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A Comparative Study of AZF Deletions and TSPY Gene Variation in Czech and Indian Infertile Men

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ABSTRACT The human Y chromosome harbours genes that are essential for spermatogenesis. Most of these genes lie in the male-specific region (MSY) of Y chromosome. Microdeletions of AZF within the MSY have been reported in infertile men. Widely different frequencies of such deletions (0-55%) have been reported from different populations. TSPY is another gene located in the MSY region that plays a significant role in spermatogenesis. It is a multi-copy gene but the role its gene copy numbers play in infertility is not yet evaluated. The present study was undertaken on infertile men from Czech Republic and India to ascertain the relative and comparative role of AZF deletions and of TSPY copy numbers.

INTRODUCTION

Male infertility can be caused by a variety of factors that include cryptorchidism, ductal obstruction, hormonal dysfunction, retrograde ejaculation, systemic diseases, testicular cancer, testicular trauma, varicocele, etc. Apart from these in ~30% of male infertility cases, that are referred to as idiopathic, genetic abnormality at molecular level is suspected (Huynh et al. 2002). Most of the genes associated with male infertility lie in the male-specific region (MSY) of Y chromosome (Singh et al. 2005a). Micro-deletions of AZF especially of AZFa, AZFb, and AZFc regions, within the MSY have been reported in infertile men. Widely different frequencies of such deletions, ranging from 0% to 55%, have been reported (Foresta et al. 1998; Kihaile et al. 2005). This large inter-laboratory variation in the frequency of AZF deletions is probably due to the type of patients selected for Y chromosome analysis (McElreavey and Krausz 1999).

Another Y chromosome gene, TSPY (GDB: 120471), is a multicopy gene and most of its copies are located on the MSY region. This gene family has 20-40 gene copies that vary from individual to individual (Dechend et al. 2000). Its prototype coding sequence is 924 bp and a sequence divergence of 10% has been reported in human TSPY sequences (Arnemann et al. 1991; Manz et al. 1993). The function of TSPY sequences is to integrate TSPY protein into spermatogenesis (Vogel et al. 1997) and it is involved in the proliferation of germ cells (Schnieders et al. 1996). Conard et al. (1996) proposed that RBM and TSPY genes might have originated form a common organization on an ancient Y chromosome. As RBM is an AZF candidate gene, the possible role of TSPY in spermatogenesis is further strengthened. However, whether the gene copy numbers of TSPY play a role in male infertility is yet to be evaluated.

It is important to ascertain the AZF deletions and the role of TSPY copy numbers when ICSI procedures have to be followed for diagnosis, prediction of prognosis and genetic counseling of the infertile couple.

The present study was undertaken on infertile men from Czech Republic and from India (Punjab; North West India), following same procedures, to ascertain the role of AZF deletions and TSPY copy numbers in these two geographically and ethnically different populations.

MATERIALS AND METHODS

The presence or absence of micro-deletions of AZF involving the AZFa, AZFb and AZFc regions were evaluated in 219 individuals (Table 1) in the present study. The TSPY gene analysis

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Table 1: Details about the patients studied

Patient group	*Number of patients*
Czech patients	
Azoospermia	32
Oligospermia	
<5 million sperms/ml	48
>5 million sperms/ml	19
Testicular feminization syndrome (46,XY)	2
Klinefelter syndrome mosaic (47,XXY/46,XY)	2
Controls	47
Indian patients	
Azoospermia	39
Controls	30
Total	219

was undertaken on 84 cases. These included 24 azoospermics and 40 controls from Czech Republic and 20 azoospermics from India (Punjab, North West India). All Czech patients were analyzed at Department of Medical Genetics and Fetal Medicine, University Hospital, Palacky University, Olomouc, while Indian patients were analyzed at Centre for Genetic Disorders, Guru Nanak Dev University, Amritsar, India. In all the infertile cases investigated, the endocrine disorders, trauma, chronic diseases, cryptorchidism were ruled out and karyotyping had been undertaken.

DNA was isolated from peripheral blood, using Miller's method (Miller et al. 1998). Simplex PCR was used for detecting micro-deletions of the AZF region on Y chromosome in order to prevent misinterpretation of accidental false positives. Seven informative markers of sequence-tagged-sites (STS) were used (Table 2).

