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ORIGINAL ARTICLE

Male Infertility

MACS-annexin V cell sorting of semen samples with high TUNEL values decreases the concentration of cells with abnormal chromosomal content: a pilot study

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We question whether, in men with an abnormal rate of sperm DNA fragmentation, the magnetic-activated cell sorting (MACS) could select spermatozoa with lower rates of DNA fragmentation as well as spermatozoa with unbalanced chromosome content. Cryopreserved spermatozoa from six males were separated into nonapoptotic and apoptotic populations. We determined the percentages of spermatozoa with (i) externalization of phosphatidylserine (EPS) by annexin V-Fluorescein isothiocyanate (FITC) labeling, (ii) DNA fragmentation by TdT-mediated-dUTP nick-end labeling (TUNEL), and (iii) numerical abnormalities for chromosomes X, Y, 13, 18, and 21 by fluorescence *in situ* hybridization (FISH), on the whole ejaculate and selected spermatozoa in the same patient. Compared to the nonapoptotic fraction, the apoptotic fraction statistically showed a higher number of spermatozoa with EPS, with DNA fragmentation, and with numerical chromosomal abnormalities. Compared to the whole ejaculate, we found a significant decrease in the percentage of spermatozoa with EPS and decrease tendencies of the DNA fragmentation rate and the sum of disomy levels in the nonapoptotic fraction. Conversely, we observed statistically significant higher rates of these three parameters in the apoptotic fraction. MACS may help to select spermatozoa with lower rates of DNA fragmentation and unbalanced chromosome content in men with abnormal rates of sperm DNA fragmentation.

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INTRODUCTION

An increased rate of apoptotic markers in ejaculated spermatozoa of infertile men has been reported by several authors.[1–5] These data suggest a possible relationship between these markers and male infertility.[6,7] The apoptotic markers are mainly an externalization of phosphatidylserine (EPS), a decrease in mitochondrial membrane potential, a caspase activation and DNA fragmentation.[2,8,9] Numerous studies have shown the negative effects of sperm DNA fragmentation on the results of assisted reproduction outcomes concerning embryonic development, and/or fertilization rates, and/or implantation rates, and/or spontaneous miscarriages, and/or pregnancies, and/or births.[10–16]

Chromosomal abnormalities in spermatozoa also affect male fertility.[17] They mainly concern men with constitutive abnormal karyotypes. However, certain subgroups of infertile males with normal karyotypes also produce spermatozoa with abnormal chromosomal equipment.[18] From a retrospective study in 319 infertile patients, sex chromosome disomy and disomy of chromosome 21 as well as aneuploidy increased by 2- to 3-fold compared to the fertile control group.[19] These chromosomal abnormalities found in the spermatozoa of infertile men are considered to be one of the causes of pregnancy failure, implantation failure after ICSI, miscarriage, and high frequency of aneuploidy in embryos.[20–25]

Previous studies showed the presence of DNA fragmentation positively correlated with the presence of chromosomal abnormalities in the same spermatozoa of infertile patients with normal semen parameters, abnormal semen parameters, or infertile patients with a structural chromosome abnormality.[26–30] Muriel *et al.*[27] suggested that the occurrence of aneuploidy during sperm maturation could trigger DNA fragmentation via an apoptosis-like process mediated by endogenous nucleases.

Therefore, the question that arises was: how can chromosomally normal sperm without fragmented DNA be selected? Conventional methods used in assisted reproduction technique (ART) select spermatozoa according to their mobility and morphology, but those methods are not predictive of the quality of the chromosomal equipment. Another technique, magnetic sorting using activated cell

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sorting (MACS) for annexin V-negative cells, has been proposed as a method for the selection of nonapoptotic spermatozoa.[31,32] Indeed, annexin V has a high affinity for membrane phosphatidylserines,[33,34] which are externalized by apoptotic spermatozoa. Therefore, MACS technique involves incubating sperm with magnetic microbeads conjugated with annexin V. After incubation, spermatozoa were selected by going through a column placed under a magnetic field. Therefore, sperm is separated into two populations: nonapoptotic fraction (spermatozoa without EPS, or spermatozoa negative for annexin V) and apoptotic fraction (spermatozoa with EPS, or spermatozoa positive for annexin V).

