J Assist Reprod Genet (2014) 31:1045–1051
DOI 10.1007/s10815-014-0267-3

ASSISTED REPRODUCTION TECHNOLOGIES

Annexin V magnetic-activated cell sorting versus swim-up for the selection of human sperm in ART: is the new approach better then the traditional one?

Marco Nadalini · Nicoletta Tarozzi · Marlea Di Santo · Andrea Borini

Received: 23 April 2014 / Accepted: 27 May 2014 / Published online: 7 June 2014

Abstract
Purpose To investigate whether the sperm fertilizing potential can be improved by selecting a non-apoptotic fraction using magnetic activated cell sorting (MACS), and to compare the results with the conventional swim-up method.
Methods Twenty five male patients attending the andrology laboratory for sperm DNA fragmentation analysis. The sperm were prepared by density gradient centrifugation (DGC) and subsequently divided into three aliquots. The first was further separated into Annexin V-negative (non-apoptotic) fraction using MACS, the second was further processed by swim-up, while the third was left unseparated as a control. The impact of the combination of DGC with the two sperm preparation techniques on sperm quality was evaluated by comparing 'rapid progressive' motility, normal morphology according to Tygerberg's strict criteria and DNA integrity (by terminal deoxynucleotide transferase-mediated dUTP nick-end labeling [TUNEL]) for each aliquot.
Results Sperm preparation that combines DGC with conventional swim-up method can provide sperm of higher quality in terms of motility, morphology and extent of DNA fragmentation compared to the Annexin V-negative (non-apoptotic) fraction derived from the combination of DGC with MACS.
Conclusions Integrating MACS as a part of sperm preparation technique will not improve sperm fertilizing potential to the same extent as the traditional swim-up separation procedure.

Keywords Human sperm · DNA fragmentation · Motility · Morphology · MACS · Annexin V

Capsule Integrating MACS as a part of sperm preparation technique will not improve sperm fertilizing potential to the same extent as the traditional swim-up separation procedure.

M. Nadalini · N. Tarozzi · M. Di Santo · A. Borini (✉)
Tecnobios Procreazione - Centre for Reproductive Health,
Via Dante 15, I-40125 Bologna, Italy
e-mail: borini@tecnobiosprocreazione.it

Introduction

In the last two decades assisted reproductive technologies (ART) have become the treatment of choice in many cases of male and female infertility; nonetheless, the current success rates of these procedures remain suboptimal. What might be the limiting factors? One potential reason for these conditions may be the inclusion of apoptotic sperm or sperm with fragmented DNA despite a normal appearance and motility during in vitro fertilization (IVF) [1, 2]. The use of such sperm in ART may have adverse effects on the outcome of the procedure [3]. Therefore, the quality of sperm samples is one of the factors that helps to determine successful assisted reproduction [4]. Currently, a variety of sperm preparation techniques have been proven to select sperm that are characterized by superior motility and morphology, giving higher fertilization potential. Among them, the density gradient centrifugation and the swim-up method are widespread used as standard preparation techniques [5]. These procedures are based on sedimentation or migration of spermatozoa, although molecular events such as sperm apoptosis are overlooked, which may negatively impact the final outcomes [6]. Key features of activated apoptosis signalling like disruption of the mitochondrial transmembrane potential, activation of caspase-3, externalization of phosphatidylserine, and increased abnormalities such as sperm DNA fragmentation have been identified in human ejaculated spermatozoa [7–9] and directly linked to failure of fertilization, clinical pregnancy and pregnancy loss during assisted reproduction [10–13, 1]. Apoptosis is a type of programmed cellular death which leads the cell to suicide without eliciting an inflammatory response [14]. Because spermatozoa are transcriptionally inactive cells and the DNA is packed, the apoptosis process is different from that in somatic cells; additionally, mature sperm cells express different markers in response to apoptosis-related cell damage [14]. Usually, in normal eukaryotic cells the negatively-

