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Apoptosis mediated by phosphatidylserine externalization in the elimination of aneuploid germ cells during human spermatogenesis

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ABSTRACT

Aneuploidies trigger cell cycle checkpoints leading to apoptosis. The aim of the present study was to assess the relationship between the presence of chromosomal abnormalities and apoptosis in germ cells and in Sertoli cells. Fourteen diagnostic testicular biopsies from infertile patients were processed following a sequential methodology, which included enzymatic disaggregation, apoptotic staining, cell sorting, cell fixation and fluorescent in situ hybridization (FISH) analysis. The chromosome constitution of germ cells (interphase pre-meiotic germ cells, meiotic figures, post-reductional germ cells and spermatozoa) and Sertoli cells was evaluated in non-sorted and flow-sorted cell populations (apoptotic and viable). The mean percentage of aneuploidy was compared between the three fractions in each cell type using a Kruskal-Wallis test. If significant results were obtained, a two-by-two chi-square test was performed. There were significant differences between the apoptotic fraction and the viable and non-sorted fractions in the pre-meiotic germ cells (p-value < 0.01). In the remaining cell types, no association between the presence of aneuploidy and apoptotic processes was observed, even in the case of post-reductional germ cells in which we detected the highest rates of aneuploidy regardless of the fraction analyzed. From our data, it can be inferred that most of the aneuploid post-reductional germ cells are efficiently removed from the testicular epithelium without differentiating into sperm. Our results suggest that the elimination of aneuploid testicular epithelial cells is triggered by different mechanisms. Accordingly, the cellular elimination of aneuploid germ cells beyond the blood-testis barrier does not involve phosphatidylserine externalization.

KEYWORDS: Aneuploidy / Apoptosis / FISH / Germ cells / Spermatozoa

INTRODUCTION

Spermatogenesis is the process by which spermatozoa are produced from male primordial germ cells. It takes place in the seminiferous tubules of the testis and occurs continuously during the reproductive lifetime of adult men. The number of cells in the seminiferous tubules is determined by a dynamic balance between cell proliferation and degeneration; it is well-known that not all germ cells achieve maturity and that germ cell apoptosis is inherent to spermatogenesis (Sinha Hikim *et al.*, 2003).

Apoptotic activation in germ cells occurs at different steps. During embryogenesis, apoptosis plays a role in discarding primordial germ cells that do not migrate properly from the epiblast to the developing gonad (Shaha *et al.*, 2010). In the fetal and neonatal testes, apoptosis is mainly involved in the physiological adjustments needed to obtain an optimal ratio between germ cells and Sertoli cells (Boulogne *et al.*, 1999; Shaha *et al.*, 2010). During the pre-pubertal stages, apoptosis regulates the first differentiation of spermatogonial stem cells into spermatogonia and primary spermatocytes (Rodriguez *et al.*, 1997). In addition, programmed cell death is also triggered by checkpoints that control key events of spermatogenesis, such as pairing, recombination and chromosome segregation (Turner, 2007; Vogt *et al.*, 2008; Burgoyne *et al.*, 2009). When checkpoints fail to repair the abnormalities, several cell mechanisms are activated to eliminate the affected cells (Braun, 1998; Hamer *et al.*, 2008; Li *et al.*, 2009; Yan, 2009) avoiding the transmission of chromosomal abnormalities to the next generation. The key regulator of apoptotic induction in human germ cell apoptosis is the Fas-FasL system (Lee *et al.*, 1997; Francavilla *et al.*, 2002; Lin *et al.*, 2010; Shaha *et al.*, 2010). The FasL death signal is expressed by Sertoli cells in order to regulate germ cell fate (reviewed by Sofikitis *et*

al., 2008). In addition, Sertoli cells are also responsible for apoptotic germ cell phagocytosis in a phosphatidylserine-dependent manner, which has been described as the major removal system of germ cells from testicular tissue (Maeda *et al.*, 2002; The *et al.*, 2004).

