

Cryptorchidism and Semen Quality: A TEM and Molecular Study

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ABSTRACT: Cryptorchidism is a pathological condition defined as the failure of the testis to descend into the scrotum, the location of the cryptorchid testis can be in the inguinal canal or in the prescrotal and abdominal area, sometimes resulting in atrophic seminiferous tubules. The aim of this study was to analyze semen quality of men who underwent orchidopexy for unilateral or bilateral cryptorchidism during childhood. Semen quality was investigated by light microscopy to evaluate sperm concentration and motility. Sperm morphology was performed by transmission electron microscope (TEM), and the data were mathematically elaborated. The presence of Y microdeletions was investigated by polymerase chain reaction. The effect of cryptorchidism on meiosis was explored by fluorescence in situ hybridization (FISH). The incidence of azoospermia was higher in the group with bilateral compared with unilateral cryptorchidism, and semen parameters were better in the unilateral group. Sperm pathologies detected by TEM indicated a severe deterioration of

sperm quality in both groups. Necrosis and apoptosis appeared to be the most frequent pathologies, and their values reached statistical significance compared with those from fertile controls. The presence of chromosome Y microdeletions in patients with cryptorchidism and severe spermatogenic defects is controversial. No microdeletions were found in this study. FISH values indicated that the mean percentage of gonosome disomies and diploidies were generally out of normal range, indicating a severe disturbance of meiotic segregation. The effects induced by cryptorchidism resolved in childhood seem to include a spermatogenic impairment, leading to recommendation of detailed ultrastructural and chromosomal sperm analyses before undertaking assisted reproductive techniques.

Key words: Undescended testes, FISH, sperm quality, electron microscopy.

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Cryptorchidism is a congenital abnormality of the male genitalia that occurs in up to 2%–5% of newborn males, and at the age of 3 months the incidence spontaneously reduces to 1%–2% (Dohle et al, 2005). Physical examination reveals a nonpalpable testis in the scrotum. The most common location of the cryptorchid testis is in the inguinal canal (63%), followed by the ectopic (11%), the external ring (9%), and the intra-abdominal position (2%) (Gracia et al, 2000).

Cryptorchidism is a well-known cause of male factor infertility, depressing the spermatogenic process (Mieusset et al, 1997). The aetiology of cryptorchidism is multifactorial, involving endocrine regulation and several gene defects. Cryptorchidism is associated with impaired spermatogenesis (Dada et al, 2002; Dohle et al, 2005) and an increased incidence of testicular cancer (Holm et al, 2003). This impairment of spermatogenesis is more severe in patients with bilateral cryptorchidism

compared with unilateral cryptorchidism or retractile testes (Caroppo et al, 2005). The common explanation for low semen quality in infertile males with a history of testicular maldescent is irreversible damage of the tubular compartment despite orchidopexy (Toppari and Kaleva, 1999), probably related to the lack of appropriate or timely surgical correction. In recent years, a correlation between cryptorchidism and the molecular analysis of Y microdeletions has also been postulated (Dada et al, 2002; Ferlin et al, 2004).

The aim of this research was to evaluate the semen quality of selected men who underwent orchidopexy for unilateral or bilateral cryptorchidism during childhood. Semen samples were analyzed by light microscopy to evaluate sperm concentration and motility. Sperm morphology, generally observed by light microscopy, was performed by transmission electron microscope (TEM), which provides a more detailed evaluation of sperm alterations. The presence of Y microdeletion was established by polymerase chain reaction (PCR). Finally, the effect of cryptorchidism on meiotic chromosome segregation was investigated by fluorescence in situ hybridization (FISH) performed directly on sperm nuclei.

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Methods

Selection of Patients

Between January 1999 and February 2005, 44 patients (age 24–38 years) treated for cryptorchidism were recruited at the Regional Referral Center for Male Infertility. The patients were interviewed about their case histories, their reproductive problems, and their family background. They reported orchidopexy during childhood (range 1.5–9 years), also demonstrated by clinical records.