charged phospholipid phosphatidylserine (PS) is located in the inner cytosolic layer of the sperm plasma membrane lipid bilayer [15]. PS redistribution from the inner to the outer leaflet is an early and widespread event during apoptosis [16]. However, in necrosis, PS becomes accessible due to the disruption of membrane integrity, and also serves as a trigger for the recognition and removal of apoptotic cells by macrophages [17]. Recently, magnetic activated cell sorting (MACS), a new method for selection of spermatozoa that offers advantages of simplicity of operation, low cost, specificity and sensitivity [18], has been proposed: this technique typically employs several types of nanobeads particles and magnetic microbeads conjugated to proteins or antibodies to tag cells of interest. Annexin V is a phospholipid-binding protein with a high affinity to PS in the presence of physiological concentrations of Ca^{2+} and is unable to pass through intact sperm membranes [19]. Thus, Annexin V binding by a sperm indicates that its membrane integrity has been compromised, as well as its capacity to fertilize eggs [20].

Annexin V-conjugated super-magnetic microspheres, which are exposed to a magnetic field in an affinity column, can effectively separate apoptotic spermatozoa (EPS, spermatozoa-labelled positive for apoptotic markers) from non-apoptotic (intact membranes, spermatozoa free of apoptotic markers) based on the externalization of phosphatidylserine residues. Therefore, cells with externalized PS (Annexin V-positive) will bind to these micro-beads when placed into a column containing iron balls and passed through a strong magnetic field; those cells remain in the separation column and retained in the magnetic field [17, 21]. Non-apoptotic cells (Annexin V- negative) with intact membrane remain unlabelled and pass freely through the column.

Since its appearance, many studies have evaluated the use of MACS as a method to reduce apoptotic sperm and improve sperm and embryo quality. On the base of these findings, other group studied MACS as a sperm selection method for ART [22].

In the current study we aimed to investigate whether the sperm fertilizing potential can be improved by selecting a non-apoptotic fraction using MACS after DGC, and to compare the results with the combination of DGC with conventional swim-up method. The impact of these two sperm preparation techniques on sperm quality was evaluated by comparing 'rapid progressive' motility, normal morphology according to Tygerberg's strict criteria and DNA integrity.

Materials and methods

Experimental design

A total of 25 male patients attending the andrology laboratory for sperm DNA fragmentation analysis were recruited. All subjects enrolled in the study signed a written informed consent. The mean age of men was 36.56±4.25 years (range 25–44 years). Patients collected semen by masturbation into sterile cups after 3–5 days of sexual abstinence. The semen analysis procedure in this study was performed according to the WHO guidelines [23]; the basic sperm characteristics of 25 male patients are reported in Table 1. The values of sperm concentration, total sperm motility, 'rapid progressive' sperm motility and normal sperm morphology were relatively different because of the nature of our population, which consisted of men with normal and abnormal seminal parameters. Nevertheless, we included in the study only samples with ≥10 × 10^6spermatozoa/ml, at least 30 % of total sperm motility and 8 % of normal sperm morphology (according to Tygerberg's strict criteria). Semen samples were allowed to liquefy for 30 min at room temperature and then prepared using a discontinuous PureSperm gradient (Nidacon, Gothemberg, Sweden). Briefly, sperm was layered upon a 40:80 % PureSperm density gradient, processed by centrifuge at 600 × *g* for 15 min and resuspended in 1 ml of sperm culture medium (PureSperm wash, Nidacon, Gothemberg, Sweden). After density gradient separation, the samples were divided into three aliquots. The first was further separated into Annexin V-negative (non-apoptotic) fraction using MACS (group A), the second was further processed by swim-up (group B), while the third was left unseparated as a control (group C) (Fig. 1). The only exclusion criterion was the presence of less than 6 × 10^6/ml total motile spermatozoa after DGC; this was determined to have sufficient number of sperm cells to perform the MACS separation technique, as well as the swim-up procedure. The impact of the combination of DGC with the two sperm preparation techniques on sperm quality was evaluated by comparing 'rapid progressive' motility, normal morphology according to Tygerberg's strict criteria [24] and DNA integrity (by terminal deoxynucleotide transferase-mediated dUTP nick-end labeling [TUNEL]) for each aliquot. TUNEL assay protocol and related cut off values for this evaluation were performed as previously described elsewhere [10].