The PCR cocktail for SY87, SY134, SY147, and SY158 STS marker consisted of 16.15 µl deionized water; 2.50 µl 10x concentrated PCR buffer; 1.25 µl of 50 mM $MgCl_2$; 0.50 µl 100 mM dNTPs; 1.25 µl Forward Primer; 1.25 µl Backward Primer; 0.10 µl Taq polymerase (concentration 5U/µl); 2.00 µl DNA (100ng/µl). The final reaction volume used was 25µl. The PCR cocktail for SY84, SY143, and SY255 STS markers consisted of 13.65 µl deionized water and 2.50 µl DMSO. All other constituents were the same as detailed for other STS markers. The PCR program used for all the above mentioned markers was the same. It consisted of initial denaturation (94°C), denaturation (94°C), annealing (56°C) and elongation (72°C) cycles each of 1 minute. The total number of cycles was 35.

Refined Quantitative Fluorescent PCR (Vodicka et al. 2004) was used to analyze the copy numbers of TSPY gene and for this multiplex PCR was utilized. The single copy genes AMELX and AMELY (Table 2) acted as control genes to determine relative amplifications of TSPY. The PCR cocktail and conditions for multiplex PCR were as described by Singh (2005).

To ascertain the number of amplification cycles that give best results for the evaluation of various indices with respect to TSPY copy numbers, and AMELX and AMELY indices, each

Table 2: Details of the loci/STS markers studied and primers used

Locus/STS markers	*Cytogenetic localization*	*Sequence (1: forward; 2: reverse)*
AMEL X/Y	Xp22.31 – p22.1 Yp11.2	5'-ctgatggttggcctcaagcct- 3' 5 –atgaggaaaccagggttcca-3'
TSPY	Yp11.1 – q11.1	5' –cggggaagtgtaagtgaccgatggg- 3' 5' –ctgctcttccaaaaagatgccccaaa -3'
SY84	Yq11-q11	5' –agaagggtctgaaagcaggt- 3' 5' –gcctactacctggaggcttc -3'
SY87	Yq11-q11	5' –tctgttgcttgaaaagaggg- 3' 5' –actgcaggaagaatcagctg- 3'
SY134	Yq11-q11	5' –gtctgcctcaccataaaacg- 3' 5' –accactgccaaaactttcaa- 3'
SY143	Yq11-q11	5' –gcaggatgagaagcaggtag- 3' 5' –ccgtgtgctggagactaatc- 3'
SY147	Yq11-q11	5' –tttctcgtttgatgatcctag- 3' 5' –ttaatatgagaatgagaacagatgt- 3'
SY255		5' –gttacaggattcggcgtgat- 3' 5' –ctcgtcatgtgcagccac- 3'
SY 158	Yq11-q11	5' –ctcagaagtcctcctaatagttcc- 3' 5' –acagtggtttgtagcgggta- 3'

of the samples was simultaneously amplified for four different amplification cycles, i.e., 22, 24, 26 and 28.

Agarose gel electrophoresis was used to detect various deletions. The PCR products were separated on 1.5 % agarose gel (80V; 45-60 min) and stained with ethidium bromide. The analysis of TSPY gene copy numbers was undertaken with ABI PRISM 310 capillary electrophoresis. The digital data of TSPY was evaluated with ABI Prism 310 Data Collection software.

RESULTS

AZF Micro-deletions

The specificity and efficacy of PCR conditions with respect to all seven STS markers were confirmed by observing the amplification of samples from fertile men. AZF micro-deletions were detected among six azoospermic men (3 each from Czech Republic and India) only, while none was seen in any other category of infertile men or amongst the 47 Czech and 30 Indian controls.

Only one deletion for the AZFa region was detected amongst the azoospermic males from Czech Republic (case no. CZ-78/03) and no case with deletions involving only the AZFb region was seen. Two azoospermic cases each, from Czech Republic and India (case nos. CZ-304/04, CZ-333/04 and IN-5, IN-29, respectively) showed deletions involving only the AZFc region. Among these, case numbers CZ 304/04 and IN-29 showed deletions with all three STS markers, i.e., sY147, sY158 and sY255. One case (CZ-333/04), showed deletions for the markers sY147 and sY255 only (Fig. 1) and another case (IN-9), showed deletions with all seven STS markers indicating the deletion of all regions of AZF, viz., AZFa, AZFb and AZFc.

