

# Salivary testosterone concentrations in pubertal ICSI boys compared with spontaneously conceived boys

F. Belva<sup>1,\*</sup>, M. Bonduelle<sup>1</sup>, J. Schiettecatte<sup>2</sup>, H. Tournaye<sup>3</sup>, R.C. Painter<sup>4</sup>, P. Devroey<sup>3</sup>, and J. De Schepper<sup>5</sup>

<sup>1</sup>Center for Medical Genetics, UZ Brussel, Laarbeeklaan 101, 1090 Brussels, Belgium <sup>2</sup>Radioimmunology Laboratory, UZ Brussel, Brussels, Belgium <sup>3</sup>Center for Reproductive Medicine, UZ Brussel, Brussels, Belgium <sup>4</sup>Department of Obstetrics and Gynecology, AMC, Amsterdam, The Netherlands <sup>5</sup>Department of Pediatric Endocrinology, UZ Brussel, Brussels, Belgium

\*Correspondence address: Tel: +32-2-477-60-71; Fax: +32-2-477-68-60; E-mail: florence.belva@uzbrussel.be

Submitted on September 27, 2010; resubmitted on October 18, 2010; accepted on October 22, 2010

**BACKGROUND:** To date, no data exist about Leydig cell function of pubertal boys born after ICSI. To evaluate a potential risk of gonadal dysfunction in children born from fathers with compromised fertility, testicular function was assessed by the measurement of salivary testosterone.

**METHODS:** Morning salivary testosterone levels at the age of 14 years were compared between 58 ICSI teenagers who are part of the oldest ICSI cohort, and 62 boys born after spontaneous conception (SC).

**RESULTS:** Salivary testosterone levels were comparable between ICSI ( $113 \pm 42$  pg/ml) and SC ( $123 \pm 56$  pg/ml) teenagers at the age of 14 years. In the ICSI group, testosterone levels in boys from fathers with severe oligozoospermia were not different from concentrations in boys from fathers without severe oligozoospermia ( $115.5 \pm 43$  and  $109 \pm 41$  pg/ml, respectively).

**CONCLUSIONS:** At the age of 14 years, pubertal ICSI boys show testosterone levels comparable to their peers born after SC. ICSI adolescents fathered from men with severely compromised spermatogenesis show testosterone levels comparable to those from fathers with normal spermatogenesis. This notwithstanding, further follow-up of ICSI teenagers into adulthood is mandatory to confirm a normal gonadal function.

**Key words:** ICSI / testosterone / pubertal / Leydig cell / children

## Introduction

ICSI is an effective treatment option for couples facing severe male factor infertility (Palermo *et al.*, 1992; Van Steirteghem *et al.*, 1996). However, little is known about the reproductive health of male offspring after ICSI. Since familial clustering of male infertility is reported, ICSI boys may be at risk for inheriting impaired testicular function from their fathers (Lilford *et al.*, 1994; Meschede *et al.*, 2000). Previously, a higher incidence of genital malformations has been reported in ICSI children (Wennerholm *et al.*, 2000; Kallen *et al.*, 2005). In addition, reduced serum testosterone levels have been described in 3-month-old ICSI boys (Mau Kai *et al.*, 2007), but no data exist on the Leydig cell function of ICSI-conceived boys beyond childhood.

Therefore, in this prospective study, we compared salivary testosterone levels of ICSI adolescents with those of peers born after spontaneous conception (SC). We hypothesized that ICSI-conceived boys from fathers with compromised spermatogenesis are at higher risk for

Leydig cell dysfunction, since spermatogenic dysfunction in adults is reported to be associated with impaired Leydig cell function and lower testosterone levels (Andersson *et al.*, 2004).