Table 1 Basic sperm characteristics of male patients

Parameter	Value
No. of patients	25
Age (years)	36.56±4.25 (25 – 44)
Sperm concentration (10^6/ml)	45.74±34.82 (11 – 176)
Total sperm motility (%)	50.00±6.92 (35 – 65)
'Rapid progressive' sperm motility (%)[a]	6.60±5.90 (0 – 15)
Normal sperm morphology (%)[b]	21.84±9.05 (8 – 40)

Values are expressed as mean±SD (range)

[a] Grade A motility [23]

[b] According to Tygerberg's strict criteria [24]

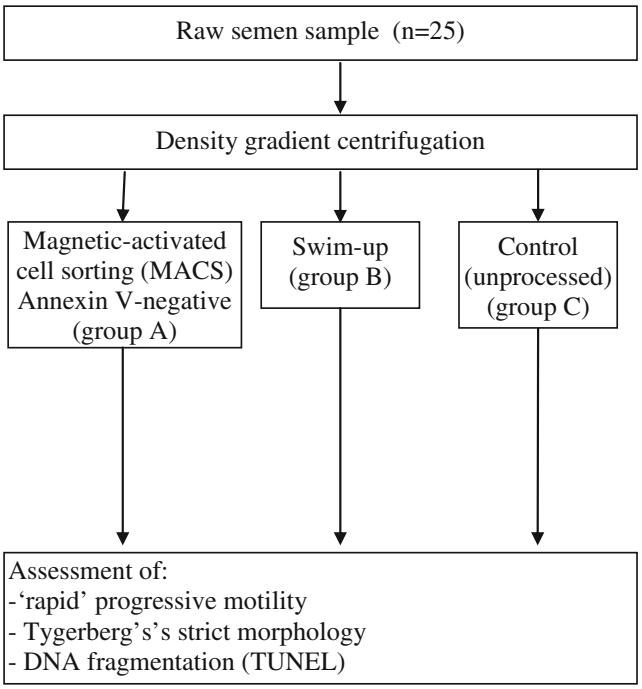

Fig. 1 Experimental design

Swim up

An aliquot of semen was washed with 0.5 ml of sperm culture medium (PureSperm wash, Nidacon, Gothemberg, Sweden) in a 15 ml Falcon conical tube and then centrifuged at 200 × *g* for 10 min. The supernatant was discarded and the pellet resuspended in 0.5 ml of PureSperm wash. Additionally, 1 ml of PureSperm wash was gently layered on the sperm suspension; the tube was inclined at an angle of 45° and incubated at 37 °C for 45 min. After that time the tube was gently set upright and the upper interface, around 0.3 ml, aspirated with a Pasteur pipette and transferred into eppendorf. A small aliquot was examined for sperm morphology and motility, and the rest was used for the DNA fragmentation analysis.

Magnetic activated cell sorting

Briefly, the washed spermatozoa were incubated with Annexin V-conjugated microbeads (Miltenyi Biotec, Auburn, CA, USA) at room temperature for 15 min. About 10 μl of microbeads were used for each one million separated cells. The spermatozoa-microbeads suspension was loaded on a separation column containing a coated cell-friendly matrix containing iron balls, which was fitted in a magnet (MiniMACS; Miltenyi Biotec). The power of magnetic field is measured as 0.5 Tesla between the poles of the magnet and up to 1.5 Tesla within the iron globes of the column. The fraction consistent of sperm with apoptotic markers was retained in the separation column and identified as Annexin V- positive (externalized PS), whereas the fraction with intact membranes (non-apoptotic) that was eluted with buffer (Binding Buffer Stock Solution, Miltenyi Biotec) through the column was identified as Annexin V-negative [17, 21].

Statistical analysis

Statistical analysis was performed with the Statistics Package for Social Sciences for Windows software package version 10.1 (SPSS, Chicago, IL, USA). Comparison of 'rapid progressive' motility, normal sperm morphology and TUNEL positivity between control group and each of the sperm fractions isolated after DGC plus magnetic-activated cell sorting and after DGC plus swim-up was carried out using the *t*-test considering two groups at a time. Intra-individual comparison between control group and each sperm fractions isolated after DGC plus magnetic-activated cell sorting and after DGC plus swim up in terms of TUNEL positivity was performed using chi-squared test considering two groups at a time. Levene's test was applied before independent-samples *t*-test to verify the equality of variances. Statistical differences were considered significant at $P < 0.05$ and highly significant at $P < 0.01$.

