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Decrease of spermatozoa with an unbalanced chromosome content after cell sorting in men carrying a structural chromosomal abnormality

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Abstract:

Background: We showed that in men with a constitutional chromosomal abnormality, DNA fragmentation was significantly higher in chromosomally unbalanced spermatozoa than in spermatozoa with a normal or balanced chromosomal content. These results could be explained by a phenomenon already described in infertile men: abortive apoptosis.

Objectives: To determine if magnetic-activated cell separation could select spermatozoa with lower levels of DNA fragmentation and unbalanced chromosome content in men carrying a structural chromosomal abnormality.

Materials and methods: The spermatozoa of ten males with a chromosomal rearrangement were separated into two populations using MACS (annexin V (-) and annexin V (+) fractions), in order to study meiotic segregation by FISH, the percentage of spermatozoa with an externalization of phosphatidylserine (EPS) by annexin V staining and DNA fragmentation by TUNEL on the whole ejaculate and on selected spermatozoa in the same patient.

Results: For all patients, the percentage of spermatozoa with EPS decreased in the annexin V (-) fraction and increased in the annexin V (+) fraction as compared to the frozen-thawed semen sample.

The rates of DNA fragmentation were statistically much lower in the annexin V (-) fraction when compared to the rate before MACS for all but one patient. Conversely, we observed a statistically significant higher rate of DNA fragmentation in the annexin V (+) fraction for 6 patients.

After MACS, there was a significant increase of normal/balanced spermatozoa in the fraction of annexin V (-) for all patients. Conversely, we observed a significant decrease in the fraction of annexin V (+) for seven patients.

Discussion and Conclusions: MACS is a promising tool for increasing the selection of healthy spermatozoa, with a decrease in the number of spermatozoa with EPS, DNA fragmentation and chromosome unbalance, for use in assisted reproductive technologies such as ICSI for males with a chromosomal structural abnormality.

Introduction

There is a 2-20 times higher probability of having a structural chromosomal abnormality in the infertile male population than in the general population (Hafez 1991; Morel *et al.* 2004). Robertsonian translocations are the most common structural chromosomal abnormalities observed with an incidence of 1/1085 newborns, and balanced reciprocal translocations are the second most common aberration with an incidence of 1/1175 births (De Braekeleer & Dao 1991). Carriers of a structural chromosome abnormality are phenotypically normal but males can have spermatogenic disruption such as decreased sperm concentration (Vincent *et al.* 2002; Morel *et al.* 2004) which directs them towards assisted reproductive technologies (ART) through in vitro fertilization (IVF) with Intracytoplasmic Sperm Injection (ICSI). However, the mobility and morphology parameters used to select spermatozoa for ICSI are not predictive of the quality of chromosomal equipment (Nicopoullos *et al.* 2008). Men with chromosomal structural abnormality are at a higher risk of producing chromosomally unbalanced gametes. These gametes are the result of adjacent and 3:0 segregation modes in men with Robertsonian translocation and the result of adjacent I, adjacent II, 3:1 and 4:0 segregation in men with balanced reciprocal translocations. Therefore, these men have at a higher risk of repeated miscarriages and the birth of children with a chromosome abnormality (Stern *et al.* 1999; Munné *et al.* 2000). It would thus be helpful to select spermatozoa with normal or balanced chromosomal content for ICSI in this group of men.

In a previously published study, we showed that the DNA fragmentation rate depended on the presence of a chromosomal abnormality, and that consequently a structural chromosomal abnormality predicted DNA fragmentation (Perrin *et al.* 2009). Some studies have observed significantly higher rates of apoptotic markers in translocation carriers as compared to sperm donors (Brugnon *et al.* 2006). Several authors have also reported higher aneuploidy levels in spermatozoa with fragmented DNA in infertile 46,XY men with disrupted semen parameters (Muriel *et al.* 2007; Perrin *et al.* 2011a; Perrin *et al.* 2011b; Brahem *et al.* 2012), suggesting that DNA fragmented spermatozoa were apoptotic cells (Muriel *et al.* 2007). More recently, we showed that in men with a constitutional chromosomal abnormality, DNA fragmentation was significantly higher in chromosomally unbalanced spermatozoa than in spermatozoa with a normal or balanced chromosomal content (Perrin *et al.* 2011a; Perrin *et al.* 2013). Different hypotheses are advanced to explain this DNA fragmentation: exposure to reactive oxygen species (Agarwal *et al.* 2008; Aitken *et al.* 2010), sub-protamination (McPHERSON & Longo 1992; McPherson & Longo 1993a; McPherson & Longo 1993b) or abortive apoptosis (Sakkas