The location of the testis was in the inguinal canal (31/44: 70.5%), prescrotal position (9/44: 20.4%) and abdominal area (4/44: 9.1%). The cryptorchid testes were considered to be of normal size (35/44: 79.5%) and of normal consistency (34/44: 77.2%). Thirty-two of these patients were unilateral and 12 were bilateral cryptorchids. Two of 44 patients with unilateral cryptorchidism showed an altered karyotype and were excluded from the group. None of the patients were siblings, sons of first degree cousins, sons of second degree cousins, or sons of third degree cousins, and none presented any genetic sperm defects.

Microbiological investigations did not reveal any urogenital infections. None of the patients had ever received hormone therapy. Sexual development was evaluated in the selected group. The patients provided a blood sample for serum follicle-stimulating hormone (FSH), luteinizing hormone (LH), and testosterone analysis, measured with the use of commercial radioimmunoassays.

Semen Analysis

Light and Electron Microscopy—Semen samples of patients treated for cryptorchidism were collected by masturbation after 4 days of sexual abstinence and examined after liquefaction for 30 min at 37°C. Volume, pH, concentration, and motility were evaluated according to World Health Organization (WHO) guidelines (1999). When possible, at least 2 semen analyses were performed (16 unilateral and 6 bilateral).

For electron microscopy, sperm samples were fixed in cold Karnovsky fixative and maintained at 4°C for 2 hours. Fixed semen was washed in 0.1 mol/L cacodylate buffer (pH 7.2) for 12 hours, postfixed in 1% buffered osmium tetroxide for 1 hour at 4°C, then dehydrated and embedded in Epon Araldite. Ultrathin sections were cut with a Supernova ultramicrotome (Reickert Jung, Vienna, Austria), mounted on copper grids, stained with uranyl acetate and lead citrate, and then observed and photographed with a Philips CM10 transmission electron microscope (TEM; Philips Scientifics, Eindhoven, The Netherlands).

Three hundred ultrathin sperm sections were analyzed for each patient. Major submicroscopic characteristics were recorded by highly trained examiners who were blind to the experiment. TEM data were evaluated by the statistical mathematical formula of Baccetti et al (1995), which calculates the number of spermatozoa free of structural defects (“healthy”) and the percentage of 3 main phenotypic sperm pathologies: immaturity, necrosis, and apoptosis (Baccetti et al, 2006), suggesting the best assisted reproductive technique

(ART) for each ejaculate. We observed that the lowest number of spermatozoa free of defects, assuring normal fertility, was slightly higher than 2 million. If the number was between 1 and 2 million, intrauterine insemination (IUI) could be suggested. When the number of healthy sperm was between 300 000 and 1 million, the best ART was in vitro fertilization (IVF; Piomboni et al, 1996), however if the number of healthy sperm was less than 300 000, intracytoplasmic sperm injection (ICSI) was recommended as the gold standard treatment (Strehler et al, 1995).

Semen samples from 20 fertile men (age 22–35 years), without anatomical problems and infections and with normal karyotype, were used as controls. These fertile men had fathered 1 or more children during the past 3 years.

FISH Analysis of Sperm—To evaluate aneuploidy frequency, FISH was performed according to Baccetti et al (2006) on the sperm nuclei of 19 patients with unilateral cryptorchidism and 8 patients with bilateral cryptorchidism. It was impossible to perform FISH in cases of azoospermia or extremely severe oligozoospermia.

A mix of α -satellite DNA probes (CEP; Chromosome Enumeration Probes, Vysis, Ill) for chromosomes 18, X, and Y and directly labeled with different fluorochromes were used. All samples were analyzed by a highly trained examiner.

Observation and scoring were performed with a Leitz Aristoplan Optical Microscope equipped with a fluorescence apparatus; a triple bandpass filter for aqua, orange, and green fluorochromes (Vysis); and a monochrome filter for 4',6-diamidino-2-phenylindole. Semen samples from 7 fertile men (age 26–39 years) were analyzed and used as controls (Baccetti et al, 2006).

PCR Analysis—PCR analysis was performed in all treated cryptorchid patients examined. DNA was extracted from peripheral blood lymphocytes with the QIAamp DNA Blood kit (QIAGEN, Valencia, Calif).