## Materials and Methods

### Subjects

We analysed data of boys born after ICSI and after SC, all included in a prospective cohort study on cardiometabolic outcome, reaching the age of 14 years between January 2008 and April 2010. From 58 ICSI boys, both hormonal and clinical data (body height, body weight, BMI and Tanner pubertal stage) were available. All included boys were singletons, Caucasian and born after at least 32 weeks' gestation. In two ICSI boys (3.4%), surgery was performed for major genital anomalies: hypospadias and bilateral cryptorchid testes. All tested boys ( $n = 54$ ) had a normal karyotype. Levels of serum inhibin B, (a marker of Sertoli cell function) were available in 51 of the 58 included ICSI boys (Belva *et al.*, 2010). ICSI was

performed at the Center for Reproductive Medicine of the UZ Brussel. Father's spermatogram ( $n = 58$ ) (according to WHO guidelines 1999) showed the following: mean (and ranges) of the analysed volume was 3.8 (0.5–8.0) ml with a mean sperm concentration of  $7.1 \times 10^6$  ( $0.01 \times 10^6$ – $52 \times 10^6$ )/ml and a mean motility count of 57 (0–100)%. In 38 fathers (65.5%), sperm count was below  $5 \times 10^6$ /ml, consistent with severe oligozoospermia. All tested fathers ( $n = 51$ ) had a normal karyotype.

Children born after SC serving as controls were recruited cross sectionally from schools in the surrounding areas. In order to be eligible, boys had to be singleton, Caucasian and born after 32 weeks of gestation. Children born after ovarian stimulation of the mother were excluded. From 62 boys born after SC, clinical and hormonal data were available. In two boys born after SC (3.2%), surgery was performed for major genital anomalies: unilateral cryptorchid testis and a chordae penis.

## Methods

Saliva samples were collected at home between 6 and 10 a.m., in a commercial salivette (Sarstedt), with an insert containing a sterile polyester swab for collection of the saliva, yielding a clear and particle-free sample. The salivettes were used according to the instructions provided by the manufacturer. Samples collected in this way are stable at room temperature for at least 1 week. Salivettes containing saliva were centrifuged at 2000g for 10 min, and the filtrates were frozen ( $-20^\circ\text{C}$ ). Before analysis, the samples were thawed and mixed.

Salivary testosterone was measured using a modified commercially available radioimmunoassay (Orion Diagnostica, Finland). Briefly, standards are prediluted (factor 10) in Tris–buffer 0.1 M (pH 7.0) and 25  $\mu\text{l}$  diluted standard or saliva were pipetted into the coated tube and incubated with  $^{125}\text{I}$ -labelled testosterone for 60 min at  $37^\circ\text{C}$ . The measuring range of the modified testosterone assay ranged from 15 to 1400 pg/ml, the analytical sensitivity is 20 pg/ml and the within- and between-run coefficients of variation were  $<10$  and  $<12\%$ , respectively. The linearity of the modified Orion testosterone RIA kit was evaluated by diluting a saliva sample in Tris–HCl buffer with 0.1% bovine serum albumin. The dilution showed a linear relationship between dilution factor and testosterone concentration with a high correlation coefficient ( $R^2 = 0.992$ ), showing no matrix effect using saliva in the modified testosterone Orion RIA assay.

## Statistical analysis

Data are expressed as mean (SD) or median. Ranges or 5–95th percentiles are given where appropriate. Body weight, body height, BMI and birthweight are expressed as Standard Deviation Scores (SDS), i.e. corrected for age and gender. Statistical analyses included Student's *t*-test and ANOVA for comparisons and Spearman's rank order test (correlation

coefficient: *r*) for correlation analysis. Multiple linear regression was used to assess associations between mode of conception and salivary testosterone, after adjustment for potential confounders (birthweight, age, BMI and pubertal stage at examination). Differences between groups were analysed using Fisher's exact test for dichotomous variables. All analyses were performed using SPSS 16.0. A difference was considered significant when the two-tailed *P* values are  $< 0.05$ .

