Human Reproduction, Vol.25, No.4 pp. 839–846, 2010
Advanced Access publication on February 10, 2010 doi:10.1093/humrep/deq009

human reproduction

ORIGINAL ARTICLE *Andrology*

Magnetic-activated cell sorting for sperm preparation reduces spermatozoa with apoptotic markers and improves the acrosome reaction in couples with unexplained infertility†

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Submitted on September 29 2009; resubmitted on December 27 2009; accepted on January 6 2010

BACKGROUND: Couples with unexplained infertility (UI) tend to have low fertilization rates with current IVF procedures. Here, we attempted to identify spermatozoa with apoptotic markers in couples with UI and unsuccessful intrauterine insemination (IUI) and we investigated the efficiency and benefit of magnetic-activated cell sorting (MACS) for sperm preparation in such patients.

METHODS: Sixty couples with UI and two IUI failures were recruited. The sperm were prepared by conventional density gradient centrifugation (DGC) and divided into two aliquots. One aliquot was used as a control and the other was further processed by MACS (D + M). Apoptotic markers were identified using fluorescence-labeled dye and flow cytometry, including externalization of phosphatidylserine (EPS), disrupted mitochondrial membrane potential (MMP) and DNA fragmentation. The fertilization potential of prepared spermatozoa was analyzed by basic semen analysis, computer-aided sperm analysis and the induced acrosome reaction test (IART).

RESULTS: After DGC, spermatozoa showed 18.6% EPS, 28.3% disrupted MMP and 13.5% DNA fragmentation. Numbers of spermatozoa with apoptotic markers were significantly reduced by D + M, versus DGC alone ($P < 0.001$). Although the motility of spermatozoa was slightly decreased after MACS, most sperm motion characteristics were not impaired. Interestingly, the IART significantly improved after D + M, versus DGC alone, especially for the couples with a normal hemizona assay ($P < 0.001$).

CONCLUSIONS: The spermatozoa prepared by D + M showed a reduced level of apoptotic markers. Improvement in the IART suggests a high fertilization potential of the processed spermatozoa. The identification of apoptotic markers and use of MACS may be helpful in directing the management plan for patients with UI and multiple IUI failures.

Key words: acrosome reaction / apoptotic markers / magnetic-activated cell sorting / semen analysis / infertility

Introduction

Unexplained infertility (UI) is a diagnosis of exclusion subsequent to a standard and comprehensive infertility evaluation, including semen analysis, tests of ovulation and tubal patency, that has failed to detect any gross abnormalities (Crosignani *et al*., 1993). Although the term UI is controversial and the possible etiology is heterogeneous (Gleicher and Barad, 2006), the initial management plan is not affected by additional invasive tests or investigations (Siristatidis and Bhattacharya, 2007). In general, stimulation of ovulation either alone or combined with intrauterine insemination (IUI) or IVF is commonly utilized for the management of UI (Guzick *et al*., 1999; Liu and Baker, 2003).

†Part of the data were presented at the Annual Meeting of the American Society for Reproductive Medicine in October 2009.

The conventional or basic semen analysis as recommended by World Health Organization (WHO) guidelines includes the assessment of sperm concentration, progressive motility and normal morphology of ejaculated spermatozoa (WHO, 1999). For general practice, all results of basic semen analysis have to be above the reference values provided by the WHO guidelines for the male partner of couples with UI. However, the sperm quality necessary for successful IUI is lower than the reference values in the WHO guidelines for assessing male fertility potential (Dickey *et al*., 1999). Furthermore, the increase in sperm quality parameters above a minimal threshold of sperm concentration (5×10^6/ml) and motility (30%) is not associated with a corresponding increase in the likelihood of pregnancy by IUI (Dickey *et al*., 1999). In contrast, DNA fragmentation in spermatozoa has been reported to be relevant to the success of IUI treatment for a cohort of infertile couples, including 41% with UI (Duran *et al*., 2002). In addition, up to 29% of couples with UI have defective zona pellucida-induced acrosome reactions subsequent to zona pellucida binding, which indicates the patients are at a risk of zero or have a low fertilization rate in a standard IVF program (Liu and Baker, 2003).