PCR was performed as described in Baccetti et al (2006). Control DNA was extracted from the blood of 10 male donors (age 30–40 years) with a documented history of fertility. DNA extracted from the blood of 2 fertile females was used as a negative control.

Statistical Analysis

Analysis was performed by the StatGraphics Plus (Ver. 5.0) statistical package. Because of the small number of patients, we used nonparametric tests to compare the values of the 3 groups (unilateral cryptorchidism, bilateral cryptorchidism, and controls) because the variables examined were normally distributed (tested by standardized skewness and standardized kurtosis), and the standard deviations were assumed to be homogeneous (evaluated by Bartlett test).

The Kruskal-Wallis test was performed to compare the groups. When a statistically significant difference was found among the 3 groups, a Mann-Whitney *U* test was then used between pairs of groups—1) unilateral vs bilateral, 2) unilateral vs control, 3) bilateral vs control—to evaluate statistical significance. *P* less than 0.05 was considered significant for all statistical evaluations.

Results

Sperm analysis was carried out on 42 patients that had undergone orchidopexy before puberty. Of the 30 patients showing unilateral cryptorchidism, 4 men (13.3%) were azoospermic and 1 showed a higher level of FSH compared with the standard range. In this group, 3 patients with treated right cryptorchidism presented a first degree varicocele in the contralateral testis, and 3 patients (10%) developed a testicular cancer in the same testis. All these patients were ruled out for semen evaluation because these pathologies can influence sperm findings.

Of the 12 patients with bilateral cryptorchidism, 3 individuals (25%) were azoospermic and 2 of 3 men presented higher levels of FSH and LH; the same hormonal pattern was observed in a case of severe oligozoospermia. In this group, 1 patient with treated right cryptorchidism presented a second degree varicocele in the contralateral testis, and he was not considered for this study.

PCR analysis was performed on peripheral blood lymphocytes from all patients recruited for this study, and it did not reveal any microdeletions of the Y chromosome. When possible, at least 2 semen analyses were performed (16 unilateral and 6 bilateral), and the semen parameters did not show relevant differences.

The means of sperm concentration in both groups were normal ($>20 \times 10^6$ sperm/mL; WHO, 1999); rapid and slow progressive motility (a + b) in cases of unilateral cryptorchidism was $24.8 \pm 17.66\%$, whereas in cases of bilateral cryptorchidism it was $26.5 \pm 9.05\%$. Progressive motility rates were lower in men with cryptorchidism vs WHO parameters ($>50\%$, a + b).

In unilateral cryptorchid patients, normal sperm concentration was observed in 50% of cases, and motility was more than 50% in only 10% of the analyzed samples (Table 1). The observed values were lower in bilateral cryptorchidism, in which only 25% of cases were normospermic and none of the patients reached a normal value of motility compared with WHO parameters (Table 1).

Submicroscopic characteristics of sperm organelles were evaluated with a statistical mathematical formula (Baccetti et al, 1995) that calculates the number of spermatozoa free of structural defects (healthy) and the percentages of 3 main phenotypic sperm pathologies: immaturity, necrosis, and apoptosis.

In the unilateral cryptorchidism group, only 1 patient out of 20 showed more than 2×10^6 healthy sperm, assuring normal fertility, whereas none of the patients in the other group reached this value. Moreover, in the unilateral cryptorchidism group, 1 patient showed

Table 1. Sperm concentration and percent motility in unilateral and bilateral cryptorchidism

	Unilateral Cryptorchidism		Bilateral Cryptorchidism	
	No. of Cases (n = 20)	%	No. of Cases (n = 8)	%
Concentration, sperm/mL ($\times 10^6$)				
Normal (>20)*	10	50	2	25
10–20	6	30	2	25
<10	4	20	4	50
Motility (a + b), %				
Normal (>50)*	2	10	0	0
30–50	3	15	3	37.5
15–30	10	50	4	50
<15	5	25	1	12.5

* World Health Organization (1999).

between 1 and 2 million healthy sperm (IUI suggested), 2 obtained a fertility index suitable for IVF, and ICSI treatment was recommended for 16 men. In the bilateral cryptorchidism group only 1 patient reached the fertility score, suggesting IVF, whereas the others all had much lower scores.