Meiotic studies on testicular biopsies from infertile males have shown that a significant percentage of individuals display errors in key meiotic processes such as synapsis, recombination and/or chromosome segregation (Egozcue *et al.*, 2005). Disruption of these processes are often linked to partial or total arrest of spermatogenesis (Gonsalves *et al.*, 2004; Guichaoua *et al.*, 2005; Topping *et al.*, 2006; Ferguson *et al.*, 2007; Sun *et al.*, 2007) and increases of chromosomal abnormalities in sperm (Hassold & Hunt, 2001; Egozcue *et al.*, 2005; Ferguson *et al.*, 2007; Martin, 2008; Sun *et al.*, 2008). Moreover, there is a clear relationship between the presence of increased incidence of apoptotic germ cells and male infertility (Lin *et al.*, 1997; Sun *et al.*, 1997; Tesarik *et al.*, 1998; Sakkas *et al.*, 1999; Sinha Hikim & Swerdloff, 1999; Paasch *et al.*, 2003; Wang *et al.*, 2003; Chen *et al.*, 2006; Zhang *et al.*, 2008; Aitken *et al.*, 2011). In addition, several studies described that a significant percentage of spermatozoa with chromosomal abnormalities (numerical or structural) also display apoptotic markers (Carrell *et al.*, 2003; Schmid *et al.*, 2003; Liu *et al.*, 2004; Muriel *et al.*, 2007; Brahem *et al.*, 2011; Enciso *et al.*, 2013; Perrin *et al.*, 2013; Rouen *et al.*, 2013).

Most of these studies have been performed in ejaculated spermatozoa; however, none of them have looked for any association in germ cells throughout the meiotic process. The objective of the present study was to evaluate the relationship between the

chromosomal abnormalities and apoptosis in different germ cell populations and in Sertoli cells from human testicular biopsies.

MATERIALS AND METHODS

Study samples

This study was undertaken using fragments of testicular biopsies obtained for meiotic analysis from 11 infertile patients. Three more samples were collected from three patients subjected to post-vasectomy TESE (Testicular Sperm Extraction) (**Table 1**). Samples were obtained under local anesthesia according to the surgical protocol of the medical center and were delivered to our laboratory in phosphate-buffered saline (PBS). The protocol was approved by the ethics committee of the collaborating center, and the patients gave their informed consent with regard to participation in the study.

Methodology

Samples were processed following a sequential methodology previously described in detail by Garcia-Quevedo et al. (2012). Tissue processing included the following five steps: enzymatic disaggregation, apoptotic staining, cell sorting, cell fixation and fluorescent in situ hybridization (FISH) analysis (**Fig. 1**).

The enzymatic disaggregation protocol was based on Brook et al. (2001) and adapted to the processing of human testicular tissue. It consisted of two successive disaggregations;

the first with RPMI 1640 medium containing L-Glutamin (Gibco, Invitrogen; Paisley, UK) supplemented with collagenase IA (Sigma-Aldrich GmbH, Steinheim, Germany) and the second with RPMI medium supplemented with Trypsin (GibCo, Invitrogen), sodium pyruvate (Sigma-Aldrich GmbH), EDTA (Fluka Chemie GmbH, Switzerland), hyaluronidase type IV (Sigma-Aldrich GmbH) and DNase I (Roche Diagnostics GmbH, Mannheim, Germany).

Specific apoptotic staining was performed with Annexin V conjugated with FITC (AV-FITC) and propidium iodide (PI) (Annexin-V-FLUOS Staining Kit; Roche Diagnostics GmbH). Cell population selection was carried out using a MoFlo flow cytometer (DakoCytomation, Fort Collins, Colorado, USA) equipped with a Coherent Enterprise II argon-ion laser. To establish the informative cell population discarding aggregates and cell fragments, we used the Side Scatter (linear SS) and the Forward scatter (FS log). We determined apoptotic, necrotic and viable cell populations using specific controls for Annexin V, PI and double negatives in each sample (**Fig. 2**). Once informative cells and fluorescence profiles of each fluorochrome were established, apoptotic (AV positive, PI negative) and viable (double negative; **Fig. 2**) populations of each sample were sorted. In all cases, a non-sorted fraction was separated prior to the sorting for further processing (**Fig. 1**).