The experiments for all those cases that

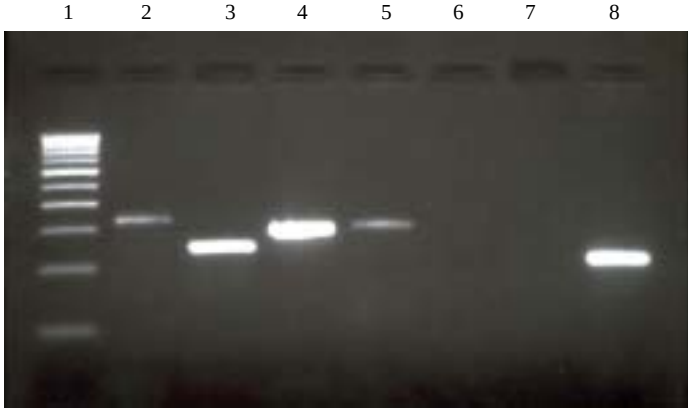

Fig. 1. AZFc deletion in CZ-333/04. Non-amplification of sY147 and sY255

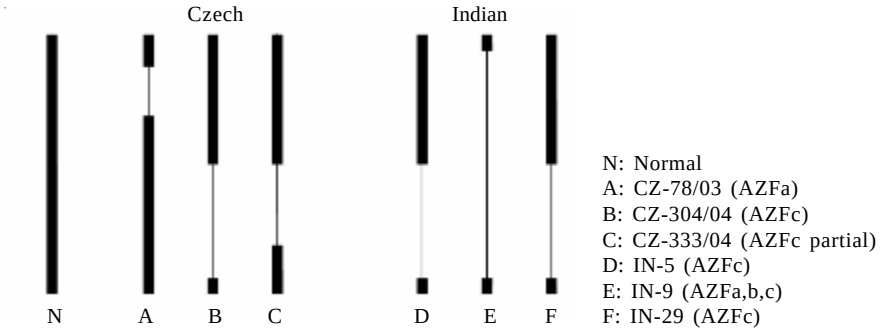

Fig. 2. Diagrammatic representation of deletions observed in AZF region

showed presence of any deletion were repeated at least three times with the fresh solutions for re-confirmation. Figure 2 depicts the diagrammatic representation of the deleted AZF regions among the Czech and Indian azoospermics.

The frequencies of various deletions seen in two groups of infertile men are depicted in Table 3. An overall frequency of 2.91% AZF deletions was observed for Czech infertile men (n=103). The relative frequencies of various deletions were 0.97% (AZFa), 0% (AZFb) and 1.94% (AZFc). If only azoospermic men were considered, then these frequencies were 3.13% (AZFa), 0% (AZFb), and 6.26% (AZFc). Among Indian azoospermics the total frequency of such deletions was 7.69%. The frequency of deletions in AZFa and AZFb regions being 0% and that for AZFc region being 5.13% and for AZFa+b+c being 2.56%.

The frequency of AZFc mutations was highest (66.6%) amongst the cases that showed any AZF deletion, both for the Czech as well as Indian azoospermic males (Table 4). The variations seen between the frequencies of deletions amongst the azoospermic men from Czech Republic and India were statistically insignificant.

Table 3: Comparison of AZF deletions among Czech and Indian infertile men

Deleted region	*Czech infertile %; n=103*	*Czech azoospermic %; n=32*	*Indian azoospermic %; n=39*
AZFa	0.97	3.13	0
AZFb	0	0	0
AZFc (complete)	0.97	3.13	5.13
AZFc (partial)	0.97	3.13	0
AZF a+b+c	0	0	2.56
Total	2.91	9.38	7.69

* No deletion seen in 47 Czech and 30 Indian controls

Table 4: Region-wise distribution of AZF deletions among azoospermic men

Region of	*Czech sample*		*Indian sample*	
AZF	*No.*	*%*	*No.*	*%*
AZFa	1/3	33.3	0/3	-
AZFb	0/3	-	0/3	-
AZFc	2/3	66.6	2/3	66.6
AZF a+b+c	0/3	-	1/3	33.3