## Results

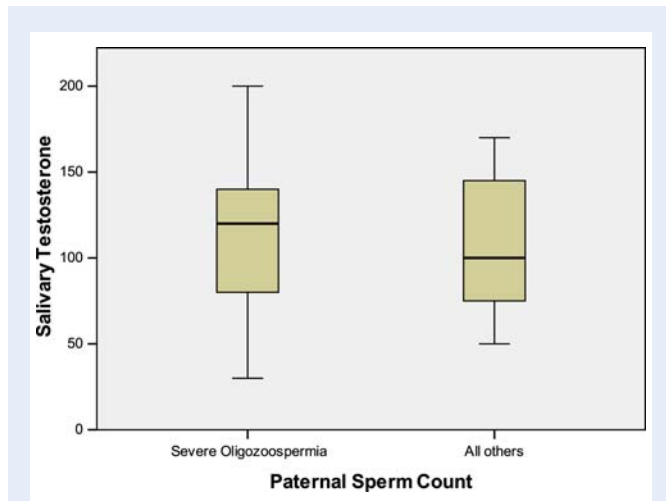
Spontaneously conceived boys were, on average, 2 months older than ICSI boys ( $14.3 \pm 0.4$  versus  $14.1 \pm 0.4$  years) ( $P < 0.001$ ). Birthweight SDS was comparable between the two groups ( $P = 0.3$ ). Low birthweight rate (defined as birthweight  $< 2500$  g) did not differ between the two groups (ICSI: 8.6%; SC: 3.3%) ( $P = 0.3$ ). Whereas body weight SDS, BMI SDS and pubertal staging were comparable between ICSI and SC children ( $P = 0.07$ ,  $P = 0.5$  and  $P = 0.17$ , respectively), body height SDS was significantly lower in ICSI compared with controls ( $0.4 \pm 1.1$  versus  $0.9 \pm 0.8$ ) ( $P = 0.004$ ).

Mean salivary testosterone did not differ between ICSI boys ( $113 \pm 42$  pg/ml) and SC boys ( $123 \pm 56$  pg/ml) ( $P = 0.3$ ). This result remained unaltered after correction for confounding characteristics (birthweight, age, BMI and pubertal stage at examination) ( $P = 0.9$ ). In ICSI boys, salivary testosterone correlated positively with weight SDS ( $r: 0.3$ ;  $P = 0.04$ ) and with age ( $r: 0.3$ ;  $P = 0.03$ ), but this positive correlation was not statistically significant in the SC group (weight SDS:  $r: 0.2$ ,  $P = 0.1$ ; age:  $r: 0.1$ ,  $P = 0.3$ ). In 1 out of the 58 of the ICSI adolescents (2%), salivary testosterone levels were below the 2.5th percentile (40 pg/ml) of levels described in the SC group. Salivary testosterone levels stratified according to pubertal stage are reported in Table 1. In the ICSI group, mean salivary testosterone increased across the four pubertal subgroups ( $P = 0.02$ ). Although a similar increase of salivary testosterone across the four pubertal stages was found in the controls, it did not reach statistical significance ( $P = 0.1$ ).

Serum inhibin B levels did not correlate with salivary testosterone levels at the age of 14 years ( $P = 0.7$ ). From two ICSI boys with below-normal serum inhibin B levels at 14 years (Belva *et al.*, 2010), salivary testosterone levels were available. In one boy, the salivary testosterone level (190 pg/ml) was above the mean; in the other boy, the salivary testosterone level (50 pg/ml) was far below the mean. Salivary testosterone levels were normal in the two ICSI boys with major genital abnormalities (150 pg/ml) as well as in the two SC boys with major genital abnormalities (90 and 140 pg/ml).

**Table 1** Salivary testosterone levels (pg/ml) stratified by pubertal stage in 14-year-old ICSI and spontaneously conceived (SC) boys.

|                               | ICSI ( $n = 58$ ) |        |        |        | SC ( $n = 62$ ) |        |        |        |
|-------------------------------|-------------------|--------|--------|--------|-----------------|--------|--------|--------|
|                               | G2                | G3     | G4     | G5     | G2              | G3     | G4     | G5     |
| Salivary testosterone (pg/ml) |                   |        |        |        |                 |        |        |        |
| Mean                          | 67                | 100    | 126    | 135    | 40              | 107    | 126    | 149    |
| Median                        | 50                | 95     | 140    | 130    |                 | 95     | 120    | 135    |
| Min.–max.                     | 50–100            | 30–190 | 50–190 | 90–200 |                 | 50–200 | 50–300 | 60–340 |
| <i>n</i>                      | 3                 | 24     | 25     | 6      | 1               | 16     | 37     | 8      |



**Figure 1** Mean salivary testosterone levels (pg/ml) in ICSI boys from fathers with ( $n = 38$ ) and without ( $n = 20$ ) severe oligozoospermia.