In this study, we question whether, in men with an abnormal rate of sperm DNA fragmentation, specific MACS by annexinV could select spermatozoa with lower levels of DNA fragmentation as well as unbalanced chromosome content. Furthermore, the levels of spermatozoa with EPS, DNA fragmentation, and chromosomal imbalance after MACS were compared to the total ejaculate population in the same patient.

PATIENTS AND METHODS

Patients

Six samples of cryopreserved sperm from infertile males with an abnormal rate of sperm DNA fragmentation were included in the study. Straws of cryopreserved ejaculated sperm were selected from the sperm bank dedicated to research at the Department of Medical Genetics and Reproductive Biology, Brest, France. The threshold value of DNA fragmentation in our Department of Medical Genetics and Reproductive Biology performed by the TdT-mediated-dUTP nick-end labeling (TUNEL) technique is 7%. A value more than or equal to 7%, in our department, orientated the patients to treatment. All six men were informed of the investigations and gave their written consent. Our study is approved by the ethics committee of Brest University Regional Hospital (ethic code: 29BRC19.0258).

Study design

The study design is presented in **Figure 1**. Briefly, the sperm was thawed for 30 min at room temperature and washed by adding 1 ml binding buffer (1×BB; Miltenyi Biotec SAS, Paris, France) to eliminate the cryoprotectant. The washed sample was then separated into 4 aliquots, for DNA fragmentation analyses by TUNEL, for aneuploidy analyses by fluorescence *in situ* hybridization (FISH), for externalization of phosphatidylserine determination by annexin V staining, and for MACS.

MACS

The sample was incubated with 100 µl 1×BB and annexin-V microbeads (Miltenyi Biotec SAS) for 15 min at room temperature. An additional aliquot was kept for the study of DNA fragmentation, and the remaining spermatozoa-microbead mix was placed on the separation column containing magnetic beads (MiniMACS, Miltenyi Biotec SAS). The spermatozoa negative for EPS passed through the column and were considered as annexin V (−) fraction. Elution using 1×BB allowed the collection of annexin V (+) spermatozoa, corresponding to sample with EPS. The externalization of phosphatidylserine, DNA fragmentation and aneuploidy rate were carried out on both annexin V (−) and annexin V (+) fractions of each patient.

Annexin V-FITC staining

The externalization of phosphatidylserine was detected by Annexin V-FITC Kit (Miltenyi Biotec SAS). The procedure has been previously published.[35] For each sample, five hundred spermatozoa were analyzed.

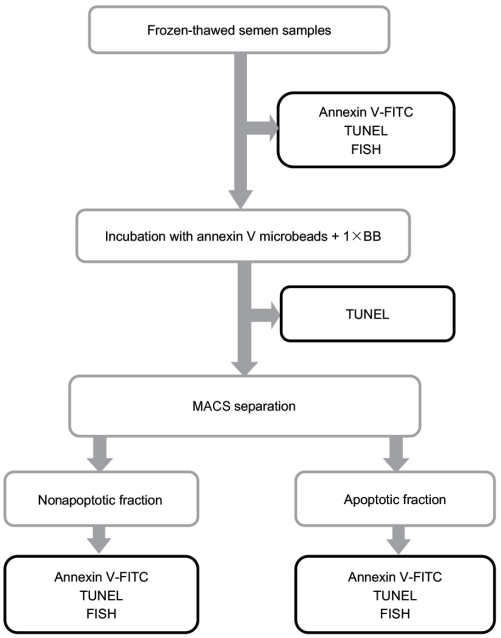

Figure 1: Flow diagram of the overall experimental design. FITC: fluorescein isothiocyanate; FISH: fluorescence *in situ* hybridization; BB: binding buffer; MACS: magnetic-activated cell sorting; TUNEL: TdT-mediated-dUTP nick-end labeling.

The spermatozoa without EPS showed blue fluorescence and those with EPS showed green fluorescence.

TUNEL assay

DNA fragmentation was detected by the ApopTag® Red Kit In Situ (Millipore, Saint-Quentin-en-Yvelines, France) according to the manufacturer's recommendations. The TUNEL assay has been described previously.[35,36] For each sample, five hundred spermatozoa were analyzed. Spermatozoa were considered to contain either fragmented (red fluorescence) or normal (blue fluorescence) DNA.[36]

FISH

Numerical abnormalities of chromosomes 13, 18, 21, X, and Y were evaluated. Triple FISH (chromosomes X, Y, and 18) was carried out with specific alphoid probes for the X chromosome (probe DXZ1, spectrum green; Abbott, Rungis, France) and Y chromosome (probe DYZ3, spectrum orange; Abbott), and a mix of bacterial artificial chromosome (BAC) clones localized in 18q11.1-q11.2 (RP11-676M19, RP11-746M23, RP11-311F3, RP11-446A4, RP11-510P5, and RP11-1076F2) labeled in spectrum aqua (Abbott).