TSPY Gene Copy Number

Four sets of electrophorograms were recorded by the ABI Prism capillary electrophoresis for each patient. These respectively exhibit the fluorescent peaks of each gene for 22, 24, 26 and 28 amplification cycles (Fig. 3) with respect to TSPY, AMELY and AMELX. All the data collected were statistically analyzed with respect to various indices, viz., AMELY/AMELX, TSPY/AMELX and TSPY/AMELY. The mean log transformed values of TSPY/AMELY index between the various infertile groups and the controls showed significant increase in TSPY/AMELY ratio in infertile men as compared to the control group (Singh et al. 2005b). Statistical analysis (ANOVA) undertaken on TSPY/AMELY ratios for 22, 24, 26

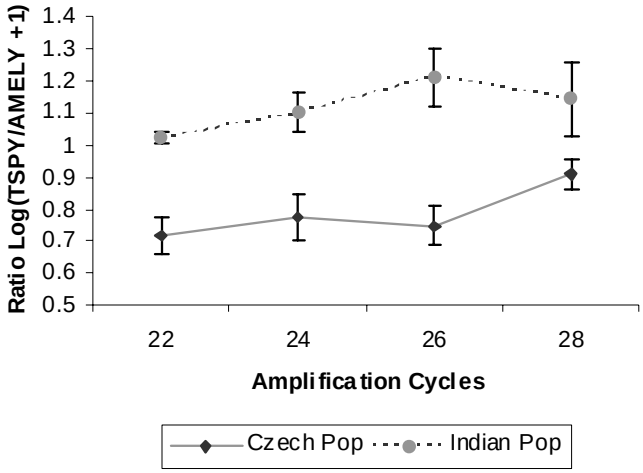

Fig. 3. Comparison of Log transformed values between Czech and Indian azoospermic patients for different amplification cycles

and 28 cycles with respect to various groups of infertile men and controls had indicated that the f-values for this parameter at all the cycles were non-significant and that the values for 22 cycles were more uniform (Singh et al. 2005b,c).

The comparison of the log transformed values for TSPY/AMELY between the azoospermic groups from Czech Republic and India (Figure 4) revealed that these values differed between the two azoospermic groups at all the amplification cycles viz. 22, 24, 26 and 28. However, these differences were statistically significantly different for 22, 24 and 26 cycles.

DISCUSSION

AZF Deletions

AZF deletions have been extensively reported to cause male infertility. In the present study an overall incidence of AZF micro-deletions of 2.9% was observed in Czech infertile men. Similar results have been reported from various populations. Sargin et al. (2004) reported 3.3% AZF deletions in Turkish infertile men; Katagiri et al. (2004) reported 3.4% among infertile North American men and a frequency of 4% was reported by Machatkova et al. (2003) in Czech infertile men while 4.5% in Croatia by Sertic et al. (2001). However, a wide variation of such micro-deletions has been observed in different geographical regions. Kihaile et al. (2005) have reported complete absence (0%) of AZF deletions in their sample of azoospermics from Africa, while frequencies of 6.7% in Brazil (Sao Pedro et al. 2003), 7.1% in Japan (Carvallo et al. 2003), and 10% in Romania (Raicu et al. 2003) have been observed. A high frequency of 19.3% was reported from India by Rao et al. (2004) and even a frequency of 55% has been reported (Foresta et al. 1998). This large inter-laboratory variation in the frequency of AZF deletions is probably due to the type of patients selected for Y-chromosome analysis (Kent-First et al. 1999; McElreavey and Krausz, 1999).

In the present study no deletions were detected amongst oligospermics. Similar observations have been made by several researchers. Calleja Macia et al. (2003) who reported an incidence 13.9% of AZF deletions in azoospermics, also did not find any deletion in their oligospermic cases. Kihaile et al. (2005) also did not find any AZF deletions among oligospermics. Many researchers have observed that frequencies of AZF deletions are lower among oligospermics as compared to azoospermics (Bor et al. 2002; Kerr et al. 2000).

9.38% and 7.69% AZF micro-deletions were observed amongst Czech and Indian azoospermics, respectively. Corroboratory observations have been made by Madgar et al. (2002) in Israel (8.2%) and Tse et al. (2002) from Hong Kong and Shanghai (8.2% and 6% respectively). However, a low incidence (2.2%) was reported by Bor et al. (2002) from Denmark and a high incidence (20%) by Kerr et al. (2000) from New Zealand.