et al. 1999a; Sakkas *et al.* 1999b; Sakkas *et al.* 2002; Sakkas *et al.* 2003). Our results support this latter hypothesis. Indeed, since there are significantly more spermatozoa with fragmented DNA among chromosomally unbalanced gametes, we can speculate that these gametes, between the spermatocyte and spermatid stages, have been tagged for elimination by apoptosis because of their abnormal chromosomal equipment. These gametes would then have undergone an apoptotic process, but it would not have been completed for a number of them. Consequently, abortive apoptosis would explain why chromosomally unbalanced gametes are more fragmented than chromosomally normal or balanced gametes (Perrin *et al.* 2011a; Perrin *et al.* 2013). In the apoptosis process, one of the earliest signs is the externalization of the phosphatidylserine (EPS) inner plasma membrane, a phospholipid with a high affinity for annexin V. This high affinity is guaranteed by the biological property of annexin V to bind to phospholipids in a Calcium (Ca2+) dependent manner (Klee 1988; Swairjo & Seaton 1994; van Heerde *et al.* 1995). Magnetic-activated cell separation (MACS) is a technique of spermatozoa selection based on super-paramagnetic microbeads conjugated with annexin V which labels the apoptotic and dead spermatozoa within an external magnetic field. MACS was used to separate sperm into 2 populations: an annexin V (-) fraction (spermatozoa without EPS) and an annexin V (+) fraction (spermatozoa with EPS) (de Vantéry Arrighi *et al.* 2009; Grunewald *et al.* 2009; Huang *et al.* 2009; Buzzi *et al.* 2010). This is a very simple, specific and rapid technique which is known to reduce a large proportion of apoptotic spermatozoa in the processed samples (Said *et al.* 2006), and also to reduce the level of DNA fragmentation (Rawe *et al.* 2009; Lee *et al.* 2010; Herrero *et al.* 2012; Delbes *et al.* 2013; Nadalini *et al.* 2014; Troya & Zorrilla 2015; Berteli *et al.* 2017). As a consequence, spermatozoa that have undergone abortive apoptosis should be retained in the column when using annexin V coupled with MACS. Therefore, if the origin of the DNA fragmentation is due to abortive apoptosis, we expect the rate of chromosomally unbalanced spermatozoa to be decreased in the annexin V (-) population.

In this study, we separated spermatozoa of males with a chromosomal rearrangement into two populations using MACS, an annexin V (-) and annexin V (+) population, in order to study meiotic segregation and DNA fragmentation on the whole ejaculate and on selected spermatozoa (annexin V (-) *versus* annexin V (+)) in the same patient. The percentage of spermatozoa with EPS was also evaluated on the whole ejaculate and on selected spermatozoa to determine the purity rate of our samples after sorting.

The aim of the present study was to determine if magnetic-activated cell separation could select spermatozoa with lower levels of DNA fragmentation and unbalanced chromosome content in men carrying a structural chromosomal abnormality.

Patients and methods

Patients and sperm parameters

Ten males with a structural chromosome abnormality were included in the study (Table 1). Karyotyping was performed on peripheral blood cultures. For this study straws of cryopreserved semen samples were obtained with the collaboration of different national centers (3 samples from University Hospital of Clermont-Ferrand and 7 samples from the GERMETHEQUE national biobank, BB-0033-00081; coordination: Toulouse, France). Sperm parameters were evaluated according to World Health Organization recommendations (WHO 2010).

Our study was approved by the regional Committee for the Protection of Persons (declaration number DC-2008-597; authorization AC-2009-886). All patients gave their informed consent for the study.

Study design and MACS separation

Spermatozoa from frozen semen samples were thawed for 30 min at room temperature, and were washed in 1 ml of BB 1X (Binding Buffer) (Miltenyi Bioec, Paris, France) at 400 x g for 10 minutes to eliminate the cryoprotectant. After centrifugation, the final pellets were resuspended in BB 1X. Sperm DNA slides were prepared for annexin V staining, TUNEL (TdT-mediated-dUTP nick-end labeling) and FISH (Fluorescence *in situ* Hybridation), and the remaining sample was processed by MACS separation.

For MACS separation, the washed spermatozoa were incubated with 100 µl of BB 1X and annexin V microbeads (Miltenyi Biotec) at room temperature for 15 minutes. Sperm DNA slides were prepared for TUNEL. The remaining spermatozoa-microbead suspension was placed on top of the separation column containing iron balls and the column placed in a

magnetic field (MiniMACS, Miltenyi Biotec). The spermatozoa with intact membranes (without EPS) passed through the column and were labeled annexin V (-). The spermatozoa with EPS retained in the separation column were eluted using BB 1X after the column was removed from the magnetic field, and were labeled annexin V (+). Sperm DNA slides from annexin V (-) and annexin V (+) solutions were prepared for annexin V staining, TUNEL and FISH. The different steps of our experiment design are illustrated in Figure 1.

Evaluation of spermatozoa with EPS using annexin V-FITC

For technical reason, to evaluate spermatozoa with EPS using annexin V-FITC, the separated spermatozoa populations were adjusted to a concentration of 200 000 spermatozoa. They were incubated in the dark with annexinV-FITC and 100µl of BB 1X for 15 minutes at room temperature, and centrifuged at 400 x g for 10 minutes. The final pellets were resuspended in 10 µl of BB 1X. Sperm slides were prepared and counterstained with DAPI. The fluorescence signals of labeled spermatozoa were analyzed using a Zeiss AxioPlan Microscope (Zeiss, Le Pecq, France). A total of 500 spermatozoa were analyzed for each sample. Spermatozoa were considered “annexin V (-)” (blue fluorescence) or “annexin V (+)” (green fluorescence). Subsequent image acquisition was performed using a CCD camera with *in situ* imaging system (ISIS) (Metasystems, Altlussheim, Germany).