For the management of patients with UI, failure of IUI necessitates further evaluation of biological properties of spermatozoa, such as apoptotic markers and DNA fragmentation (Aziz and Agarwal, 2008). An alternative approach is performing sperm functional tests, including sperm–zona pellucida binding and/or acrosome reaction tests, to direct the treatment plan into standard IVF or ICSI (Liu and Baker, 1994, 2003). The hemizona assay in addition to semen analysis has been utilized to direct the treatment of IVF or ICSI for infertile couples (Lee *et al*., 2008), which is also applicable for patients with UI and IUI failure.

In recent decades, several sperm preparation techniques have been developed for assisted reproduction treatment (ART) laboratories (Henkel and Schill, 2003). Some methods are based upon molecular attributes of spermatozoa, such as attempts to eliminate spermatozoa with apoptotic markers. Annexin V-conjugated magnetic microbeads can be utilized effectively to separate spermatozoa without apoptotic markers from those with deteriorated plasma membranes based on the externalization of phosphatidylserine (EPS) using magnetic-activated cell sorting or separation (MACS; Said *et al*., 2005). Such preparation techniques might be helpful to obtain spermatozoa with high fertilization potential for patients with UI and IUI failure, and then to improve the outcome of subsequent ART management.

In the current study, patients with UI who had undergone IUI twice, but failed to conceive, were recruited. The goal of this study was to survey the incidence of spermatozoa with apoptotic markers and also investigate the efficiency of the MACS technique for sperm preparation in these patients. The fertilizing potential of spermatozoa after MACS was further evaluated by computer-aided sperm motion analysis (CASA) and the induced acrosome reaction test (IART).

Materials and Methods

Patient selection

The study was approved by the Institutional Review Board of Chung Shan Medical University (CS07162) and Lee Women's Hospital (LWH08001) in Taiwan. Signed informed consent for study participation was obtained from all participants.

The initial survey for the etiology of infertility for patients seeking treatment included the following: semen analysis, hysterosalpingography or laparoscopy for tubal patency and assessing serum hormone levels of prolactin, estradiol, testosterone, FSH, LH and TSH. The infertile couples with the following characteristics were included: ovulating women with bilateral patent oviducts, the presence of bilateral ovaries, no endocrine disorders (polycystic ovary syndrome or hyperprolactinemia) and a male partner with normal parameters on semen analysis. Exclusion criteria were as follows: females $\geq$38 years of age; baseline FSH $>$12 mIU/ml; endometriosis; and uterine synechiae. The couples recruited for this study were classified as UI. In addition, the couples had undergone ovarian stimulation combined with IUI twice, but failed to conceive.

The basic semen analysis procedure in this study was performed according to the WHO guidelines (1999). As a case involving UI, the basic semen analysis for the male partner should reveal results above the reference values provided by WHO guidelines (sperm concentration exceeding 20×10^6/ml and percentage of motile sperm $>$50%) and by Tygerberg strict criteria (normal morphology $>$14%; Kruger *et al*., 1986) for at least two of three tests at the time of the initial sperm evaluation and on the day of IUI.

The female partner of couples with UI underwent ovarian simulation using clomiphene citrate plus recombinant FSH, with the patients receiving clomiphene citrate (50 mg/day) on days 3–7 of the stimulation cycle and receiving recombinant FSH (Gonal-F, 150-IU sc injection; Serono, Frankfurt, Germany) on stimulation cycle days 6, 8 and 10 and then daily until the administration of hCG. Further, 250 μg of hCG (Ovitrelle; Serono) was administered to all patients in whom two leading follicles reached $\geq$18 mm in diameter, and IUI was arranged 18–24 h subsequent to hCG administration.

Semen sample collection and preparation

From June 2008 to June 2009, a total of 60 semen samples were obtained from male partners of couples with UI who had failed IUI on two attempts. Following a period of 3–5 days of sexual abstinence, fresh semen samples were collected by masturbation into sterile plastic jars on the day of the hemizona assay. After liquefaction at room temperature (25°C) within 1 h of ejaculation, the ejaculated spermatozoa were prepared by density gradient centrifugation (DGC) at 300*g* with PureSperm (Nicadon, Gothenberg, Sweden) 90−45% in tubes for 5 min. The pellet obtained from the 90% fraction was resuspended in IVF medium (Scandinavian IVF, Gothenburg, Sweden).

One aliquot of the sperm suspension served as a control, and the other aliquot was subjected to MACS (Fig. 1). Spermatozoa were incubated with annexin V-conjugated microbeads (Miltenyi Biotec, Auburn, CA, USA) for 15 min at room temperature. About 100 ml of microbeads were used for each 10 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 fraction composed of spermatozoa with apoptotic markers was retained in the separation column and labeled as annexin-positive, whereas the fraction with intact membranes that was eluted through the column was labeled as annexin-negative. The power of the magnetic field was measured as 0.5 T between the poles of the magnet and up to 1.5 T within the iron globes of the column.