The mean number of healthy sperm in either group did not reach the value for natural fertility, and the score related to cases of bilateral cryptorchidism was less than for the unilateral cases (Table 2). A significant difference among the groups of unilateral cryptorchidism, bilateral cryptorchidism, and controls was observed ($P = 0.000026$) by the Kruskal-Wallis test.

TEM analysis confirmed the presence of different alterations affecting sperm organelles related to immaturity, apoptosis, and necrosis. Altered acrosomes and misshapen, round, or elliptical nuclei with uncondensed chromatin and the presence of cytoplasmic droplets were the characteristic features of immaturity (Figure 1). Marginated chromatin, cytoplasmic translucent vacuoles, and swollen and badly assembled mitochondria were the typical ultrastructural markers of apoptosis (Figure 2). Spermatozoa with broken plasma membranes, reacted or absent acrosomes, misshapen nuclei with disrupted chromatin, and poor axonemal and periaxonemal cytoskeletal structures were affected by necrosis (Figure 1). The statistical mathematical formula was also used to calculate the percentage of these phenotypic sperm pathologies, and the results are reported in Table 2.

Necrosis ($P = 0.006325$) and apoptosis ($P = 0.009187$) differed significantly according to the Kruskal-Wallis test. For pathologies in which a statistical difference was detected, we applied the Mann-Whitney *U* test to compare the pairs: 1) unilateral vs bilateral, 2) unilateral vs control, and 3) bilateral vs control (Table 2). Significant differences were found in the

Table 2. Number of healthy sperm (mean \pm SD) and percentage of sperm pathologies (mean \pm SD) in the groups of unilateral and bilateral cryptorchidism compared with controls*

	No. of Healthy Sperm	Immaturity, %	Necrosis, %	Apoptosis, %
Unilateral cryptorchidism (n = 20)	384,047 \pm 889,522	65.63 \pm 10.39	47.99 \pm 25.16	10.37 \pm 7.42
Bilateral cryptorchidism (n = 8)	117,695 \pm 283,938	64.43 \pm 9.38	50.26 \pm 13.00	8.23 \pm 4.38
Fertile controls (n = 20)	$>2 \times 10^6$	55.10 \pm 10.74	21.00 \pm 14.94	4.80 \pm 3.40
Kruskall-Wallis test	$P = 0.000026$	$P = 0.6423$	$P = 0.006325$	$P = 0.009187$

* In cases of significant differences among the 3 groups, the Mann-Whitney *U* test was applied: unilateral vs bilateral: healthy sperm ($P = 0.6655$), necrosis ($P = 0.6655$), apoptosis ($P = 0.5585$); unilateral vs control: healthy sperm ($P = 0.000017$), necrosis ($P = 0.01006$), apoptosis ($P = 0.0034$); bilateral vs control: healthy sperm ($P = 0.000448$), necrosis ($P = 0.002174$), apoptosis ($P = 0.0368$).

comparison of the 2 treated cryptorchidism groups compared with the control group (Table 2, footnote), whereas no difference was detected between unilateral and bilateral groups.

Meiotic segregation was investigated by FISH on the sperm nuclei of 19 patients with unilateral and 8 with bilateral cryptorchidism. A total number of 118,831 sperm nuclei was scored. The mean of frequencies related to the aneuploidy of chromosomes 18, X, and Y are summarized in Table 3. Out of 27 patients, only 1 from the bilateral group showed all FISH values within the normal range. Generally, the mean of the frequencies of sex chromosome disomy and diploidy was higher in the 2 examined groups than in the control group.

Moreover, the mean of values related to chromosome 18 disomy was also out of the range in the bilateral group; to date, all the aneuploidy values have been slightly higher compared with those of the unilateral group; however, they were not significantly different (Table 3).

Discussion

Cryptorchidism is a pathologic condition in which the testes fail to descend into the scrotum, sometimes resulting in atrophic seminiferous tubules. Semen parameters are often impaired in men with a history of cryptorchidism (Hadziselimovic, 2002). Yavetz et al (1992), studying a large population of men with disturbances of the descent of the testes into the scrotum, reported a general oligoteratoasthenozoospermia, and the degree of spermatogenic damage was higher in bilateral cryptorchidism than in unilateral cryptorchidism. Severe impairment of semen quality in bilateral forms has also been reported by other authors (Gracia et al, 2000; Vinardi et al, 2001; Lee, 2005).