Dual FISH (chromosomes 13 and 21) was carried out with a mix of BAC specific for 13q33.3 (RP11-1122H21, RP11-158B21, RP11-468K20, RP11-1104E18, RP11-974B24, and RP11-183A20) labeled in spectrum green and BAC for 21q22.11 (RP11-698D15, RP11-760B14, RP11-159L9, RP11-30C7, RP11-369E2, and RP11-1071P10) labeled in spectrum orange. The procedure has been previously published.[35,37] For each sample, a minimum of 1000 spermatozoa was examined using a Zeiss Axioplan microscope (Zeiss, Le Pecq, France).

Statistical analyses

An independent Chi-square test was used to compare the percentages before and after MACS of spermatozoa with EPS, DNA fragmentation, and numerical abnormalities for chromosomes 13, 21, X, Y, and 18. An independent Chi-square test was also used to compare the frequencies

of the different chromosomal abnormalities before and after MACS. The data were studied online using the BiostaTGV software (http://marne.u707.jussieu.fr/biostatgv/). $P < 0.05$ was considered statistically significant.

RESULTS

Annexin V-FITC staining

Table 1 shows the percentage of spermatozoa presenting an externalization of phosphatidylserine before and after MACS in apoptotic and nonapoptotic fractions. The percentage (mean ± standard deviation [s.d.]) of spermatozoa with EPS after thawing is 36.1% ± 10.1%. After selection, the percentage (mean ± s.d.) of spermatozoa with EPS decreases significantly in nonapoptotic fraction (19.4% ± 1.7%, $P < 0.05$), while increasing significantly in apoptotic fraction (65.0% ± 13.0%, $P < 0.05$). We observed a statistically significant difference between the nonapoptotic and apoptotic fractions ($P < 0.05$).

TUNEL assay

Table 2 presents the rate of DNA fragmentation in a frozen-thawed semen sample, after incubation, and in apoptotic and nonapoptotic fractions. Regarding the mean value of sperm DNA fragmentation, the percentage (mean ± s.d.) of DNA fragmentation after thawing is 24.8% ± 5.6%; whereas after incubation with microbeads and 1×BB, it is significantly higher at 30.7% ± 7.8% ($P < 0.05$). After passage through the column, the fragmentation percentage (mean ± s.d.) in the nonapoptotic fraction is 27.4% ± 8.7%. A decreasing trend is observed in the majority of patients in the nonapoptotic fraction. This rate (mean ± s.d.) increases significantly to 44.1% ± 11.3% in the apoptotic fraction ($P < 0.05$). We also observed a significant difference between the nonapoptotic and apoptotic fractions ($P < 0.05$).

Table 1: Percentage of spermatozoa in annexin V-FITC (+), in frozen-thawed semen samples, in nonapoptotic and apoptotic fractions for each patient

Patient	*Frozen-thawed semen sample (%)*	*Nonapoptotic fraction (%)*	*Apoptotic fraction (%)*	*Between nonapoptotic and apoptotic fractions*
P1	29.0	20.0*	68.0*	#
P2	41.0	17.8*	86.1*	#
P3	21.0	17.0	NA	NA
P4	47.8	21.6*	61.0*	#
P5	33.6	19.8*	55.2*	#
P6	44.4	20.2*	54.5*	#
Mean±s.d.	36.1±10.1	19.4±1.7*	65.0±13.0*	#

*$P<0.05$, significant difference when the indicated item compared to the frozen-thawed semen sample; #$P<0.05$, significant difference when the indicated item compared to the nonapoptotic and apoptotic fractions. NA: not available; s.d.: standard deviation; FITC: fluorescein isothiocyanate

FISH

The results for FISH of chromosomes 13 and 21 are shown in **Table 3**. The percentage (mean ± s.d.) of normal spermatozoa after thawing is 96.9% ± 1.9%. Compared to frozen-thawed semen samples, there was a significant increase ($P < 0.05$) in the percentage (mean ± s.d.) of normal spermatozoa in nonapoptotic fraction (98.4% ± 0.9%). In contrast, we did not observe any significant difference in the apoptotic fraction (mean ± s.d.: 96.1% ± 1.9%). However, we observed a significant difference ($P < 0.05$) between the nonapoptotic and apoptotic fractions, with a majority of normal spermatozoa in the nonapoptotic fraction versus unbalanced spermatozoa in the apoptotic fraction.