Results

Sperm characteristics of 'rapid progressive motility', normal sperm morphology and TUNEL positivity of control group and each of the sperm fractions isolated after combination of DGC plus magnetic-activated cell sorting and after combination of DGC plus swim up are showed in Table 2.

The percentage of 'rapid progressive' motility compared to DGC control (group C), was significantly increased by 18.23 % in group B (34.00±15.00 % vs. 40.20±11.04 %, $p < 0.05$) and by 7.65 % in group A (34.00±15.00 % vs. 36.60±14.27 %, $p < 0.05$); similarly, compared to control group C the mean percentage extent of DNA fragmentation was significantly reduced by 39.66 % in group B (3.48±4.54 % vs. 2.10±2.75 %, $p < 0.05$) and by 30.75 % in group A (3.48±4.54 % vs. 2.41±2.72 %, $p < 0.05$). Otherwise, compared to control, no significant percentage variation was achieved for normal sperm morphology in both group B and group A (−0.18 and + 0.94 %, respectively; 27.54±9.14 % vs. 27.59±9.92 % and 27.85±8.86 % vs. 27.59±9.92 %, respectively) (Table 2). Noticeably, the mean percentage of sperm DNA fragmentation in group B was also significantly reduced by 12.86 % when compared with group A (2.10±2.75 % vs. 2.41±2.72 %, respectively, $p < 0.05$), whereas the mean percentage of 'rapid' progressive motility and of morphologically normal sperm was not significantly different between group B and group A (36.60±14.27 % vs. 40.20±11.04 % and 27.54±9.14 % vs. 27.85±8.86 %, respectively) (Table 3).

Table 2 Sperm characteristics of 'rapid progressive motility', normal sperm morphology and TUNEL positivity of control group and sperm fractions isolated after DGC+MACS and after DGC+swim-up

	group A	group B	group C
'Rapid progressive' motility (%)[a]	36.60±14.27 (0 – 60)	40.20±11.04 (20 – 60)	34.00±15.00 (5 – 65)
Normal sperm morphology (%)[b]	27.85±8.86 (11.9–44.8)	27.54±9.14 (10.3 – 46.6)	27.59±9.92 (7.6 – 45)
TUNEL positivity (%)	2.41±2.72 (0.1 – 9.6)	2.10±2.75 (0.1 – 10.2)	3.48±4.54 (0.1 – 17.8)

DGC density gradient centrifugation, *MACS* magnetic-activated cell sorting

Values are expressed as mean±SD (range)

[a] Grade A motility [23]

[b] According to tygerberg's strict criteria [24]

Considering intra-individual differences, the DGC combined with swim-up procedure significantly reduced the percentage of DNA fragmentation in eight patients out of 25, while the DGC combined with MACS procedure significantly reduced the percentage of DNA damage in only three patients out of 25 (Table 4). In addition, interesting results came from the Annexin V-positive fraction, (externalized PS, apoptotic sperm) which was retained in the magnetic field: compared to control (group C), significant differences regard all the previously considered parameters were found (data not shown). Briefly, the percentage of 'rapid progressive' motility in the Annexin-V positive group compared to group C was significantly reduced (0.00±0.00 % vs. 34.00±15.00 %, respectively, p <0,05); similarly, the mean percentage of morphologically normal sperm was significantly different between Annexin V-positive fraction and group C (12.90± 4.91 % vs. 27.59±9.92 %, respectively, p <0,05). Finally, the mean percentage of sperm DNA fragmentation in the Annexin-V positive fraction was significantly increased compared to control group (6.71±7.00 % vs. 3.48±4.54 %, respectively, p <0,05).