No isolated deletion of AZFa was observed amongst the Indian azoospermic men, while the detection of one AZFa deletion in the Czech group gave a frequency of 0.97% among infertile men and a frequency of 3.13% among azoospermics. Concurrent to these, Okutman-Emonts et al. (2004) and Machatkova et al. (2003) also did not find any AZFa deletion among their 71 Turkish and 198 Czech cases, respectively. Least frequencies of AZFa deletions among azoospermic men have been reported among Russians (Loginova et al. 2003), Chinese (Yang et al. 2003), North Americans (Hopps et al. 2003), Indians (Thangaraj et al. 2003) and Japanese (Kihaile et al. 2005).

Micro-deletions in the AZFa region are thought to be the result of intra-chromosomal homologous recombinations between highly homologous sequences present on both sides of this region. These recombinations occur between two highly homologous human endogenous retrovirus (HERV) sequences (Patience et al. 1997). Such intra-chromosomal recombination between these two HERV sequences can result either in homogenizing sequence conversion or in a micro-deletion in AZFa region.

USP9Y is the candidate gene for AZFa region. The other genes localized in AZFa region that have possible role in spermatogenesis are DBY (Lahn and Page 1997) and UTY (Mazeyrat et al. 1998). DBY is more frequently deleted than USP9Y and its expression is also specific for testes. However, the exact function of these genes still remains unknown (Huynh et al. 2002).

The non-observation of any deletions involving only the AZFb region in the present study is in pattern with the observations of Machatkova et al. (2003) and Loginova et al. (2003).

On the other hand, many authors have reported a higher incidence of AZFb deletions as compared to AZFa deletions (Yang et al. 2003; Hopps et al. 2003).

In accordance to the findings of the present study, wherein a frequency of 1.94% of AZFc deletions was observed for Czech infertile men and 6.26% amongst Czech azoospermics, Lynch et al. (2005) have reported an incidence of 2.27% among Australian infertile men, whereas, Yang et al. (2003) reported an incidence of 12.7% AZFc deletions among their Chinese patients. 66.6% AZFc deletions amongst the cases with AZF deletions, as seen for both population groups, is in agreement with the reports of Hopps et al. (2003)**.** However, Thangaraj et al. (2003) reported high incidence of 82.8%. McElreavey et al. (2000) have observed that deletions involving the AZFc region are the most frequent amongst all the AZF deletions.

Kuroda-Kawaguchi and co-workers (2001) observed that a mechanism similar to the one proposed for AZFa deletions also exists for AZFc region. The stretch homology in AZFc region is bigger (229 kb) than that of AZFa (10 kb). Silber and Repping (2002) hypothesized that as the frequency of deletions corresponds to the length of stretch homology, therefore the AZFc deletions are more common.

In the AZFc region, five genes, DAZ, CDY1, BPY2, PRY and TTY2 have been mapped (Vogt et al. 1996; Lahn and Page, 1997) of which DAZ is the candidate gene for AZFc. DAZ encodes for a testes specific protein containing a single RNA-binding motif with 8-24 copies of 24 amino acid sequence, known as 'DAZ repeat' (Reijo et al. 1995; Yen et al. 1997) and at least three functional copies of this gene viz., DAZ1, DAZ2 and DAZ3 have been reported (Saxena et al. 2000).

The partial deletion observed in the AZFc region in one of the Czech azoospermic males probably involves the partial deletion of the candidate gene DAZ. Men with AZFc deletions usually lack all copies of the DAZ gene. However, partial deletions for DAZ have also been reported. de Vries and co-workers (2002) suggested that there may be gene dosage effect e.g., men with 2 deleted DAZ genes may be less affected than those with loss of all DAZ genes. Writzl et al. (2004) have reported a frequency of 2% deletions of two of four DAZ gene copies in the Slovenian population. Ferlin et al. (2005) reported that only partial AZFc deletions removing DAZ1/DAZ2 seemed to be associated with spermatogenic impairment, whereas those removing DAZ3/DAZ4 had no or little effect on fertility.