The results for FISH of chromosomes X, Y and 18 are shown in **Table 4**. The percentage (mean ± s.d.) of normal spermatozoa after thawing is 97.1% ± 1.0%, whereas it is 97.5% ± 1.1% in nonapoptotic fraction. The rate (mean ± s.d.) is 95.8% ± 1.4% in the apoptotic fraction. No significant difference was found between before and after MACS. However, we observed a significant difference between the nonapoptotic and apoptotic fractions ($P < 0.05$), with a majority of normal spermatozoa in the nonapoptotic fraction versus unbalanced spermatozoa in the apoptotic fraction.

The results concerning the frequencies of the different chromosomal abnormalities are shown in **Table 5**. Data from all patients were grouped according to the type of numerical chromosomal abnormality. The frequencies of chromosomal abnormalities are significantly higher in the apoptotic fraction compared to the frozen-thawed semen sample ($P < 0.05$), except for disomy 13 and disomy 21. Compared to the frozen-thawed semen sample, there was a significant increase in the sum of the disomy and the sum of diploidy in the apoptotic fraction ($P < 0.05$). Moreover, we observed a significant difference between the nonapoptotic and apoptotic fractions ($P < 0.05$), with a majority of normal spermatozoa in the nonapoptotic fraction and unbalanced spermatozoa in the apoptotic fraction concerning the levels of disomy Y, disomy XY, disomy 21, diploidy, the sum of disomy, and the sum of diploidy.

DISCUSSION

In this study, we determined the percentages of spermatozoa with EPS, with DNA fragmentation, and with numerical abnormalities of chromosomes 13, 18, 21, X and Y on total ejaculate and on MACS-sorted spermatozoa, from 6 men presenting an abnormal rate of sperm DNA fragmentation.

Analysis of the average of externalization of phosphatidylserine showed a significant decrease between the spermatozoa negative for EPS in the nonapoptotic fraction versus the frozen-thawed semen samples. On the contrary, the externalization of phosphatidylserine significantly increased in the apoptotic fraction compared to the frozen-thawed semen samples. Those trends were also identified in

Table 2: Percentage of DNA fragmentation in frozen-thawed semen samples, after incubation, in nonapoptotic and apoptotic fractions for each patient

Patient	*Frozen-thawed semen sample (%)*	*After incubation (%)*	*Nonapoptotic fraction (%)*	*Apoptotic fraction (%)*	*Between nonapoptotic and apoptotic fractions*
P1	31.0	29.0	32.0	46.0#	†
P2	17.3	22.6*	18.0	57.6#	†
P3	25.5	35.0*	33.0	53.0#	†
P4	29.0	43.8*	37.8	46.4	†
P5	27.2	29.8	27.8	33.6	†
P6	18.8	23.8	16.0#	28.0	†
Mean±s.d.	24.8±5.6	30.7±7.8*	27.4±8.7	44.1±11.3#	†

*$P<0.05$, significant difference when the indicated item compared to the frozen-thawed semen sample; #$P<0.05$, significant difference when the indicated item compared to that after incubation; †$P<0.05$, significant difference when the indicated item compared to the nonapoptotic and apoptotic fractions. s.d.: standard deviation

Table 3: Percentage of normal spermatozoa after fluorescence *in situ* hybridization of chromosomes 13 and 21 in frozen-thawed semen samples, in nonapoptotic and apoptotic fractions for each patient

Patient	*Frozen-thawed semen sample (%)*	*Nonapoptotic fraction (%)*	*Apoptotic fraction (%)*	*Between nonapoptotic and apoptotic fractions*
P1	97.8	98.7	96.5	#
P2	97.9	98.9	98.2	NS
P3	97.7	98.7	97.3	#
P4	95.5	97.9*	93.6	#
P5	93.8	96.8*	93.7	#
P6	99.0	99.2	97.1*	#
Mean±s.d.	96.9±1.9	98.4±0.9*	96.1±1.9	#