The detrimental effect of surgically induced bilateral cryptorchidism on testicular function and sperm motility was also investigated in adult male rats (Ren et al, 2006). Moreover, sperm morphology was evaluated by light microscopy and TEM in healthy boars with

spontaneous abdominal cryptorchidism, and disturbances in the late stages of spermiogenesis at the testicular level were observed. Alterations were not found in the sperm maturation process at the epididymal level (Pinart et al, 1998). In humans, a study of prepubertal testicular biopsies in cases of cryptorchidism highlighted a possible correlation between testicular lesions and spermiograms performed in adults (Nistal et al, 2000).

In this study we divided the patients into classes for concentration and motility showed in Table 1. In this classification, it was evident that the carriers of bilateral cryptorchidism showed a worse seminal profile. Moreover, the incidence of azoospermia was higher in the bilateral than unilateral group, as reported by other authors (Hadziselimovic, 2002). Despite the overall normality of hormonal values, some men had azoospermia or severe oligozoospermia associated with elevated gonadotropin levels.

The better sperm quality in the unilateral group was also demonstrated by TEM analysis. Applying the statistical mathematical formula to TEM data, the number of healthy sperm—the index of sperm quality—was 3 times higher in the unilateral compared with the bilateral group, without however reaching statistical significance. The incidence of sperm pathologies, such as apoptosis, necrosis, and immaturity, was also determined. Apoptosis, a physiologically programmed cell death, was found by Kerr (1992) to occur spontaneously in the cycle of the rat seminiferous epithelium. Apoptosis is characterized by typical ultrastructural features and biochemical events; it is determined by a precise genetic and molecular induction, and it is observed in various percentages in ejaculates from infertile patients (Baccetti et al, 1996). Necrosis is a cell death, known in sperm as necrozoospermia, a common pathology observed in various percentages in semen samples from infertile individuals. The necrosis could be considered to be the most common sperm pathology in cases of infections or inflammations of the male genital tract (Moretti et al, 2005). Moreover, immaturity is due to incomplete sperm maturation related to the presence

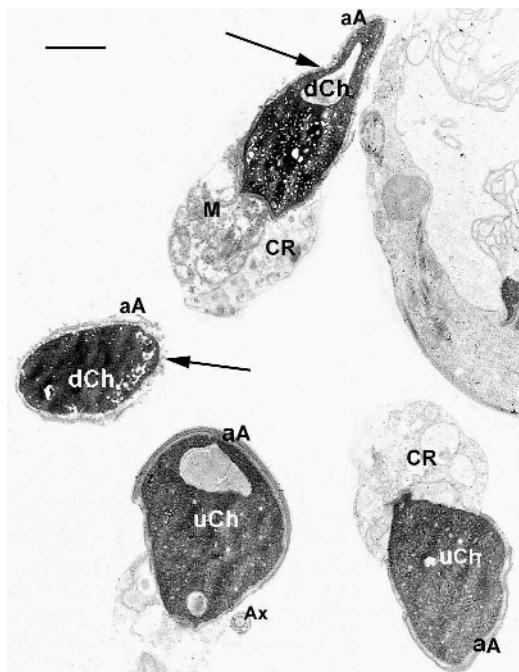


Figure 1. TEM micrograph of longitudinal and cross sections of immature and necrotic sperm characterized by irregular nuclei with uncondensed (uCh) and disrupted (dCh) chromatin. The acrosomes (aA) appear generally altered. Cytoplasmic residue (CR) is present, sometimes embedding swollen mitochondria (M), axonemes (Ax). Necrotic sperm show broken plasma membrane (arrows). Scale bar = 1 μ m.

of cytoplasmic retention and meiotic defect (Kovanci et al, 2001), and it is characterized by typical ultrastructural markers, such as poorly condensed chromatin.