Analysis of DNA fragmentation using TUNEL assay

Sperm DNA slides were fixed with Carnoy solution (methanol + acetic acid). The slides were immersed in a jar containing a 2x SSC (Saline Sodium Citrate) /0.4% NP40 (Nonidet P40) solution and then immediately passed through an ethanol series of increasing concentration (70%/90%/100%). Sperm DNA fragmentation was detected by the ApopTag kit (Millipore, Saint Quentin en Yvelines, France) according to the manufacter’s recommendations. Briefly, equilibration buffer was put on the slides and a mix of a reaction buffer and terminal deoxyribonucleotidyl transferase was added. The slides were incubated in a dark humidified chamber at 37°C. They were then incubated in a stop/wash buffer and washed with 1x phosphate-buffered saline (PBS). A mix of blocking solution and anti-digoxigenin-rhodamine was added to the slides before incubation at 37°C. The slides were washed in 1x PBS. They were air-dried and counterstained with DAPI (4’, 6-diamidino-2-phenyl-indole). A total of 500 spermatozoa from each sample were analyzed using a Zeiss AxioPlan Microscope.

Spermatozoa were considered to contain either normal (blue fluorescence) or fragmented (red fluorescence) DNA (Perrin *et al.* 2009).

Analysis of meiotic segregation by FISH

Meiotic segregation was analyzed by FISH with a mix of appropriate probes according to the chromosomal breakpoints involved in the rearrangement (Table 2). The hybridization techniques have been previously published (Morel *et al.* 2004; Perrin *et al.* 2009). Sperm DNA preparations were fixed on a slide with Carnoy solution. Before hybridization sperm nuclei were decondensed in 1M NaOH solution and then washed in SSC 2X for 5-10 min at room temperature. They were immersed in a jar of SSC 2X/0.4%NP40 solution for 30 min at 37°C and then passed through an ethanol series of increasing concentration (70%/90%/100%) and allowed to air dry. The denaturation was performed simultaneously on spermatozoa and probes for 1 min at 70°C using a ThermoBrite System (Abbott, Rungis France). The slides were incubated overnight in a dark humidified chamber at 37°C. They were washed for 30 s in SSC 0.4X /0.3% NP40 at 72°C and 15 s in SSC 2X /0.1% NP40 at room temperature. Finally, they were counterstained with DAPI. The chromosomal content in spermatozoa was determined according to the combination of fluorescent spots observed. A minimum of 2000 spermatozoa were analyzed for each sample using a Zeiss AxioPlan Microscope.

Statistical analyses

An independent chi-square test was used to compare the percentages of spermatozoa stained with annexin V-FITC before and after MACS separation. The same test was used to compare:

- the percentage of spermatozoa with DNA fragmentation
- the number of spermatozoa with normal/balanced chromosomal content and chromosomally unbalanced content before and after MACS separation.

To compare the number of chromosomally unbalanced spermatozoa produced by different segregation modes we used an independent chi-square test or the Fisher's exact test if the expected numbers were below 5. The data was analyzed online using the BiostaTGV software (http://marne.u707.jussieu.fr/biostatgv/). A value of $p < 0.05$ was considered to be significant.

Results

Patients

Ten male carriers of a structural chromosomal abnormality were included in the study. The karyotypes of the patients are shown in Table 1. There were 3 patients with a Robertsonian translocation (P1 to P3), and 7 patients with a simple balanced reciprocal translocation (P4 to P10).

Semen analysis

The summary of the basic semen parameters on raw semen samples before freezing are presented in Table 3. Sperm concentration ranges were between 14.4 millions/mL (P9) and 151 millions/mL (P2), the percentage of motility (a+b+c) ranged from 35% (P3, P7, P9 and P10) to 60% (P8), the percentage of normal forms according to the classification of David ranged from 10% (P3 and P4) to 70% (P8).

Evaluation of spermatozoa with EPS using annexin V-FITC

Data concerning the percentage of spermatozoa with EPS before MACS separation and in the annexin V (-) and V (+) fractions after MACS separation are summarized in Table 4. For all patients, the percentage of spermatozoa with EPS is lower in the annexin V (-) fraction (average: 29.72 ± 7.45%) and higher in the annexin V (+) fraction (average: 80.58 ± 7.44) as compared to the frozen-thawed semen sample (average: 59.32 ± 9.34).

Analysis of DNA fragmentation using TUNEL assay

Data concerning the percentage of DNA fragmentation for each patient at each step of the study are summarized in Table 5. For the 10 male carriers of a structural chromosomal abnormality, the percentage of DNA fragmentation after freeze-thaw varied between 7.2% (P2) and 20.6% (P6). For 6 patients, the rates of DNA fragmentation were statistically significantly higher after incubation with BB 1X + annexin V microbeads than for the frozen-thawed semen sample. The rates of DNA fragmentation were statistically significantly lower in the annexin V (-) fraction than after incubation with BB1X + annexin V microbeads except for P6, and conversely, we observed a statistically significantly higher rate in the annexin V (+) fraction than after incubation with BB 1X + annexin V microbeads for 6 patients (P2, P3, P4, P7, P9 and P10) and a significant decrease for one patient (P5). There was a statistically

significant difference for DNA fragmentation rates in the annexin V (-) fraction as compared to the annexin V (+) fraction except for P6.