Discussion

The use of assisted reproductive technologies has increased dramatically worldwide since the birth of the first IVF-conceived child in 1978. While these technologies have revolutionised the treatment of infertile couples, sperm preparation techniques still require improvements and development of advanced and molecular selection strategies. Furthermore, the conventional semen analysis does not adequately represent sperm functional status [25]. Current pregnancy and live birth success rates of assisted reproduction technologies are not completely satisfactory; the use of apoptotic sperm during ART may be one of the causes for these suboptimal results [3]. Therefore, the selection of non-apoptotic sperm should be one of the prerequisites for achieving optimal conception rates after ART. This supports the hypothesis that the sperm selection methods currently used prior to ART are inappropriate and that other methods need to be considered. Recently, a novel system of sperm selection has been introduced: magnetic activated cell sorting is considered a flexible, fast and simple cell sorting system for separation of large numbers of cells according to specific cell surface markers [6]. The procedure employs a relatively inexpensive technology that could be easily applied in andrology laboratories, efficiently reduces sperm DNA fragmentation levels [26–32] and effectively separates apoptotic from non-apoptotic spermatozoa [22]. MACS has further benefit of the efficient removal of the caspases that are present in human spermatozoa, which represent the main pathway of apoptosis [8]. Their removal enhances human sperm motility and cryosurvival rates following cryopreservation [22]. However, some

Table 3 Comparison and percentage variation of 'rapid progressive' motility, normal sperm morphology and TUNEL positivity between control and the sperm fractions isolated after DGC+MACS and after DGC+swim-up

	'Rapid progressive' motility[a]	Normal sperm morphology[b]	TUNEL positivity
group A vs group C	+ 7.65 % (p=0.018)	+ 0.94 % (p=0.343)	−30.75 % (p=0.016)
group B vs group C	+ 18.23 % (p=0.002)	−0.18 % (p=0.469)	−39.66 % (p=0.002)
group A vs group B	+ 9.86 % (p=0.107)	−1.11 % (p=0.271)	−12.86 % (p=0.017)

DGC density gradient centrifugation, *MACS* magnetic-activated cell sorting, *ns* not significant

[a] Grade A motility [23]

[b] According to Tygerberg's strict criteria [24]

Statistical differences were considered significant at p<0.05

Table 4 Intra-individual comparison between control and sperm fraction isolated after DGC+MACS and between control and sperm fraction isolated after DGC+swim-up in terms of TUNEL positivity

Patient No.	TUNEL positivity (%)			*p*-value (χ-square)
	group A	group B	group C	
1	2,7	0,6	1,3	ns
2	1,3[a]	0,4[b]	3,8[a, b]	<0,05[a, b]
3	5,2	3,7[b]	7,9[b]	<0,05[b]
4	0,2	0,2	0,2	ns
5	0,1[a]	0,1[b]	1,3[a, b]	<0,05[a, b]
6	7,2	5,4[b]	10,6[b]	<0,05[b]
7	2,7	2,1	3,8	ns
8	0,6	0,8	0,1	ns
9	0,8	0,6[b]	2,1[b]	<0,05[b]
10	8,9[a]	9,6[b]	17,8[a, b]	<0,05[a, b]
11	0,6	0,8	0,1	*ns*
12	0,6	0,6	1,2	*ns*
13	3,4	2,9[b]	5,7[b]	<0,05[b]
14	0,6	0,6	0,1	*ns*
15	1,0	0,6	1,7	*ns*
16	9,6	10,2	12,2	*ns*
17	0,2	0,2	0,1	*ns*
18	2,1	1,5	0,5	*ns*
19	0,8	1,0	0,2	*ns*
20	3,2	2,6[b]	5,1[b]	<0,05[b]
21	0,1	0,2	0,1	*ns*
22	4,2	4,6	6,7	*ns*
23	1,4	1,2	0,3	*ns*
24	0,2	0,1	0,1	*ns*
25	2,7	2,1	2,0	*ns*

DGC density gradient centrifugation, *MACS* magnetic-activated cell sorting, *ns* not significant

Statistical differences were considered significant at $p < 0.05$

concerns about the safety of this technology have arisen, especially if the selected spermatozoa are to be used for ICSI. In 2003 Paasch and colleagues [21], using transmission electron microscopy, demonstrated that the Annexin V-negative fraction did not have microbeads attached to the plasma membrane. Also scanning transmission electron microscopy is currently being performed to study the presence of Annexin V-binding to the eluted and ready to inject spermatozoa.