Flow-sorted and non-sorted fractions of each sample were spread with a cytocentrifuge (Cytospin 3; Shandon Scientific Limited, UK) that allows a controlled cell number spread in a 28-mm^2 area. Subsequently, cells were fixed for 10 minutes in 4% paraformaldehyde solution in PBS, pH 7.4 (Sigma-Aldrich GmbH). Prior to FISH, three treatments were performed: membrane permeabilization with Triton X-100 (Sigma-Aldrich GmbH),

followed by elimination of cytoplasmic components with RNase A (Roche Diagnostics GmbH) and Pepsin (Sigma-Aldrich GmbH), and re-fixation of the sample with 1% formaldehyde (Sigma-Aldrich GmbH); full details in Garcia-Quevedo *et al*., (2012).

The chromosomal constitution of germ cells and Sertoli cells was evaluated by FISH using centromeric DNA probes for chromosomes X, Y and 18 (CEP Y Spectrum Orange; CEP X Spectrum Green, CEP 18 Spectrum Aqua; AneuVysion multicolor DNA Probe kit; Vysis, Abbott Molecular, Abbott Park, IL, USA). Prior to sperm hybridization, a decondensation treatment with DTT (Dithiothreitol; Roche Diagnostics GmbH) was applied. Cytogenetic evaluations were undertaken using an Olympus BX-60 fluorescent microscope equipped with specific filters for FITC, Cy3 and Aqua, and a multiband pass filter (DAPI/FITC/Texas Red). In each fraction (non-sorted, apoptotic and viable), we assessed the number of aneuploid and euploid cells for each cell category: interphase pre-meiotic germ cells (which includes spermatogonia and primary spermatocytes), meiotic figures, post-reductional germ cells (which includes secondary spermatocytes and spermatids) and spermatozoa. Furthermore, we also assessed the chromosome constitution of Sertoli cells in each fraction and patient sample. Cell identification and cell cytogenetic analysis was performed according to unequivocal and strict nuclear and chromosome morphology criteria described by our group (full details in Garcia-Quevedo *et al.*, 2012).

Statistical analysis

The statistical analysis software used was SAS v9.2 (SAS Institute Inc., Cary, NC, USA), the significance level was set at 0.05 with two degree of freedom.

The number of euploid and aneuploid cells of all 14 samples was pooled separately in the three fractions (non-sorted, apoptotic and viable). This was carried out for the five cell categories analyzed: pre-meiotic germ cells, meiotic figures, post-reductional germ cells, spermatozoa and Sertoli cells.

The percentage of chromosome abnormalities was compared between the three fractions in each cell category using a nonparametric model (Kruskal-Wallis test). If significant results were obtained, a two-by-two chi-square test (χ^2) was performed. This allows knowing which fractions display significant differences and their level of significance.

The analyses of percentages do not consider differences in cell numbers among samples. To overcome this limitation, a generalized linear model with repeated measures was applied, correcting the overdispersion problem of the response variable. For each fraction, the percentage of abnormal cells was estimated with a confidence interval of 95.

RESULTS

The enzymatic disaggregation allowed obtaining between 1.6 x 10^6 and 5.5 x 10^6 cells per testicular biopsy (**Table 2**). The percentage of informative cells stood around 60% except in 6BT (26.9%) and 9BT (36.4%). The percentage of viable cells ranged from 24 to 63.5%, while the percentage of apoptotic cells was between 4.6 and 30.6% (**Table 2**).

Cytogenetic analysis performed in each sample and fraction allowed the identification of aneuploid cells. Total numbers of euploid and aneuploid cells are detailed in **Table 3**. Overall, 32,902 cells were evaluated: 14,379 cells in the non-sorted fractions, 6,983 cells

in the viable fractions and 11,540 cells in the apoptotic fractions. One thousand cells per fraction and patient sample were selected for evaluation; however, in a few cases, samples obtained did not allow achieving this number (full detailed descriptions of the cytogenetic analyses are reported in **Tables S1–3**).