At 14 years, testosterone levels in the ICSI boys did not correlate with paternal sperm parameters (concentration:  $P = 0.9$ ; motility:  $P = 0.5$ ). Boys of men with severe oligozoospermia ( $n = 38$ ) had testosterone levels comparable to those born from men with sperm concentrations above  $5 \times 10^6/\text{ml}$  ( $115.5 \pm 43$  versus  $109 \pm 41$  pg/ml) ( $P = 0.6$ ; Fig. 1).

## Discussion

In the present study, Leydig cell function of pubertal boys conceived after ICSI has been investigated for the first time. We found similar salivary testosterone concentrations in a group of 58 pubertal boys aged 14 years born after ICSI compared with boys born after SC.

Mau Kai et al. (2007) reported that, at the age of 3 months, serum levels of testosterone were significantly lower in 72 boys conceived by ICSI compared with boys born after SC. The validity of serum total testosterone levels during the first 3 months ('mini-puberty') can be questioned since at that age, not only the precise timing of the hormonal peak is unknown (Forest et al., 1973, 1976; Grumbach, 2005) but also serum total testosterone levels are influenced by perinatal factors (Pierik et al., 2008).

Measurement of morning serum testosterone in combination with serum SHBG and LH concentration is the most sensitive way of testing Leydig cell function; however, there are practical issues in obtaining samples. In order to overcome these practical matters (different settings for clinical testing and at different time periods of the day) and to assure maximal participation, we decided to measure morning testosterone concentration in saliva samples taken at home. The measurement of salivary testosterone, which reflects the free fraction of testosterone in circulation, can be used as an index of serum testosterone due to their strong correlation (Sannikka et al., 1983; Vittek et al., 1985; Rilling et al., 1996). In addition, since a nocturnal rise of testosterone during early and mid-puberty is well established (Wu et al., 1989), peak salivary testosterone concentrations were investigated by performing the at home sampling in the early morning in both the ICSI and the control group.

Although data on salivary testosterone are difficult to compare, due to racial diversity (Rilling et al., 1996), different (pre-pubertal) focus group (Granger et al., 1999), stratification by age or self-reported pubertal developmental scales (Granger et al., 2004) or inconsistency between applied measurement procedures (Albrecht and Styne, 2007), the salivary testosterone values observed in the ICSI as well as in the SC boys in our study are in line with the previous reports (Matchock et al., 2007). Salivary testosterone values during puberty have to be interpreted in relation to the pubertal stage. We also observed, in accordance with other published data (Albrecht and Styne, 2007), that the steepest increase in testosterone occurs between pubertal Tanner Stages 2 and 3. The significant increase in salivary testosterone levels across the pubertal stages observed in the ICSI children is an additional observation in favour of a normal Leydig cell function observed in the studied cohort.

It is well established that children born after ICSI are at risk for low birthweight (Jackson et al., 2004; McDonald et al., 2009) and genital malformations such as hypospadias and cryptorchidism (Wennerholm et al., 2000; Kallen et al., 2005) and hence could be at risk for impaired spermatogenesis later in life. We did not find an increased low birthweight rate or higher frequency of hypospadias/cryptorchidism in ICSI boys in the current study, probably due to our small sample size. Interestingly, salivary testosterone values in those small subgroups were normal (data not shown).