Several authors reported an improvement in fertilization rates [33, 34] and embryo quality [35, 36, 30] with sperm selected using MACS compared with standard selection methods, while other studies did not find any differences in terms of fertilization potential [35–37, 30]. Actually, promising results of MACS were observed in the outcomes of couples with previous assisted reproduction failure. Studies that included IUI in couples with unexplained infertility [38, 39] and ICSI in patients with high sperm DNA fragmentation [40, 41, 32] argued that the use of MACS would improve the results for couples with repeated assisted reproduction failure. Anyway, despite the absence of significant discrepancies between their results, these studies demonstrated a considerable variability: this variability, the relatively poor number of samples included and the lack of female factor consideration could represent a limitation. Moreover, even if a considerable improvement in pregnancy rates was observed, the implantation and miscarriage rates did not vary between MACS or standard sperm selection methods in the outcomes of couples with previous assisted reproduction failure [22].

In the current study, the sperm fertilizing potential has been assessed following two preparation protocols that combine Annexin V-MACS and swim-up with density gradient centrifugation, respectively. DGC has been currently established as gold standard in sperm preparation protocols prior to assisted reproductive techniques [42] and standardized to complement MACS [43]. On the other hand, the swim-up procedure uses the active motion of spermatozoa, and avoid repeated steps of centrifugation and re-suspension, which might be detrimental when applied to semen samples characterized by limited sperm counts, as low sperm recovery may be expected [21].

Several studies report that the combination of MACS with DGC yields a clean sperm population characterized by higher motility, viability and morphology. Therefore, their combination is actually considered one of the most advantaged sperm selection method [18]. In this study, MACS, when performed after density gradient centrifugation, resulted in the separation of a sperm sub-population (Annexin V-negative, non-apoptotic) that displayed a good fertilization potential, which was reflected by significantly higher 'rapid progressive' motility (+7.65 %, $p < 0.05$) and normal sperm morphology (+ 0.94 %, *ns*) values as well as significantly lower expression of DNA fragmentation (−30.75 %, $p < 0.05$). In addition, the results obtained were significantly different compared with the values detected in the Annexin V-positive sperm subpopulation in all the assessed parameters (data not shown). According to these results it seems possible that the current standard protocols for sperm preparation can still be improved by technical additions such as MACS. Anyway, in the current experiment, the combination of DGC with traditional swim-up method was superior to MACS in terms of providing motile, viable and non-apoptotic spermatozoa. As a matter of fact, in the swim-up procedure, when performed after DGC, the percentage of 'rapid progressive' motile spermatozoa was significantly increased (+18.23 %, $p < 0.05$), with the presence of a significantly lower extent of DNA damage (−39.66 %, $p < 0.05$). Most important, the mean percentage of sperm DNA fragmentation after DGC plus swim-up was also significantly reduced when compared MACS (−12.86 %, $p < 0.05$). These results are consistent with those recently published in the study by Grunewald [44], in which preparation by DGC and

swim-up resulted in improvement of progressive motility, reduction of spermatozoa with disrupted mitochondrial membrane potential and activated Caspase-3, and more recently by Jackson [45], whose data indicated that density gradient centrifugation followed by swim-up can be used to select a postincubation population of spermatozoa with both high DNA integrity and high motility.

We could conclude arguing that the ability of MACS as a molecular preparation technique to isolate sperm populations with reduced DNA damage and improved motility and morphology should be taken into account when selecting sperm processing protocols. Nevertheless, further research that includes live birth rates, in addition to well designed prospective studies under controlled conditions, should lead to better evidence regarding the usefulness of the MACS method as a clinically beneficial sperm selection method in ART.

Acknowledgments -Marco Nadalini, B.Sc.: important contributions to conception, review of manuscript and final approval of the version to be published.

-Nicoletta Tarozzi, PhD: important contributions to acquisition and analysis of data, review of manuscript and final approval of the version to be published.

-Marlea Di Santo, B.Sc: important contributions to acquisition and analysis of data, review of manuscript and final approval of the version to be published.

-Andrea Borini, M.D.: important contributions to review of manuscript and final approval of the version to be published.