The mean data for each fraction and cell category was obtained from the pooled percentage of abnormalities in the fourteen patients (**Fig. 3**). The analysis of pre-meiotic germ cells showed significant differences in the percentage of aneuploid cells among the three fractions (χ^2 = 14.29, p-value = 0.0008). Specifically, the apoptotic fraction showed a significant increase of aneuploidy compared to viable and non-sorted fractions (**Table 4**). The percentage of abnormalities observed in meiotic figures (only prophases I were observed) was not different among the three fractions (χ^2 = 0.96, p-value = 0.6185). The same result was observed in post-reductional germ cells (χ^2 = 0.073, p-value = 0.9642) and spermatozoa (χ^2 = 3.14, p-value = 0.2075).

Regarding Sertoli cells, statistical analysis shows no association between the presence of aneuploidy and apoptotic processes (χ^2 = 2.16, p-value = 0.3430).

DISCUSSION

In this study, we report the usefulness of a previously described protocol (Garcia-Quevedo *et al.*, 2012) to investigate the correlation between chromosome aneuploidy and apoptosis in human testicular cells.

Our results clearly indicate that aneuploid pre-meiotic cells are eliminated by an apoptotic PS externalization-mediated process. Although the mechanisms that regulate this depletion in germ cells remain unknown, the high rate of spermatogonium proliferation, as well as the aneuploid cell depletion observed in this study, may indicate an elimination pathway similar to the mechanism described in mitotic cells. In these cells, it has been observed that the presence of aneuploidies would increase the cellular metabolism (Torres *et al.*, 2007; Williams *et al.*, 2009). Moreover, a relationship has been described between the gain of chromosomes and gene overexpression, which should lead to cellular metabolic alterations. These changes are associated with alterations that affect protein expression related to the synthesis and repair of DNA and with increases of ROS (Reactive Oxygen Species) causing apoptosis of aneuploid cells via ATM and P53 (reviewed by Fang & Zhang, 2011; Gordon *et al.*, 2012). Although additional analyses, as transcriptional studies, are needed to confirm it, our results suggest the involvement of a similar mechanism in the removal of pre-meiotic aneuploid germ cells.

Regarding meiotic figures, the apoptosis-dependent mechanisms to eliminate pre-meiotic germ cells with aneuploidies described above, leads to a small number of aneuploid prophases I (32/2290) and especially pachytenes (n = 10). This situation makes it difficult to determine the association between the presence of aneuploidy and the activation of apoptotic processes. In addition, it has been reported that elimination via apoptosis of cells recognized by the synapsis and recombination checkpoint is conditioned by the accumulation of arrested cells (Hamer *et al.*, 2008). Thus, the presence of a reduced population of pachytene cells with aneuploidies would not reach

the threshold to trigger cell elimination via apoptosis (Hamer *et al.*, 2008). Moreover, the lack of apoptosis-aneuploidies association may also be determined by the occurrence of well-known meiotic rescue mechanisms to avoid the exposure of asynaptic regions (Blanco *et al.*, 2001; Turner *et al.*, 2005; Burgoyne *et al.*, 2009) that allow aneuploid pachytene cells to escape elimination.

The high percentage of aneuploid post-reductional germ cells observed in our study contrasts with the small number of aneuploid prophase I cells. Consequently, these cells could only have originated during the homologous segregation at meiosis I. There are no studies describing the possible molecular pathway of cell elimination associated with the meiotic Spindle Assembly Checkpoint (SAC). However, our results and others (Eaker *et al.*, 2001; Ferguson *et al.*, 2007; Sun *et al.*, 2008) suggest that although the SAC checkpoint detects chromosome misalignments, it has a limited depletion efficiency leading to an increase in chromosomal abnormalities in post-reductional cells. Although statistical analysis in post-reductional germ cells did not show an association between the presence of chromosomal abnormalities and apoptosis, about half of these cells did not differentiate into sperm. This suggests the presence of other cell elimination pathways, such as precocious spermiation of degenerating germ cells or degeneration en masse as multinucleated symplasts (Francavilla *et al.*, 2002; Sofikitis *et al.*, 2008; Yan, 2009). These pathways will discard degenerating germ cells independently of apoptosis mediated by phosphatidylserine (PS) externalization and consequently, they could not be identified by the apoptotic marker used in this study.