Analysis of meiotic segregation by FISH

Regarding the normal/balanced spermatozoa products of alternate segregation, the frequencies after freeze-thaw varied from 78.06% (P3) to 83.37% (P2) for patients with a Roberstonian translocation and from 36.39% (P10) to 48.36% (P5) for patients with a balanced reciprocal translocation (Table 6). When compared to raw semen, there was a statistically significant increase of normal/balanced spermatozoa in the annexin V (-) fraction for all patients ($p < 0.05$) after MACS. On the other hand, we observed a statistically significant decrease in the annexin V (+) fraction for P1, P4, P6, P7, P8, P9 and P10. For all patients, we observed a statistically significant difference ($p < 0.05$) between the annexin V (-) and annexin V (+) fractions with a majority of normal or balanced spermatozoa in the first group, and a majority of unbalanced spermatozoa in the second group.

Concerning the population of unbalanced spermatozoa, the results from FISH studies are summarized in Table 6. After MACS, the proportion of unbalanced spermatozoa from each of the segregation modes decreased significantly in the annexin V (-) fraction and increased significantly in the annexin V (+) fraction.

Discussion

In this study, we analyzed spermatozoa with an externalization of the phosphatidylserine using annexin V-FITC, DNA fragmentation and the meiotic segregation on total ejaculate and on selected spermatozoa after MACS, in 10 men with a structural chromosomal abnormality.

Concerning the analysis using annexin V-FITC, we found an average of 29.72% spermatozoa with EPS in the annexin V (-) fraction *versus* 80.58% in the annexin V (+) fraction with a significant difference when compared to the frozen-thawed semen samples. Even if for certain patients the annexin V-FITC spermatozoa percentage was still high in the annexin V (-) fraction, on average MACS reduced this percentage by 49.90%. These data obtained considering them as a simple good functioning check tool of MACS. Moreover, our results are similar to those described in the literature (de Vantéry Arrighi *et al.* 2009; Lee *et al.* 2010;

Vendrell *et al.* 2014). Paasch et al. analyzed the separation effect of MACS in donor sperm with flow cytometry using FITC-conjugated anti-annexin V antibodies and found an average of 5.2% ± 1.0% labeled spermatozoa in the annexin V (-) fraction *versus* 72.2% ± 2.7% labeled spermatozoa in the annexin V (+) fraction (Paasch *et al.* 2003). The presence of annexin V-FITC+ spermatozoa in the annexin V (-) fraction could be due to unspecific binding or an insufficient number of beads to retain them in the column. Glander and Schaller showed that the primary structural membrane of spermatozoa influences the ability of annexin V to detect externalization of phosphatidylserine (Glander & Schaller 2000).

Concerning the analysis of DNA fragmentation, some authors have reported that cryopreservation might induce DNA damage (Di Santo et al. 2012). In particular, many authors support the hypothesis of less susceptibility to freezing DNA damage in the sperm cells of fertile men, than those of infertile men (Donnelly *et al.* 2001). In order to be able to compare the data of the different patients in this study, we used cryopreserved samples for all patients.

Data showed that for 6 patients the rates of DNA fragmentation were statistically significantly higher after incubation with BB 1X (+ annexin V microbeads) than for the frozen-thawed semen sample. These results confirm those previously obtained (El Fekih 2018). In a previous study, we evaluated the rate of spermatozoa with EPS and the DNA fragmentation at each stage of magnetic sorting (after frozen-thawed semen sample, after incubation with BB 1X (+ annexin V microbeads) and after sorting in the annexin V (-) fraction) (El Fekih 2018). Concerning the rate of spermatozoa with EPS, we have shown that it remains unchanged between frozen-thawed semen sample and after incubation with BB 1X (+ annexin V microbeads). After sorting, the rate significantly decreases in the annexin V (-) fraction. Concerning the rate of DNA fragmentation, we have identified a significant increase of this rate between frozen-thawed semen sample and after incubation with BB 1X (+ annexin V microbeads). Moreover, we have identified a significant decrease after sorting in the annexin V (-) fraction compared to after incubation with BB 1X (+ annexin V microbeads). Thus, these results show that the BB1X has no influence on apoptosis - it does not induce DNA fragmentation. BB1X allows better accessibility and better integration of TdT and labeled dUTP during TUNEL (El Fekih 2018).

Although for certain patients the percentage of DNA fragmentation was still not low in the annexin V (-) fraction after column selection, on average MACS reduced the percentage by 46.15%. Our results are comparable with those described in the literature, with the rate of DNA fragmentation statistically significantly lower after MACS separation in the annexin V (-) fraction than before MACS separation and conversely, statistically significantly higher in the annexin V (+) fraction than before MACS separation (Rawe *et al.* 2009; Lee *et al.* 2010; Herrero *et al.* 2012).

The presence of fragmented DNA spermatozoa in the annexin V (-) fraction may be due to an apoptotic process sequence different from that known in somatic cells. In fact, in previous studies, we showed that chromosomally unbalanced spermatozoa are more fragmented than chromosomally normal or balanced sperm in men with chromosomal abnormalities and we hypothesized that abortive apoptosis might explain this association (Perrin *et al.* 2011a; Perrin *et al.* 2013). Nevertheless, the sequence of the apoptotic process in spermatozoa is still unclear. A hypothetical sequence was recently developed by Vendrell et al. (Vendrell *et al.* 2014). Indeed, these authors hypothesized that during spermatogenesis an alteration of meiotic segregation would trigger an apoptotic process leading to the fragmentation of DNA. Nevertheless, the early stages of apoptosis do not involve externalization of phosphatidylserine there are therefore fragmented spermatozoa without externalization of phosphatidylserine. These spermatozoa would thus not be retained in the column and be found in annexin V (-) (Vendrell *et al.* 2014).