References

1. Henkel R, Hajimohammad M, Stalf T. Influence of deoxyribonucleic acid damage on fertilization and pregnancy. Fertil Steril. 2004;81:965–72.
2. Seli E, Gardner DK, Schoolcraft WB. Extent of nuclear DNA damage in ejaculated spermatozoa impacts on blastocyst development after in vitro fertilization. Fertil Steril. 2004;82:378–83.
3. Sun JG, Jurisicova A, Casper RF. Detection of deoxyribonucleic acid fragmentation in human sperm: correlation with fertilization in vitro. Biol Reprod. 1997;56:602–7.
4. Ombelet W, Deblaere K, Bosmans E. Semen quality and intrauterine insemination. Reprod Biomed Online. 2003;7:485–92.
5. Paasch U, Grunewald S, Glander HJ. Sperm selection in assisted reproductive techniques. Soc Reprod Fertil Suppl. 2007;65:515–25.
6. Makker K, Agarwal A, Sharma RK. Magnetic activated cell sorting (MACS): utility in assisted reproduction. Indian J Exp Biol. 2008;46:491–7.
7. Evenson DP, Larson KL, Jost LK. Sperm chromatin structure assay: its clinical use for detecting sperm DNA fragmentation in male infertility and comparisons with other techniques. J Androl. 2002;23:25–43.
8. Paasch U, Grunewald S, Agarwal A. Activation pattern of caspases in human spermatozoa. Fertil Steril. 2004;81 Suppl 1:802–9.
9. Tarozzi N, Bizzaro D, Flamigni C. Clinical relevance of sperm DNA damage in assisted reproduction. Reprod Biomed Online. 2007;14:746–57.
10. Borini A, Tarozzi N, Bizzaro D. Sperm DNA fragmentation: paternal effect on early post-implantation embryo development in ART. Hum Reprod. 2006;21:2876–81.
11. Duran EH, Morshedi M, Taylor S. Sperm DNA quality predicts intrauterine insemination outcome: a prospective cohort study. Hum Reprod. 2002;17:3122–8.
12. Gandini L, Lombardo F, Paoli D. Study of apoptotic DNA fragmentation in human spermatozoa. Hum Reprod. 2000;15:830–9.
13. Huang CC, Lin DP, Tsao HM. Sperm DNA fragmentation negatively correlates with velocity and fertilization rates but might not affect pregnancy rates. Fertil Steril. 2005;84:130–40.
14. Shen HM, Dai J, Chia SE. Detection of apoptotic alterations in sperm in subfertile patients and their correlations with sperm quality. Hum Reprod. 2002;17:1266–73.
15. Vermes I, Haanen C, Steffens-Nakken H. A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. J Immunol Methods. 1995;184:39–51.
16. Martin SJ, Reutelingsperger CP, Mc Gahon AJ. Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl. J Exp Med. 1995;182:1545–56.
17. Grunewald S, Paasch U, Glander HJ. Enrichment of non-apoptotic human spermatozoa after cryopreservation by immunomagnetic cell sorting. Cell Tissue Bank. 2001;2:127–33.
18. Said TM, Agarwal A, Zborowski M. Utility of magnetic cell separation as a molecular sperm preparation technique. J Androl. 2008;29:134–42.
19. van Heerde WL, de Groot PG, Reutelingsperger CP. The complexity of the phospholipid binding protein Annexin V. Thromb Haemost. 1995;73:172–9.
20. Glander HJ, Schaller J. Binding of annexin V to plasma membranes of human spermatozoa: a rapid assay for detection of membrane changes after cryostorage. Mol Hum Reprod. 1999;5:109–15.
21. Paasch U, Grunewald S, Fitzl G. Deterioration of plasma membrane is associated with activated caspases in human spermatozoa. J Androl. 2003;24:246–52.
22. Gil M, Sar-Shalom V, Melendez SY. Sperm selection using magnetic activated cell sorting (MACS) in assisted reproduction: a systematic review and meta-analysis. J Assist Reprod Genet. 2013;30:479–85.
23. World Health Organization. WHO laboratory manual for examination of human semen. Cambridge: Cambridge University Press; 1999.
24. Kruger TF, Menkveld R, Stander FS. Sperm morphologic features as a prognostic factor in in vitro fertilization. Fertil Steril. 1986;46:1118–23.
25. Ricci G, Perticarari S, Boscolo R. Semen preparation methods and sperm apoptosis: swim-up versus gradient-density centrifugation technique. Fertil Steril. 2009;91:632–8.
26. Herrero MB, Delbes G, Troueng E. Annual meeting of the european society on human reproduction and embryology (ESHRE). differential enrichment of sperm with no DNA strand breaks using magnetic activated cell sorting (MACS) in men with various categories of semen parameters. Hum Reprod. 2012;27:P-034.
27. Losada C, Ortega I, Pacheco A. 28th annual meeting of the european society on human reproduction and embryology (ESHRE). Can MACS as a sperm preparation technique improve clinic results in patients with kartagener syndrome? Hum Reprod. 2012;27:S2.
28. Rawe VY, Alvarez CR, Uriondo H. Separacion magnetica por columnas de anexinas V:"filtrado molecular" para la seleccion de espermatozoides no apoptoticos. Reproduccion. 2009;24:104–14.
29. Rawe VY, Alvarez CR, Uriondo H. ICSI outcome using Annexin V columns to select non-apoptotic spermatozoa. Fertil Steril. 2009b;92(3):S73–4.
30. Said TM, Grunewald S, Paasch U. Effects of magnetic-activated cell sorting on sperm motility and cryosurvival rates. Fertil Steril. 2005;83:1442–6.
31. Winkle T, Ditzel N, Gagsteiger F. Possibilities of sperm isolation by the MACS-System. Fertil Steril. 2006;86(3):S130–1.