The presence of these pathologies in our study group indicated a severe deterioration of sperm quality in the case of cryptorchidism; however, the values of pathologies did not reach statistical significance when the 2 treated groups were compared. On the contrary, compared with fertile controls, the values of necrosis and apoptosis were statistically significant. An explanation for the high presence of sperm necrosis could be that necrosis is a final step of the apoptotic process, which has been demonstrated at morphologic and molecular levels by different authors in animal models (Chaki et al, 2005; Dundar et al, 2005) and by Baccetti et al (1996) in cases of human cryptorchidism.

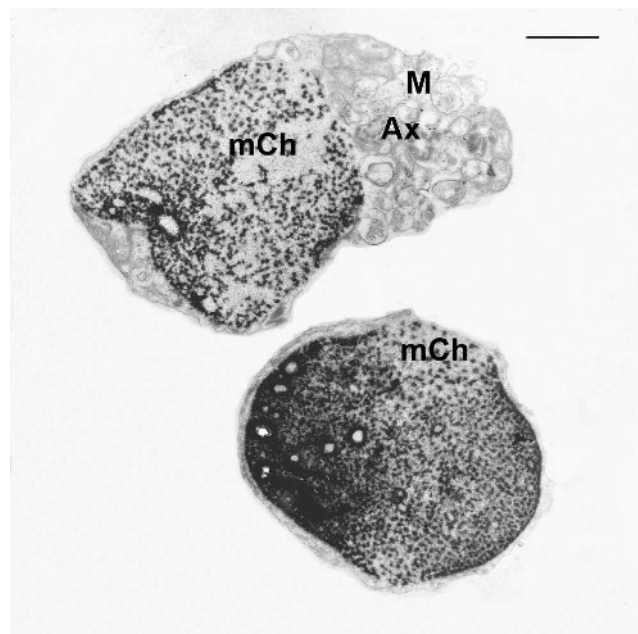


Figure 2. TEM micrograph of apoptotic sperm characterized by misshapen nuclei with marginated chromatin (mCh). A cytoplasmic residue embedding swollen mitochondria (M) and a coiled axoneme (Ax) is present. Scale bar = 1 μ m.

In the literature, microdeletions of Y chromosome have been reported in patients with cryptorchidism and severe defects of spermatogenesis (Ferlin et al, 2004; Song et al, 2005); however, none were found in our study according to other authors (Fagerli et al, 1999; Castro et al, 2004).

The data concerning meiotic segregation of analyzed chromosomes are of particular interest because, to our knowledge, FISH values related to sperm from patients treated for cryptorchidism have, up until now, not been reported. It is known that infertile patients with poor sperm quality, for a number of causes, seem to have an increase in the frequency of aneuploidy (Bernardini et al, 1997), and the presence of compromised testicular environments could also favor meiotic errors.

We analyzed, by FISH, spermatozoa from patients treated for cryptorchidism with different semen profiles. The mean frequencies of diploidies and sex chromosome disomies were generally out of the normal range,

Table 3. Percentage (mean \pm SD) of disomies and diploidies of the examined chromosomes (18, X, Y) in 19 males with unilateral cryptorchidism, in 8 males with bilateral cryptorchidism, and in 7 healthy men of proven fertility

	Mean Diploidy Frequency, %	Mean Disomy Frequency, %	
		18	Gonosomes
Unilateral cryptorchidism (n = 19)	0.478 \pm 0.36	0.114 \pm 0.06	0.403 \pm 0.23
Bilateral cryptorchidism (n = 8)	0.669 \pm 0.74	0.218 \pm 0.33	0.491 \pm 0.60
Fertile controls (n = 7)	0.280 \pm 0.006	0.110 \pm 0.003	0.230 \pm 0.004
Kruskal-Wallis test	P = 0.2503	P = 0.6445	P = 0.3857

indicating a disturbance of meiotic segregation; however, no statistically significant differences were obtained.

As a consequence, the complex of effects induced by cryptorchidism resolved in childhood seem to include a severe spermatogenetic impairment, leading us to recommend detailed sperm ultrastructural and chromosomal analyses before undertaking assisted reproductive techniques, particularly intracytoplasmic sperm injection.

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