Aneuploid testicular spermatozoa were not significantly associated with the externalization of the phosphatidylserine, suggesting that this condition is not an

apoptotic inductor at this spermatic stage. This lack of correlation has been also described recently in ejaculated spermatozoa (Vendrell *et al.*, 2013). However, a clear relationship between ejaculated sperm aneuploidy and DNA fragmentation, which is a common feature of apoptosis, has been reported by several authors (Schmid *et al.*, 2003; Muriel *et al.*, 2007; Perrin *et al.*, 2011; Enciso *et al.*, 2013; Rouen *et al.*, 2013; Vendrell *et al.*, 2013). This association has been explained in different ways: Rouen *et al*. (2013) suggested that aneuploid sperm disturb the fine three-dimensional architecture of the sperm nucleus, leading to a higher sensitivity to exogenous factors of apoptosis and the increase of DNA fragmentation during the transit through the genital tract. Vendrell *et al*. (2013) described a two-stage process occurring also during epididymal sperm maturation; an early destabilizing stage concomitantly with DNA fragmentation and a second stage characterized by phosphatidylserine externalization. Aitken *et al*. (2011) reported that DNA fragmentation could also reflex the induction of an incomplete, apoptotic response (abortive apoptosis) during spermatogenesis that results in endonuclease-mediated DNA cleavage but fails to compromise cell viability. If this is the case, DNA fragmentation could not be directly linked to the expression of apoptotic markers like PS externalization (Sakkas *et al.*, 2002; Reviewed by Perrin *et al.*, 2013). Analyzing all this information together, our results are consistent with those advocating for a mechanism of elimination of aneuploid spermatozoa occurring preferentially during the epididymal transit. However, more studies are needed to perform a consistent hypothesis.

Finally, a low percentage of Sertoli cells with aneuploidies contribute to the lack of association between aneuploidy and apoptosis observed in this study. Furthermore,

these cells are quiescent and remain in an irreversible G0 stage, and an absence of apoptotic markers has been described (Tay *et al.*, 2007; Hassan *et al.*, 2009; Stiblar-Martincic, 2009) possibly due to a mechanism that protects them against programmed cell death (Johnson *et al.*, 2008) and explaining our results.

Overall, it can be suggested that elimination mechanisms of aneuploid germ cells are determined by different mechanisms that take into account the specialization of the testicular epithelium and the cells that form it. In our study, we have observed that aneuploid cell elimination though an apoptotic PS externalization process occurs in pre-meiotic cells located in the basal compartment of the seminiferous tubules. This compartment is separated from the adluminal compartment through the Blood-Testis Barrier (BTB), which isolates germ cells situated in the basal compartment from the systemic circulation (Holstein *et al.*, 2003; Cheng & Mruk, 2010). Therefore, our results indicate that beyond the Blood-Testis Barrier, aneuploid germ cells and spermatozoa elimination does not involve PS externalization.

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AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: FV, JB. Performed the experiments: LGQ, ZS. Analyzed the data: FV, JB, LGQ. Wrote the manuscript: LGQ, FV, JB. Final approval of the manuscript: LGQ, ZS, FV, JB.

CONFLICT OF INTERESTS

None

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FIGURE LEGENDS

Figure 1: Experimental design diagram.

Figure 2: Graphical representation of the FITC/PI fluorescence ratio showing the three cell populations: Apoptotic (green), viable (blue), and necrotic (red) populations.

Figure 3: Box plot of the percentage of aneuploid cells observed in each cell category for the three fractions analyzed. Outliers are represented with circles, and the median is represented with diamonds inside each box (Pre-M: pre-meiotic cells; Post-R: post-reductional cells; Spz: spermatozoa).