To date, limited data exist on the Leydig cell and Sertoli cell function in pubertal boys conceived after assisted reproductive technology. In adult men whose mothers had received hormonal stimulation, a lower sperm count and concentration, fewer motile and morphologically normal spermatozoa and lower serum testosterone levels have been found (Jensen et al., 2007). Furthermore, an association between impaired spermatogenesis and impaired Leydig cell function has been reported in adult men with idiopathic oligozoospermia (de Kretser et al., 1972; Glass and Vigersky, 1980; Giagulli and Vermeulen, 1988; Andersson et al., 2004). The finding that ICSI-conceived boys from fathers with severe oligozoospermia had salivary testosterone concentrations comparable to those born from men with sperm concentrations above  $5 \times 10^6/\text{ml}$  is thus reassuring.

Taken together, since we found both a normal Sertoli cell function (Belva et al., 2010) and normal Leydig cell function in the majority of ICSI-conceived boys, we suspect, on the basis of these preliminary findings, that ICSI offspring may display a normal seminal testicular function in adulthood. However, before definite conclusions can be drawn, our results need to be reproduced in larger cohorts.

In conclusion, no difference in salivary testosterone levels was found between ICSI and SC boys at the age of 14 years. No lower testosterone levels were documented in boys with genital malformations or in boys from fathers with severely compromised spermatogenesis. Further follow-up of the spermatogenic potential of ICSI teenagers is mandatory to confirm the perspective of a normal reproductive capacity.

## Authors' roles

All authors qualify for authorship by having contributed substantially to this work, as specified by criteria (a), (b) and (c) of the Uniform Requirements for Manuscripts Submitted to *Biomedical Journals*. All

authors have reviewed the final version of the manuscript and approved it for publication.

## Funding

This study was supported by research grants from Fonds voor Wetenschappelijk Onderzoek Vlaanderen and Onderzoeksraad Vrije Universiteit Brussel.