Carriers of a constitutional structural chromosomal abnormality are a particular group of infertile men who produce a high rate of chromosomally unbalanced spermatozoa in their ejaculate (Vegetti *et al.* 2000). For Robertsonian translocations the alternate mode, which is the only mode which leads to the production of chromosomally normal or balanced spermatozoa, prevailed. Our results are comparable to the more than 75% normal or balanced spermatozoa produced reported in the literature (Scriven *et al.* 2001; Simpson & Elias 2003; Anton *et al.* 2004; Roux *et al.* 2005). Reciprocal translocation carriers produced between 18.6% and 80.7% normal or balanced spermatozoa (Morel *et al.* 2004; Anton *et al.* 2007; Wiland *et al.* 2008; Perrin *et al.* 2009) which corroborates our result for our seven other patients. FISH technique is frequently used for the analysis of meiotic segregation in spermatozoa of translocation carriers (Estop *et al.* 1997; Van Assche *et al.* 1999; Escudero *et al.* 2000; Frydman *et al.* 2001) to estimate the number of unbalanced spermatozoa in each

patient, thus providing information about the reproductive risks. Indeed translocation carriers are at a high risk of repeated miscarriages and the birth of children with chromosomal abnormalities (Stern *et al.* 1999; Munné *et al.* 2000). It thus seems important to eliminate unbalanced spermatozoa in order to reduce the reproductive risk associated with repeated miscarriages and the birth of children with chromosomal abnormalities in this population of infertile men.

To the best of our knowledge, only five publications have reported techniques for the selection of normal or balanced spermatozoa in translocation carriers. Two studies evaluated the potential of motile sperm organelle morphology examination (MSOME) for selecting normal or balanced spermatozoa (Cassuto *et al.* 2011; Chelli *et al.* 2013). Both studies concluded that MSOME could not be used to select normal or balanced spermatozoa in men with a constitutional chromosomal abnormality. In 2012, Vozdova *et al*. did not find a difference in mean frequencies of unbalanced segregation products in the ejaculated and swim-up processed samples in men with a constitutional chromosomal abnormality but they found a significantly lower frequency of unbalanced spermatozoa after selection by the hyaluronan-binding technique (Vozdova *et al.* 2012). More recently, Rouen's teams obtained a decrease in the proportion of unbalanced spermatozoa in translocation carriers after selection using discontinuous gradient centrifugation (Rouen *et al.* 2014) or the hypo-osmotic swelling test (Rouen *et al.* 2017).

To the best of our knowledge, only two studies have worked on the effects of MACS on sperm aneuploidy rate in patients with a normal karyotype (Vendrell *et al.* 2014; Esbert *et al.* 2017). These studies reported that processing samples through MACS decreased sperm aneuploidy rates in the annexin V (-) fraction. Moreover they compared the aneuploidy rate in the annexin V (-) and V (+) fractions and concluded that aneuploid sperm cells are preferentially retained in the MACS column (Esbert *et al.* 2017). Our study is the first of its kind to evaluate the rate of normal or balanced spermatozoa and unbalanced spermatozoa after MACS in the two fractions of annexin V (-) and annexin V (+) for patients carrying a constitutional structural chromosomal abnormality. We observed a significant increase of normal or balanced spermatozoa in the annexin V (-) fraction and a significant decrease in the annexin V (+) fraction and there is always a significant difference when comparing the two fractions. Even if unbalanced spermatozoa are found in the annexin V (-) fraction, on average MACS reduced this percentage by 32.14%. The presence of unbalanced spermatozoa in the

annexin V (-) fraction could be explained by the hypothetical sequence recently developed by Vendrell et al. (Vendrell *et al.* 2014)

We also analyzed the impact of MACS on the different segregation modes in unbalanced spermatozoa. In the literature, for Robertsonian translocation carriers the segregation mode producing unbalanced spermatozoa with disomies and nullisomies for chromosomes involved in the translocation are adjacent and 3:0 (Morel *et al.* 2004). In reciprocal translocation carriers, the unbalanced spermatozoa result mainly from the adjacent I, adjacent II, 3:1 and 4:0 (Morel *et al.* 2004). Our results are concordant with these data, except for P5 and P8, where the predominant segregation mode for unbalanced spermatozoa was 3:1. This phenomenon has already been reported in previous studies (Morel *et al.* 2004; Brugnon *et al.* 2006). Regarding the different meiotic segregation modes producing unbalanced spermatozoa, we always found a significant difference between the annexin V (-) and annexin V (+) fractions, always in favor of annexin V (-) with a significant decrease of unbalanced spermatozoa. We concluded that there is no preferential selection depending on the type of chromosomal imbalance.