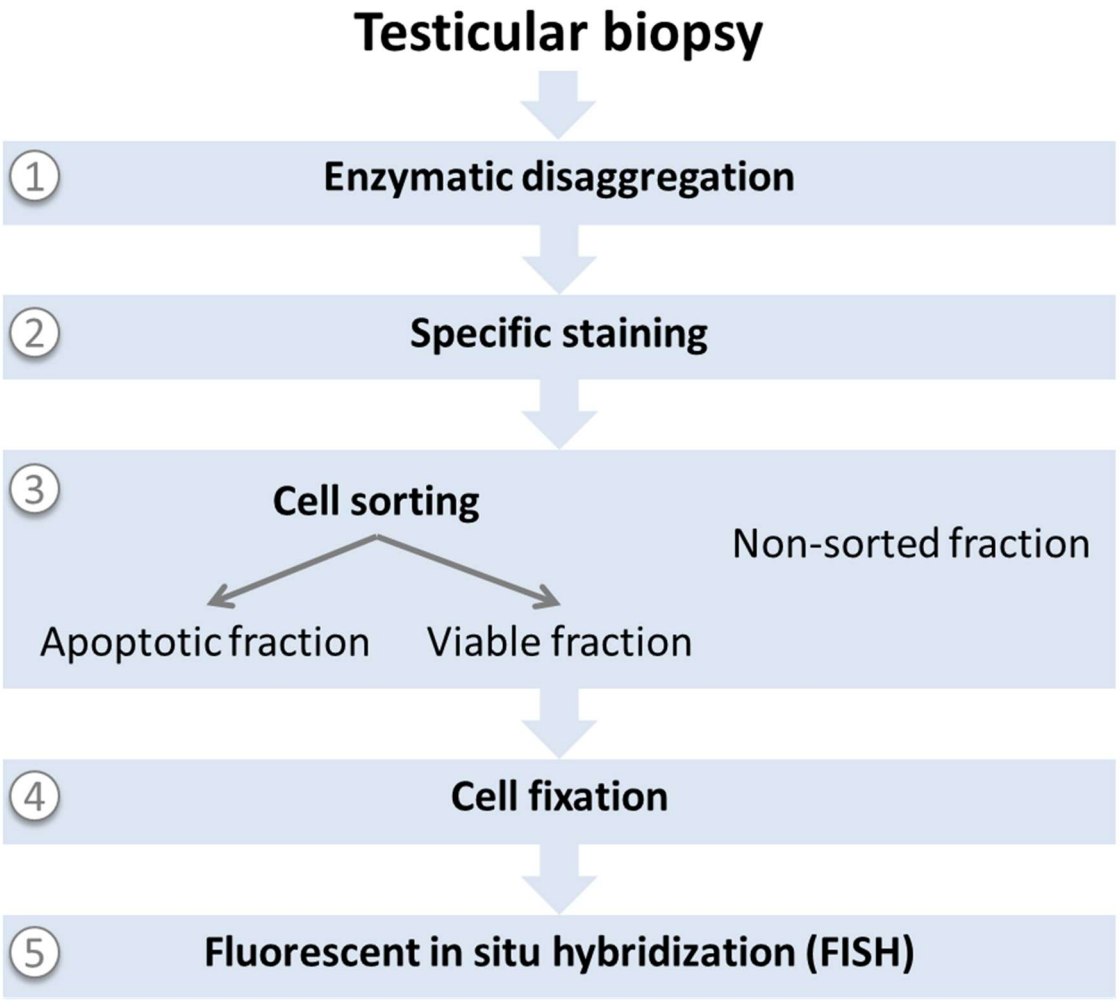
Testicular biopsy
1
Enzymatic disaggregation
2
Specific staining
3
Cell sorting
Non-sorted fraction
Apoptotic fraction
Viable fraction
4
Cell fixation
5
Fluorescent in situ hybridization (FISH)

Figure 1.