*$P<0.05$, significant difference when the indicated item compared to the frozen-thawed semen sample; #$P<0.05$, significant difference when the indicated item compared to the nonapoptotic and apoptotic fractions. NS: not significant; s.d.: standard deviation

Table 4: Percentage of normal spermatozoa after fluorescence *in situ* hybridization of chromosomes X, Y and 18 in frozen-thawed semen samples, in nonapoptotic and apoptotic fractions for each patient

Patient	*Frozen-thawed semen sample (%)*	*Nonapoptotic fraction (%)*	*Apoptotic fraction (%)*	*Between nonapoptotic and apoptotic fractions*
P1	97.2	98.0	96.5	#
P2	98.7	99.0	97.2*	#
P3	96.1	95.6	93.7*	NS
P4	96.2	97.2	94.9	#
P5	96.6	97.1	95.3	#
P6	97.7	97.9	97.0	NS
Mean±s.d.	97.1±1.0	97.5±1.1	95.8±1.4	#

*$P<0.05$, significant difference when the indicated item compared to the frozen-thawed semen sample; #$P<0.05$, significant difference when the indicated item compared to the nonapoptotic and apoptotic fractions. NS: not significant; s.d.: standard deviation

Table 5: Percentage of the different chromosomal abnormalities in frozen-thawed semen samples, in nonapoptotic and apoptotic fractions for each patient

Chromosomal abnormality	*Frozen-thawed semen sample, n/total (%)*	*Nonapoptotic fraction, n/total (%)*	*Apoptotic fraction, n/total (%)*	*Between nonapoptotic and apoptotic fractions*
Disomy 18	9/6216 (0.1)	13/6144 (0.2)	20/6317 (0.3)*	NS
Disomy X	5/6216 (0.1)	6/6144 (0.1)	14/6317 (0.2)*	NS
Disomy Y	3/6216 (0.1)	2/6144 (0)	17/6317 (0.3)*	#
Disomy XY	41/6216 (0.7)	46/6144 (0.7)	69/6317 (1.1)*	#
Disomy 13	29/6115 (0.5)	13/6092 (0.2)*	17/5978 (0.3)	NS
Disomy 21	45/6115 (0.7)	30/6092 (0.5)	57/5978 (0.9)	#
Diploidy (assessed by chromosomes 18, X and Y)	33/6216 (0.5)	26/6144 (0.4)	56/6317 (0.9)*	#
Diploidy (assessed by chromosomes 13 and 21)	39/6115 (0.6)	36/6092 (0.6)	78/5978 (1.3)*	#
Sum of disomy	132/12 331 (1.1)	110/12 236 (0.9)	194/12 295 (1.6)*	#
Sum of diploidy	72/12 331 (0.6)	62/12 236 (0.5)	134/12 295 (1.1)*	#

*$P<0.05$, significant difference when the indicated item compared to the frozen-thawed semen sample; #$P<0.05$, significant difference when the indicated item compared to the nonapoptotic and apoptotic fractions. NS: not significant

our previously published study in men with a structural chromosomal abnormality[35] and were similar to that found by other teams.[35,38–40] Moreover, our results confirm that MACS technique can effectively eliminate spermatozoa with EPS caused by apoptotic processes or caused by the premature acrosomal reaction.[35,38–40] However, in previous studies, we hypothesized that the number of beads or unspecific binding could be limited and these parameters could explain the presence of spermatozoa with EPS in the nonapoptotic fraction and/or the presence of spermatozoa negative for EPS in the apoptotic fraction.[35,41]

Concerning the analysis of DNA fragmentation, numerous studies have tried to select sperm without fragmented DNA in different types of populations, especially in infertile men,[42,43] in men with normal and abnormal semen parameters,[44–49] in cases of high sperm DNA fragmentation levels,[50–52] in cases of varicocele,[53] and in cases of idiopathic implantation failure.[38] The majority of these studies reported a significant decrease in fragmented spermatozoa levels, but the majority of authors tested the efficiency of MACS in association with the swim-up and/or density gradient.

Few studies have assessed the efficacy of the MACS technique alone.[43,46,48,54] These studies reported a significant decrease in fragmented spermatozoa levels after selection by MACS in the nonapoptotic fraction. However, the decrease of the percentage of DNA fragmentation varies from one study to another. Tavalaee *et al*.[46] showed that MACS technique reduces the DNA fragmentation rate by 26.9%. More recently, Berteli *et al*.[43] have shown that MACS technique makes it possible to reduce by 66.7% DNA fragmentation rate.