Lynch et al. (2005) have recently described gr/gr sub-deletion of AZFc that removes 2 of 4 copies of DAZ. Lin et al. (2005) have also reported reciprocal duplication product of gr/gr deletion in DAZ gene in the AZFc region. Lapretre et al. (2005) did not find any partial DAZ deletion among fertile men signifying the role of such partial deletions in the causation of infertility.

The combination deletions like AZFa+b, AZFb+c, or AZFa+b+c are much less frequent than the single region deletions (Hopps et al., 2003). Only one case of AZFa+b+c was observed amongst 39 Indian azoospermic men. The frequency of AZFa+b+c deletions is relatively low in all populations. Kihaile et al. (2005) also observed only one such case among 47 azoospermics from Japan and Africa. El Awady et al. (2004) and Loginova et al. (2003) each reported one such case amongst infertile men studied by them.

Sargin et al. (2004) did not find any case of AZFa+b+c deletion amongst 61 Turkish infertile males. Similar to the present study, wherein no AZFa+b+c deletion was observed in 103 Czech infertile men, Yang et al. (2003) also did not find any such case in 134 azoospermics from China. Amongst 78 men with AZF deletions, Hopps et al. (2003) observed 6 men (7.7%) with such deletions. Ferlin et al. (1999) observed that deletions involving more than one AZF-candidate gene are associated with a more severe testicular phenotype.

The wide variation among geographically and ethnically different populations indicates the need of further in depth evaluation, with well defined parameters, of infertile men with respect to micro-deletions involving AZFa, AZFb, and AZFc regions of the Y chromosome. The relative uniformity in the percentages of deletions seen in the present study among geographically and ethnically different populations from Czech Republic and India highlights the importance of following the uniform patient selection criteria.

TSPY Gene

The present study is probably the first study to ascertain the geographic and ethnic differences associated with TSPY gene copy number in relation to male infertility.

TSPY gene was selected for evaluation as the function of this gene, which is only expressed in testes, is to integrate TSPY protein into spermatogenesis (Vogel et al. 1997). Its role is specific for the proliferation of germ cells (Schnieders et al. 1996). The homology of TSPY with members of the TTSN gene family that are involved in cell cycle control, also suggest its role in spermatogonial proliferation (Chai et al. 2001; Ozbun et al. 2001).

Single copy genes, AMELY and AMELX were selected for determining various indices like AMELY/AMELX, TSPY/AMELX and TSPY/AMELY. AMELY lies close to TSPY (Lau, 1999) and like its X-linked homologue AMELX codes for an enamel protein (Lau et al. 1989). To determine the ideal number of cycles at which all the analyzed genes get simultaneously and comparably amplified for quantitative analysis, amplifications were done for 22, 24, 26 and 28 cycles. The values of 22nd cycle indicated more uniformity and sensitivity (Singh et al. 2005b,c). For the quantification of TSPY values, the RQF PCR method of Vodicka et al. (2004) was followed. The results validated the utility of RQF PCR method as statistically analyzable data, with respect to different groups were successfully realized.

Significant differences were observed for TSPY/AMELY values between the azoospermic men from Czech Republic and those from India (Punjab; North West India). Geographical and ethnic variations with respect to various Y chromosomal genes are well known (Santos et al. 2000). Some authors have reported a divergence of up to 10% in human TSPY (Arnemann et al. 1991; Manz et al. 1993). Schnieders et al. (1996) had also reported the diversity of human TSPY at the transcript level with at least nine different splice variants. These reports coupled with the present observations highlight the need to establish base line data for geographically and ethnically different populations with respect to TSPY copy numbers.

Carlsen et al. (1992) reported that in the past 50 years there has been almost 50% decline in the sperm concentration. Increase in the TSPY copy numbers over generations might be one factor contributing to such deterioration. The increase in the gene copy number, as observed for TSPY, primarily occurs through gene duplication for which several mechanisms have been reported (Ohno 1970; Lewin 2000). Many authors (Chang et al. 1999; Page et al. 1999; Dada et al. 2004) have observed that AZF micro-deletions could result in progressive worsening of sperm production and that with time the oligozoospermic men may become azoospermic. Therefore, the evaluation of TSPY copy numbers and AZF micro-deletions has immense implications for ICSI. These have great value for the understanding of the prognosis and also for better management and counseling of patients with oligospermia or azoospermia.

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