control group, which has also been reported in other studies of lean and obese non-diabetic adolescents (53). The fact that we included only non-obese healthy adolescents limits the generalization of this lack of effect of BMI on testosterone levels.

The normal levels of AMH and inhibin B levels together with the normal testicular volume progression observed in T1D boys during middle and late puberty indicate that the function of the seminiferous tubules, including Sertoli and germ cells, is not affected during this period of life. Normal function of the tubular compartment persists in uncomplicated young adult males with T1D receiving multiple daily insulin doses, as shown by the existence of normal inhibin B levels and normal semen analysis (14, 54, 55). Conversely, sperm abnormalities have been described in older subjects with T1D (56, 57).

Low levels of inhibin B observed in early pubertal T1D patients may reflect an altered endocrine testicular milieu that affects the normal inhibin B production by the Sertoli cell. Even though it is widely accepted that FSH is the main regulatory factor of Sertoli cell inhibin production (58), the serum inhibin B determined in normal boys from birth to puberty does not follow the FSH pattern. During the post natal period and childhood normal adult levels of inhibin B are detected in circulation associated with FSH levels in the prepubertal range (59). It has been proposed that in this period of life the Sertoli cell is able to synthesize inhibin B independently of the gonadotrophic stimulus and its production may be mainly driven by gonadal factors (60). After the onset of puberty, normal inhibin B production requires FSH and germ cell stimulatory factors (61). Low levels of inhibin B observed in early pubertal T1D patients may reflect an altered endocrine testicular milieu that affects the normal inhibin B production by the Sertoli cell. As soon as the seminiferous epithelium develops and provides the required stimulatory factors in the presence of FSH, inhibin B production is recovered and reaches the pubertal normal range.

This study has the strength of the simultaneous evaluation of a large group of T1D and healthy boys matched by pubertal development with a complete evaluation of gonadal function. However, some limitations must be considered. The sample was obtained at 8:00–9:00 hours, which may correspond to a late time of the day for evaluating testosterone levels in early puberty, which may limit the conclusions for that subgroup of the study.

Collectively, these results show that adolescents with T1D treated with multiple daily doses do not exhibit hypogonadism, as shown by normal gonadotropin, testosterone, inhibin B, and AMH levels. However, differences in the endocrine profile may explain the previously reported pattern of delayed onset of pubertal development and later catch-up with a normal age of completion of puberty (1).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Androgens levels in boys with T1D and a healthy control group measured with two different testosterone assays

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