In our study, as some authors have demonstrated that freezing process could increase sperm fragmentation, to avoid this bias, we decided to use cryopreserved samples for all patients. We observed a significant increase in the detection of DNA fragmentation rate (mean ± s.d.) after incubation with BB (30.7% ± 7.8%) in comparison to the frozen-thawed semen sample (24.8% ± 5.6%). The BB does not

induce DNA fragmentation but increases the accessibility and the integration of TdT and labeled dUTP during TUNEL.[35,41]

The passage through a MACS-annexin V column allowed a moderate enrichment of nonfragmented spermatozoa in the nonapoptotic fraction in the majority of patients (5/6 patients). Conversely, a significant increase in the rate of fragmented DNA is demonstrated in the apoptotic fraction after MACS-annexin V-sorting. Our results are coherent with those published by Martinez *et al*.[54] These authors examined the rate of sperm DNA fragmentation before and after MACS in patients with low (<30%) and high (≥30%) levels of sperm DNA fragmentation.[54] The proportion of sperm with DNA damage in nonapoptotic fraction was reduced but heterogeneous across the cohort.[54] Sperm DNA fragmentation in nonapoptotic fraction was higher than that observed before MACS for 20% of the samples.[54]

Indeed, DNA fragmentation in mature spermatozoa may not be directly related to an apoptotic process.[1] Vendrell *et al*.[38] studied the relationship between DNA fragmentation, EPS and mitochondrial membrane potential in samples sorted by MACS; they did not find a correlation between the presence of early apoptotic markers and DNA fragmentation. This suggests that the presence of apoptotic markers in ejaculated spermatozoa could be associated with abnormalities in chromatin remodeling during later stages of spermatogenesis.[1] Other authors suggest that a phenomenon of early necrotic DNA degradation could explain the presence of fragmented spermatozoa in the nonapoptotic fraction.[54]

About chromosome number abnormalities, since 2002, 12 studies[38,55–65] analyzed the chromosomal content before and after spermatic selection in fertile and/or infertile men. Reported results vary according to the team, the selection technique used, the chromosomes analyzed, and the number of spermatozoa studied. To our knowledge, only two teams have attempted to select sperm in 46,XY infertile men by MACS.[38,64] MACS was used in combination with the density gradient (density gradient followed by MACS) for both teams. Vendrell *et al*.[38] showed a decrease in the rates of nullisomy and disomy 18 and a decrease in the rate of total aneuploidy. Esbert *et al*.[64] compared the rate of aneuploidy in nonapoptotic and apoptotic fractions, and they concluded that the aneuploid spermatozoa are preferably retained in the MACS column.

However, it is original and clinically relevant to study specifically men with elevated DNA fragmentation levels. In our study, we have studied the rate of aneuploidy in this population, and we have identified a significant increase in the rate of disomy Y, disomy XY, disomy 21, diploidy (18, X, and Y), diploidy (13, 21), the sum of disomy, and the sum of diploidy in the apoptotic fraction with respect to the nonapoptotic fraction. In addition, we have also found a tendency toward a decrease of the sum of disomy in the nonapoptotic fraction and a significant increase in the frequencies of different chromosomal numerical anomalies in the apoptotic fraction compared to frozen-thawed semen samples with the exception of disomy 13 and disomy 21. These encouraging results were obtained after the study of only 5 chromosomes (X, Y, 18, 13, and 21) and the analysis of other chromosomes would be interesting. Nevertheless, we have to keep in mind that aneuploidy evaluation is particularly tedious.

CONCLUSION

In conclusion, despite the limited number of cases in this study, we consider that cell sorting using annexin V-conjugated magnetic microbeads is a promising technique to efficiently select spermatozoa with lower levels of DNA fragmentation and unbalanced chromosome content in men with an abnormal rate of sperm DNA fragmentation. These findings could have the potential to affect clinical practice.

AUTHOR CONTRIBUTIONS

SEF performed technical studies, analyzed data, and wrote the manuscript. NG and CT participated in the technical studies. HBA, MA, NDG, HD, and DB participated in the study design and coordination. AP participated in the study design and result analysis, and helped to draft the manuscript. FM conceived and supervised the study, designed and analyzed data, and wrote the manuscript. All authors read and approved the final manuscript.

COMPETING INTERESTS

All authors declared no competing interests.

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