MACS is a simple and inexpensive technique, without deleterious or magnetic effects detectable on the spermatozoa due to their passage in the separation columns (Grunewald *et al.* 2009). Microbeads do not affect cell viability and are biodegradable (Miltenyi *et al.* 1990). Since 2010, many healthy offspring have been reported with the use of MACS as a sperm selection technique with ICSI (Polak de Fried & Denaday 2010; Rawe *et al.* 2010; Herrero *et al.* 2013; Ugozzoli *et al.* 2013). Moreover in 2017 Romany et al. did a randomized control trial on 237 couples with liveborn children conceived through the association of MACS and ICSI. They concluded that there were no noticeable differences in obstetric or perinatal events when MACS is used (Romany *et al.* 2017). However, further research is needed to verify the presence of freely floating microbeads in the annexin V (-) fraction on the plasma membrane of the spermatozoa, and long term effects on the health and development of children.

Despite the small number of cases in our study we conclude that after MACS separation we observed a significant decrease of spermatozoa with an externalization of phosphatidylserine, DNA fragmentation and chromosomally unbalanced spermatozoa in the annexin V (-) fraction and inversely a significant increase in the annexin V (+) fraction. In order to improve this selection, in a future study it would be interesting to combine the MACS technique with another selection method such as density gradient centrifugation. The results obtained in this study consolidate the hypothesis that MACS tends to retain unbalanced

spermatozoa and improve the selection of normal or balanced spermatozoa for ART. It strengthens our hypothesis of an abortive apoptosis initiated in the testis.
MACS is thus a promising tool for use in selecting healthy spermatozoa with a normal or balanced chromosomal content which can be used in assisted reproduction technique (ART) such as ICSI in males with a structural chromosomal abnormality and also for use in selecting spermatozoa with a normal or balanced chromosomal content before preimplantation genetic diagnosis in these patients.

The risk of picking up an unbalanced spermatozoon for ICSI after MACS separation remains and selection by MACS cannot replace preimplantation diagnosis which is the best method for embryo selection after ICSI. However, MACS carries the benefit of selecting spermatozoa of better quality with respect to apoptosis, DNA fragmentation and chromosomal content and thus increasing the chance of achieving a successful pregnancy.

A study on the data of offspring obtained through this technology would certainly be interesting but remains subject to prevailing legislation and at the present time French legislation does not authorize this kind of study.

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The authors have no conflict of interest to declare.

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The authors have all contributed to the study (Sahar El Fekih and Elodie Caire-Tetauru: technical assistance, result analysis and article writing; Corinne Tous and Nadia Gueganic: technical assistance; Florence Brugnon, Habib Ben Ali, Louis Bujan, Nathalie Moinard, Mounir Ajina, Nathalie Douet-Guilbert, Frédéric Morel and Aurore Perrin: expertise and article review).

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FIGURES

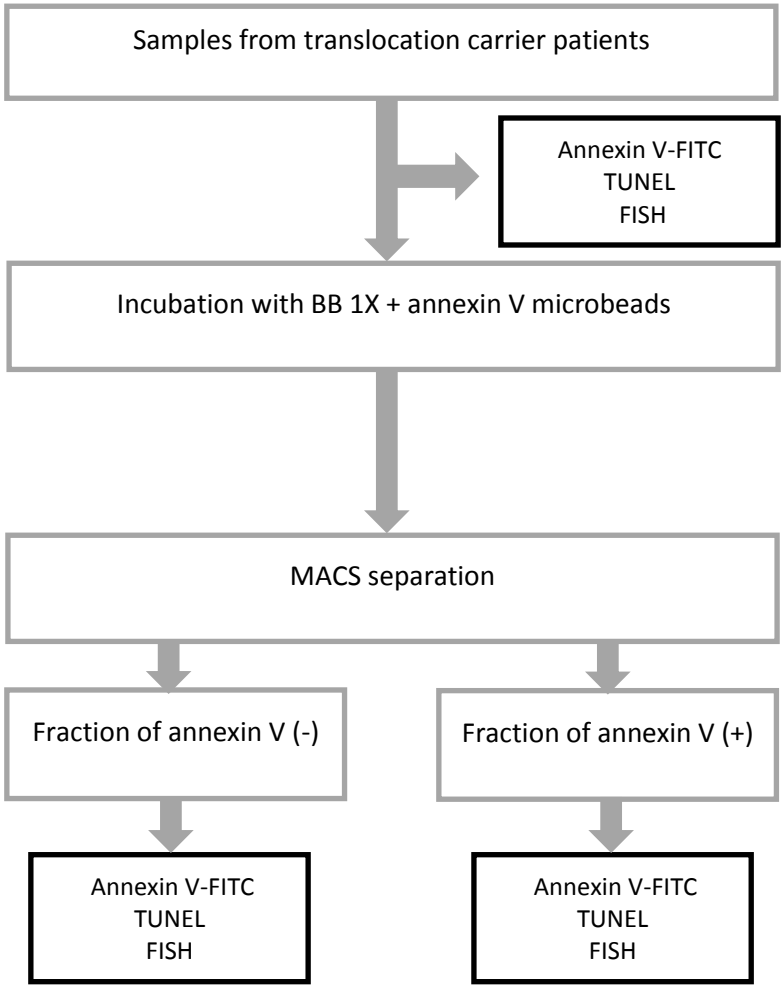

Figure 1: Flow diagram of the overall experiment design

Table 1: Karyotype performed on peripheral blood cultures of ten male carriers of Robertsonian or reciprocal translocations

Table 2: Mix, spectrums and localization of probes used to perform FISH on spermatozoa of ten male carriers of Robertsonian or reciprocal translocations