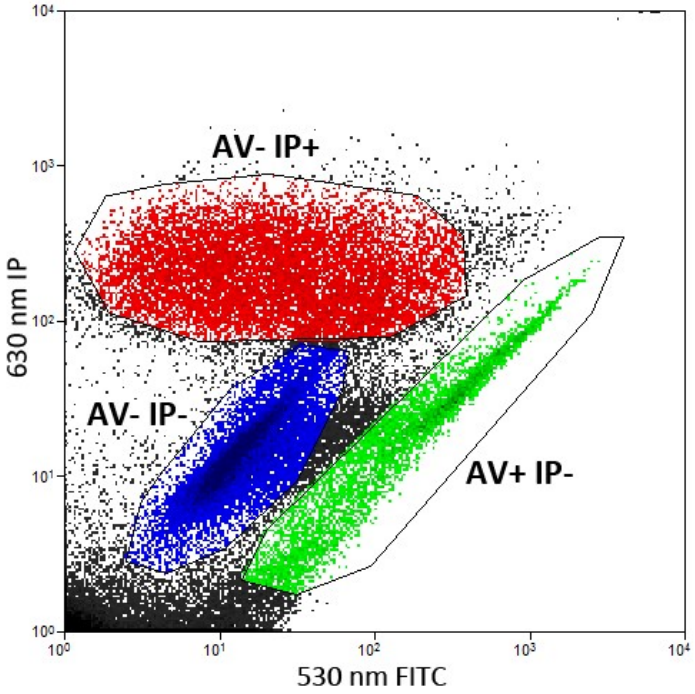

Figure 2

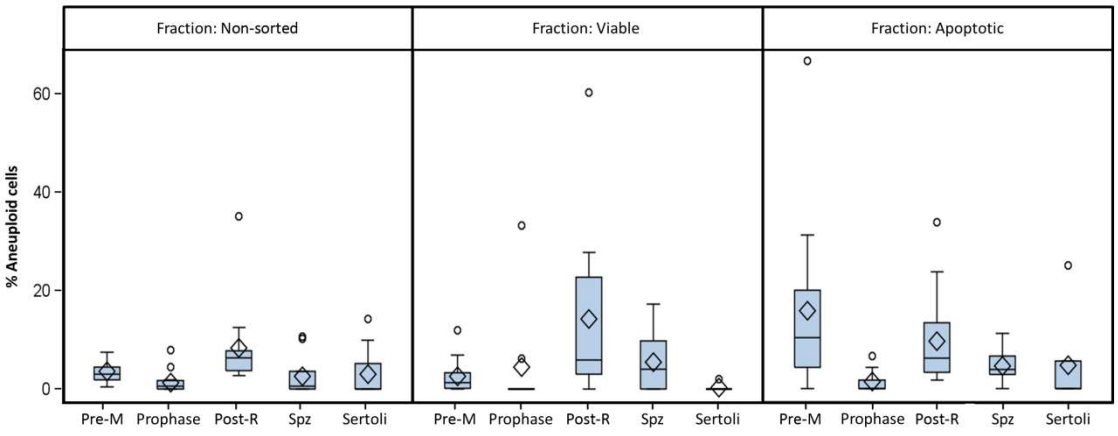
Fraction: Non-sorted
Fraction: Viable
Fraction: Apoptotic
% Aneuploid cells
0
20
40
60
Pre-M
Prophase
Post-R
Spz
Sertoli

Figure 3.

TABLES

Table 1: Patient characteristics (N.A.: Non applicable)

	Age	Clinical indications	Seminogram	Meiotic study result
1BT	52	Post-vasectomy TESE	Azoospermic	N.A.
2BT	46	Post-vasectomy TESE	Azoospermic	N.A.
3BT	41	Meiotic study	Oligoasthenoteratozoospermic	Altered
4BT	37	Meiotic study	Normozoospermic	Altered
5BT	39	Meiotic study	Normozoospermic	Normal
6BT	36	Meiotic study	Azoospermic	Altered
7BT	36	Meiotic study	Azoospermic	Altered
8BT	50	Post-vasectomy TESE	Azoospermic	N.A.
9BT	38	Meiotic study	Asthenoteratozoospermic	Normal
10BT	35	Meiotic study	Normozoospermic	Normal
11BT	35	Meiotic study	Asthenozoospermic	Normal
12BT	non reported	Meiotic study	Normozoospermic	Altered
13BT	38	Meiotic study	Oligozoospermic	Normal
14BT	40	Meiotic study	Oligoasthenozoospermic	Altered

Table 2: Disaggregation and flow cytometry sorting results.