32. Young Obejero E, De Caro R, Marconi G. Reproductive outcome using Annexin V columns for non-apoptotic sperm selection. Hum Reprod. 2010;25:i7.
33. Grunewald S, Reinhardt M, Blumenauer V. Increased sperm chromatin decondensation in selected nonapoptotic spermatozoa of patients with male infertility. Fertil Steril. 2009;92:572–7.
34. Romany L, Meseguer M, Garcia Herrero JL. Magnetic activated sorting of non-apoptotic sperm result in improved embryo quality in ovum donation cycles with intracytoplasmic sperm injection. Hum Reprod. 2010;25:i8.
35. Alvarez Sedò C, Uriondo H, Lavolpe M. Clinical outcome using non-apoptotic sperm selection for ICSI procedures: report of 1 year experience. Fertil Steril. 2010;94:S232.
36. Dirican EK, Ozgun OD, Akarsu S. Clinical outcome of magnetic activated cell sorting of non-apoptotic spermatozoa before density gradient centrifugation for assisted reproduction. J Assist Reprod Genet. 2008;25:375–81.
37. Buzzi J, Valcarcel A, Lombardi E. Magnetic activated cell sorting (MACS) improves oocyte donation results associated to severe male factor infertility. Hum Reprod. 2010;25:i124.
38. Lee H, Liu CH, Lee MS. Sperm preparation by magnetic activated cell sorting (MACS) reduced apoptotic sperm and improved acrosome reaction for unexplained infertility. Fertil Steril. 2009;92:S143.
39. Lee TH, Liu CH, Shih YT, et al. Magnetic-activated cell sorting for sperm preparation reduces spermatozoa with apoptotic markers and improves the acrosome reaction in couples with unexplained infertility. Hum Reprod. 2010;25:839–46.
40. Polak De Fried E, Denaday F. Single and twin ongoing pregnancies in two cases of previous ART failure after ICSI performed with sperm sorted using annexin V microbeads. Fertil Steril. 2010;94(1):351e15.
41. Van Thillo G, Guidobono M, Young E. Biological safety and live birth after selection of non- apoptotic spermatozoa during ICSI. Fertil Steril. 2011;96(3):S160–1.
42. Chen MJ, Bongso A. Comparative evaluation of two density gradient preparations for sperm separation for medically assisted conception. Hum Reprod. 1999;14:759–64.
43. Said TM, Grunewald S, Paasch U, et al. Advantage of combining magnetic cell separation with sperm preparation techniques. Reprod Biomed Online. 2005;10:740–6.
44. Grunewald S, Reinhardt M, Blumenauer V. Effects of post-density gradient swim-up on apoptosis signalling in human spermatozoa. Andrologia. 2010;42:127–31.
45. Jackson RE, Bormann CL, Pericles AH, et al. Effects of semen storage and separation techniques on sperm DNA fragmentation. Fertil Steril. 2010;94(7):2626–30.