## References

- Albrecht L, Styne D. Laboratory testing of gonadal steroids in children. *Pediatr Endocrinol Rev* 2007;**5**(suppl 1):599–607.
- Andersson AM, Jørgensen N, Frydelund-Larsen L, Rajpert-de Meyts E, Skakkebaek NE. Impaired Leydig cell function in infertile men: a study of 357 idiopathic infertile men and 318 proven fertile controls. *J Clin Endocrinol Metab* 2004;**89**:3161–6167.
- Belva F, Bonduelle M, Painter RC, Schiettecatte J, Devroey P, De Schepper J. Serum inhibin B concentrations in pubertal boys conceived by ICSI: first results. *Hum Reprod* 2010; Sep 16. Epub ahead of print.
- de Kretser DM, Burger HG, Fortune D, Hudson B, Long AR, Paulsen CA, Taft HP. Hormonal, histological and chromosomal studies in adult males with testicular disorders. *J Clin Endocrinol Metab* 1972;**35**:392–401.
- Forest MG, Cathiard AM, Bertrand JA. Evidence of testicular activity in early infancy. *J Clin Endocrinol Metab* 1973;**37**:148–151.
- Forest MG, de Peretti E, Bertrand JA. Hypothalamic-pituitary-gonadal relationships in man from birth to puberty. *Clin Endocrinol (Oxf)* 1976;**5**:551–569.
- Giagulli VA, Vermeulen A. Leydig cell function in infertile men with idiopathic oligospermic infertility. *J Clin Endocrinol Metab* 1988;**66**:62–67.
- Glass AR, Vigersky RA. Leydig cell function in idiopathic oligospermia. *Fertil Steril* 1980;**34**:144–148.
- Granger DA, Schwartz EB, Booth A, Arentz M. Salivary testosterone determination in studies of child health and development. *Horm Behav* 1999;**35**:18–27.
- Granger DA, Shirtcliff EA, Booth A, Kivlighan KT, Schwartz EB. The 'trouble' with salivary testosterone. *Psychoneuroendocrinology* 2004;**29**:1229–1240.
- Grumbach MM. A window of opportunity: the diagnosis of gonadotropin deficiency in the male infant. *J Clin Endocrinol Metab* 2005;**90**:3122–3127.
- Jackson RA, Gibson KA, Wu YW, Croughan MS. Perinatal outcomes in singletons following *in vitro* fertilization: a meta-analysis. *Obstet Gynecol* 2004;**103**:551–563.
- Jensen TK, Jørgensen N, Askund C, Carlsen E, Holm M, Skakkebaek NE. Fertility treatment and reproductive health of male offspring: a study of 19 325 young men from the general population. *Am J Epidemiol* 2007;**165**:583–590.
- Kallen B, Finnstrom O, Nygren KG, Olausson PO. *In vitro* fertilization (IVF) in Sweden: risk for congenital malformations after different IVF methods. *Birth Defects Res A Clin Mol Teratol* 2005;**73**:162–169.
- Lilford R, Jones AM, Bishop DT, Thornton J, Mueller R. Case-control study of whether subfertility in men is familial. *Br Med J* 1994;**309**:570–573.
- Matchock RL, Dorn LD, Susman EJ. Diurnal and seasonal cortisol, testosterone and DHEA rhythms in boys and girls during puberty. *Chronobiol Int* 2007;**24**:969–990.
- Mau Kai C, Main KM, Andersen AN, Loft A, Skakkebaek NE, Juul A. Reduced serum testosterone levels in infant boys conceived by intracytoplasmic sperm injection. *J Clin Endocrinol Metab* 2007;**92**:2598–2603.
- McDonald SD, Han Z, Mulla S, Murphy KE, Beyene J, Ohlsson A; Knowledge synthesis group. Preterm birth and low birth weight among *in vitro* fertilization singletons: a systematic review and meta-analyses. *Eur J Obstet Gynecol Reprod Biol* 2009;**146**:138–148.
- Meschede D, Lemcke B, Behre HM, Geyter CD, Nieschlag E, Horst J. Non-reproductive heritable disorders in infertile couples and their First degree relatives. *Hum Reprod* 2000;**15**:1609–1612.
- Palermo G, Joris H, Devroey P, Van Steirteghem AC. Pregnancies after intracytoplasmic sperm injection of single spermatozoon into an oocyte. *Lancet* 1992;**340**:17–18.
- Pierik FH, Diddens JA, Burdorf A, de Muinck Keizer-Schrama SM, de Jong FH, Weber RA. The hypothalamus-pituitary-testis axis in boys during the first six months of life: a comparison of cryptorchidism and hypospadias cases with controls. *Int J Androl* 2008;**31**:1–9.
- Rilling JK, Worthman CM, Campbell BC, Stallings JF, Mbizva M. Ratios of plasma and salivary testosterone throughout puberty: production versus bioavailability. *Steroids* 1996;**61**:374–378.
- Sannikka E, Terho P, Suominen J, Santti R. Testosterone concentrations in human seminal plasma and saliva and its correlation with non-protein-bound and total testosterone levels in serum. *Int J Androl* 1983;**6**:319–330.
- Van Steirteghem A, Nagy P, Joris H, Verheyen G, Smits J, Camus M, Tournaye H, Ubaldi F, Bonduelle M, Silber S et al. The development of intracytoplasmic sperm injection. *Hum Reprod* 1996;**11**(suppl 1):59–72.
- Vitteck J, L'Hommedieu DG, Gordon GG, Rappaport SC, Southren AL. Direct radioimmunoassay of salivary testosterone: correlation with free and total serum testosterone. *Life Sci* 1985;**37**:711–716.
- Wennerholm UB, Bergh C, Hamberger L, Lundin K, Nilsson L, Wikland M, Kallen B. Incidence of congenital malformations in children born after ICSI. *Hum Reprod* 2000;**15**:944–948.
- WHO. *WHO Laboratory Manual for the Examination of Human Semen and Sperm-cervical Mucus Interaction*, 1999.
- Wu FCW, Borrow SM, Nicol K, Elton R, Hunter WM. Ontogeny of pulsatile gonadotropin secretion and pituitary responsiveness in male puberty in man: a mixed longitudinal and cross-sectional study. *J Endocrinol* 1989;**123**:347–359.