Table 3: Data on sperm parameters for raw semen samples from each individual (volume, concentration, mobility, normal forms)

Table 4: Percentage of spermatozoa with externalization of phosphatidylserine in frozen-thawed semen sample, in the annexin V (-) and V (+) fractions for each patient

Table 5: Percentage of DNA fragmentation in frozen-thawed semen sample, after incubation, in the annexin V (-) and V (+) fractions for each patient

Table 6: Different modes of meiotic segregation in semen samples, in the annexin V (-) and V (+) fractions for each patient

Table 1: Karyotype performed on peripheral blood cultures of ten male carriers of Robertsonian or reciprocal translocations

Patient	Karyotype
P1	45,XY,rob(13;14)(q10;q10)
P2	45,XY,rob(14;15)(q10;q10)
P3	45,XY,rob(13;14)(q10;q10)
P4	46,XY,t(4;12)(q31;q23)
P5	46,XY,t(9;12)(q31;p12)
P6	46,XY,t(1;15)(p22.2;q21.1)
P7	46,XY,t(11;13)(q22.1;q21.2)
P8	46,XY,t(13;19)(q12;q13.3)
P9	46,XY,t(1;22)(p11;p11.2)
P10	46,XY,t(2;9)(p24;p21)

Table 2: Mix, spectrum and localization of probes used to perform FISH on spermatozoa of ten male carriers of Robertsonian or reciprocal translocations

Patient	Mix of probes used for FISH analyses
P1	BACs pool on 13q33.3 (Green), BACs pool on 14q32.33 (Orange), CEP18 (Aqua)
P2	BACs pool on 14q32.33 (Green), BACs pool on 15q26.3 (Orange), CEP8 (Aqua)
P3	BACs pool on 13q33.3 (Green), BACs pool on 14q32.33 (Orange), CEP8 (Aqua)
P4	BACs pool on 4q22.1 (Aqua), BACs pool on 4q34.1 (Green), CEP12 (Orange)
P5	BACs pool on 9q34 (Green), BACs pool on 12p13.33 (Aqua), CEP12 (Orange)
P6	BACs pool on 1p36.33 (Orange), BACs pool on 1q24.1-24.2 (Aqua), BACs pool on 15q26.3 (Green)
P7	BACs pool on 11q25 (Orange), BACs pool on 13q14.3 (Green), BACs pool on 13q33.3 (Aqua)
P8	BACs pool on 13q33.3 (Green), BACs pool on 19p12-p13.11 (Orange), BACs pool on 19q13.41 (Aqua)
P9	BACs pool on 1p36.33 (Orange), BACs pool on 1q24.1-24.2 (Aqua), BACs pool on 22q11.23 (Green)
P10	BACs pool on 2p25.3 (Green), BACs pool on 2q37.3 (Orange), BACs pool on 9q34.12-q34.13 (Aqua)

Table 3: Data on sperm parameters for raw semen samples from each individual (volume, concentration, motility, normal forms)

Patient	Volume (mL)	Concentration (M/mL)	Motility (a+b+c)	Normal forms (%)
P1	3.2	50	55	4*
P2	2.1	151	45	13**
P3	3.2	19.6	35	10**
P4	3.2	117	41	10**
P5	5.3	50	55	26**
P6	2.8	108	40	31**
P7	4.2	42	35	14**
P8	2.5	49	60	70**
P9	8.1	14.4	35	NR
P10	3.5	15	35	15**

*according to the classification of Kruger, ** according to the classification of David, NR: unrealized

Table 4: Percentage of spermatozoa with externalization of phosphatidylserine in frozen-thawed semen sample, in the annexin V (-) and V (+) fractions for each patient

Patient	Frozen-thawed semen sample (%)	Fraction of annexin V (-) (%)	Fraction of annexin V (+) (%)	Between fraction of annexin V (-) and V (+)
P1	57.4	19.6*	84.2*	**
P2	56.0	32.6*	83.6*	**
P3	54.6	33.2*	71.4*	**
P4	38.0	14.2*	85.6*	**
P5	72.8	36*	88*	**
P6	67.2	35.4*	83.4*	**
P7	58.0	30.6*	69.8*	**
P8	63.2	28.4*	84.8*	**
P9	64.8	37.6*	86.2*	**
P10	61.2	29.2*	68.8*	**
Average ± SD	59.32 ± 9.34	29.72 ± 7.45*	80.58 ± 7.44*	**

* Significant difference when compared to the frozen-thawed semen sample ($p < 0.05$), ** significant difference when comparing the annexin V (-) and V (+) fractions ($p < 0.05$)

Table 5: Percentage of DNA fragmentation in frozen-thawed semen sample, after incubation, in the annexin V (-) and V (+) fractions for each patient

Patient	Frozen-thawed semen sample (%)	After Incubation (%)	Fraction of annexin V (-) (%)	Fraction of annexin V (+) (%)	Between fraction of annexin V (-) and V (+)
P1	15	23*	9.2**	28	***
P2	7.2	21.8*	7.4**	31.2**	***
P3	18.8	20.6	14**	34.8**	***
P4	16.2	23.4*	16.2**	36.2**	***
P5	18.4	37*	18.6**	25.4**	***
P6	20.6	23.8	20	23.4	NS
P7	18.2	22.8	9.4**	34**	***
P8	15	21.2*	10**	24	***
P9	14.8	25.4*	13**	37.6**	***
P10	14	17.2	9.4**	32.8**	***
Average ± SD	15.82 ± 3.93	23.62 ± 5.19	12.72 ± 4.36**	30.74 ± 5.20**	***