	Disaggregated cells	Flow sorted cells	Informative cells[1]	Sorted viable cells	Sorted apoptotic cells
1BT	2.3×10^6	$1.4 \cdot 10^6$	$8.5 \cdot 10^5$ (60.4%)	$2 \cdot 10^5$ (63.5%)	$2.3 \cdot 10^5$ (16.3%)
2BT	2.7×10^6	1.2×10^6	6.5×10^5 (54.1%)	$5 \cdot 10^5$ (62.5%)	$3.4 \cdot 10^5$ (9.8%)
3BT	4.6×10^6	3.1×10^6	2.3×10^6 (75.2%)	$1.6 \cdot 10^6$ (60.8%)	$7.3 \cdot 10^5$ (28.8%)
4BT	4.4×10^6	2.9×10^6	1.6×10^6 (56.6%)	$8 \cdot 10^5$ (42.8%)	$4.3 \cdot 10^5$ (30.6%)
5BT	5.5×10^6	4×10^6	2.5×10^6 (62.7%)	$1.2 \cdot 10^6$ (44.2%)	$7.8 \cdot 10^5$ (20.3%)
6BT	1.6×10^6	1×10^6	2.7×10^5 (26.9%)	$1.3 \cdot 10^5$ (52.1%)	$1.3 \cdot 10^5$ (14.3%)
7BT	1.7×10^6	1.3×10^6	8.6×10^5 (66.6%)	$2.5 \cdot 10^5$ (41.5%)	$1.2 \cdot 10^5$ (12.1%)
8BT	3.4×10^6	3.1×10^6	2×10^6 (63.8%)	$8.5 \cdot 10^5$ (44.6%)	$2.5 \cdot 10^5$ (25.3%)
9BT	2.3×10^6	2×10^6	7.3×10^5 (36.4%)	$3.6 \cdot 10^5$ (54.1%)	$1 \cdot 10^5$ (10.3%)
10BT	1.8×10^6	1.5×10^6	1×10^6 (69.7%)	$5.2 \cdot 10^5$ (43.5%)	$1.8 \cdot 10^5$ (8.4%)
11BT	2.1×10^6	2×10^6	1.3×10^6 (64.1%)	$4.4 \cdot 10^5$ (24,0%)	$1.5 \cdot 10^5$ (8.7%)
12BT	2.3×10^6	2×10^6	1.1×10^6 (54.5%)	$5.1 \cdot 10^5$ (38.3%)	$4 \cdot 10^5$ (6.3%)
13BT	2×10^6	1.8×10^6	1.1×10^6 (61.4%)	$6.3 \cdot 10^5$ (40.8%)	$3 \cdot 10^5$ (4.6%)
14BT	3.4×10^6	3.1×10^6	2.5×10^6 (79.5%)	$3.4 \cdot 10^5$ (26.3%)	$3.7 \cdot 10^5$ (12.3%)
Mean	2.9×10^6	2.2×10^6	1.3×10^6 (59.4%)	$5.5 \cdot 10^5$ (45.6%)	$3.5 \cdot 10^5$ (14.9%)
SD	1.2×10^6	9.1×10^5	7.2×10^5 (13.9%)	$5.4 \cdot 10^5$ (12.1%)	$5.9 \cdot 10^5$ (8.3%)

[1] Cells processed for sorting after discarding aggregates and cell fragments

Table 3: Cytogenetic results in the three fractions and for the five cell categories in all samples analyzed.

Fraction	Pre-meiotic		Meiotic figures *		Post-reductional		Spermatozoa		Sertoli cells		Others**	Total
	Euploid	Aneuploid	Euploid	Aneuploid	Euploid	Aneuploid	Euploid	Aneuploid	Euploid	Aneuploid		
Non-sorted	4,913	152	1,443	21	5,422	1,263	779	15	269	7	95	14,379
Viable	3,448	68	297	2	1,703	691	420	62	248	2	42	6,983
Apoptotic	1,697	181	518	9	6,349	1,069	1,416	86	119	4	92	11,540

* Only Prophases I were found.

** Polyploid cells and non-expected signal combinations

Table 4: Aneuploid pre-meiotic germ cells. Statistical comparisons between fractions (bold p-values indicate significant differences).

Fraction	Fraction	Estimate	Chi-Square	P-value	Error rate
Apoptotic	**Viable**	1.5871	38.74	**<.0001**	4.89
Apoptotic	**Non-sorted**	1.1340	36.36	**<.0001**	3.11
Viable	**Non-sorted**	-0.4531	2.38	0.1227	0.64

1

2