* Significant difference when compared to the frozen-thawed semen sample ($p < 0.05$) ** significant difference when compared to after incubation ($p < 0.05$) *** significant difference when comparing the annexin V (-) and V (+) fractions ($p < 0.05$) and NS: no significant

Table 6: Different modes of meiotic segregation in semen samples, in the annexin V (-) and V (+) fractions for each patient

	Modes of meiotic segregation	Semen sample	Fraction of annexin V (-)	Fraction of annexin V (+)	Between fraction of annexin V (-) and V (+)
P1	Alternate (%)	79.99	88.03*	75*	**
	Adjacent (%)	18.82	11.14*	21.77*	**
	3 : 0 (%)	0.47	0.43	1.87*	**
	Diploid (%)	0.47	0.2*	1.1*	**
	Others (%)	0.24	0.2	0.26	NS
No of spermatozoa analysed		2534	2531	2728	
P2	Alternate (%)	83.37	90.2*	82.43	**
	Adjacent (%)	14.4	8.5*	14.23*	NS
	3 : 0 (%)	0.2	0.1	0.15	NS
	Diploid (%)	1.29	1.05	2.1*	**
	Others (%)	0.74	0.15*	1.1	**
No of spermatozoa analysed		2021	2002	2003	
P3	Alternate (%)	78.06	88.64*	80.93*	**
	Adjacent (%)	19.55	10.72*	16.72*	**
	3 : 0 (%)	0.4	0.4	0.83	NS
	Diploid (%)	1.74	0.1*	1.03	**
	Others (%)	0.25	0.15	0.49	NS
No of spermatozoa analysed		2010	2024	2045	
P4	Alternate (%)	44.27	62.77*	31.63*	**
	Adjacent I (%)	36.43	27.58*	51.99	**
	Adjacent II (%)	8.64	4.77*	7.19	**
	3 : 1 (%)	7.5	3.66*	6.81*	**
	Diploidy or 4 : 0 (%)	0.91	0.59	2.16*	**
	Others (%)	2.25	0.63*	0.21*	**
No of spermatozoa analysed		2627	2705	2864	
P5	Alternate (%)	48.36	66.16*	50.77	**
	Adjacent I (%)	0.15	0.04	0.16	NS
	Adjacent II (%)	0.18	0.19	0.2	NS
	3 : 1 (%)	50.83	33.38*	48.64*	**
	Diploidy or 4 : 0 (%)	0.07	0.19	0.24	NS
	Others (%)	0.41	0.04*	0*	NS
No of spermatozoa analysed		2707	2651	2531	NS

P6	Alternate (%)	37.75	54.76*	27.63*	**
	Adjacent I (%)	32.49	30.13	36.21	**
	Adjacent II (%)	16.12	10.06*	22.15*	**
	3 : 1 (%)	12.75	4.76*	12.64*	**
	Diploidy or 4 : 0 (%)	0.79	0.19 *	1.08	**
	Others (%)	0.1	0.1	0.29	NS
No of spermatozoa analysed		2016	2078	2041	
P7	Alternate (%)	43.13	60.54*	33.07*	**
	Adjacent I (%)	35.63	31.84*	43.6*	**
	Adjacent II (%)	7.35	3.47*	6.95	**
	3 : 1 (%)	12.04	3.47*	14.44*	**
	Diploidy or 4 : 0 (%)	1.45	0.53*	1.52	**
	Others (%)	0.4	0.14	0.41	NS
No of spermatozoa analysed		2001	2073	2431	
P8	Alternate (%)	37.24	59.84*	30.17*	**
	Adjacent I (%)	1.12	0.1*	0.35*	NS
	Adjacent II (%)	5.2	2.42*	9.76*	**
	3 : 1 (%)	54.01	35.77*	54.61	**
	Diploidy or 4 : 0 (%)	2.14	1.58*	3.37*	**
	Others (%)	0.29	0.3	1.73*	**
No of spermatozoa analysed		2057	2027	2254	
P9	Alternate (%)	44.07	58.02*	39.21*	**
	Adjacent I (%)	20.1	18.42	17.31*	NS
	Adjacent II (%)	19.66	16.93*	24.67*	**
	3 : 1 (%)	14.9	6.19*	17.18*	**
	Diploidy or 4 : 0 (%)	0.49	0.35	1.19*	**
	Others (%)	0.78	0.1*	0.44	**
No of spermatozoa analysed		2040	2020	2270	
P10	Alternate (%)	36.39	53.78*	31.65*	**
	Adjacent I (%)	31.51	26.41*	32.28	**
	Adjacent II (%)	21.01	15.44*	24.33*	**
	3 : 1 (%)	9.86	4.04*	10.86	**
	Diploidy or 4 : 0 (%)	0.5	0.05*	0.39	**
	Others (%)	0.75	0.28*	0.48	NS
No of spermatozoa analysed		2009	2105	2063	

* Significant difference when compared to the semen sample ($p < 0.05$) ** significant difference when comparing the annexin V (-) and V (+) fractions ($p < 0.05$) and NS: no significant.