The extent of EPS, the integrity of the mitochondrial membrane potential (MMP) and DNA fragmentation were assessed in the control aliquot (DGC group) and in the MACS preparation samples eluted through the column, labeled as annexin-negative (D + M group). In addition, basic semen analysis, sperm motion characteristics by CASA and the IART were performed for both groups. The morphology of spermatozoa was checked according the WHO guidelines (1999) with Diff-Quick stain (Baxter Scientific Products, McGaw Park, IL, USA).

Evaluation of EPS with Annexin-V

Annexin-V is a calcium-dependent phospholipidic union protein with a high affinity for phosphatidylserine (PS). The translocation of PS from the

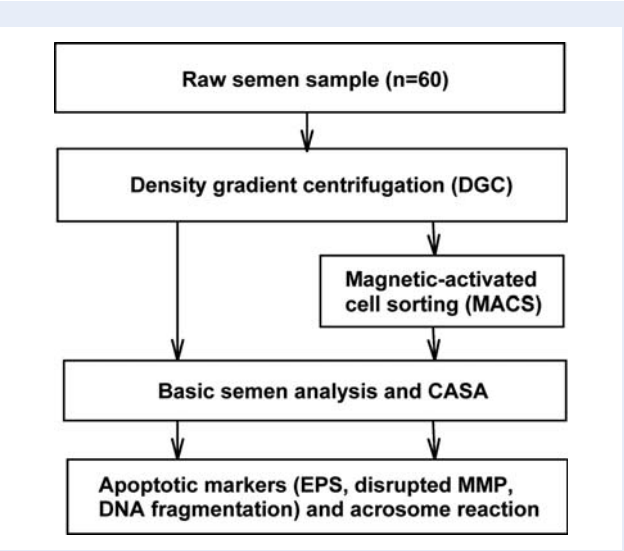

Figure 1 Flow chart of the preparation of sperm from couples with UI. The sperm sample prepared by DGC was divided into two aliquots: a control and one which underwent MACS. CASA, EPS and MMP denote computer-aided sperm analysis, externalization of phosphatidylserine and mitochondrial membrane potential, respectively. CASA, computer assisted sperm analysis; EPS, externalization of phosphotidylserine, MMP, mitochondrial membrane potential.

internal membrane to the external membrane is reflective of early apoptotic events. Binding of annexin-V to externalized PS typically shows a fluorescent green stain. Propidium iodide (PI; red staining) was added to differentiate viable from necrotic cells escaping apoptotic analysis; this allows the exclusion of those cells showing PS translocation that are dead, and therefore the identification of live cells without (normal spermatozoa) and with (live, but apoptotic) PS translocation.

The annexin V assay was performed according to a previous report with minor modifications (Oosterhuis *et al*., 2000). The spermatozoa were washed twice with annexin V binding buffer and then incubated with 0.1 mg/ml fluorescein isothiocyanate (FITC)–labeled annexin V at room temperature for 30 min in the dark, followed by the addition of 50 mg/ml PI (Sigma, St Louis, MO, USA). The sperm were then analyzed in a fluorescence-activated cell sorter (FACS, Becton Dickinson, Oxford, UK). A minimum of 10 000 sperm were examined for each experimental group. The FITC-labeled annexin V-positive sperm cells were measured in the FL1 channel, and the PI-labeled cells were measured in the FL2 channel of the flow cytometer.

Using the annexin V–FITC binding assay coupled to the vital dye PI, we were able to identify four subpopulations of human DGC- and MACS-prepared spermatozoa: (i) live, intact spermatozoa with no EPS (no fluorescence), namely annexin V-negative and PI-negative (AV^-/PI^-), (ii) live spermatozoa with EPS (green), namely AV^+/PI^-, corresponding to spermatozoa with apoptotic markers, (iii) dead spermatozoa with no EPS, but positive for PI (red), namely AV^-/PI^+ and (iv) spermatozoa with EPS and positive for PI (red and green), namely annexin AV^+/PI^+, corresponding to necrotic spermatozoa.

Determination of sperm mitochondrial functional integrity

Sperm mitochondria functional integrity (assessed as MMP) was determined using an ApoalertTM Mitochondrial Membrane Sensor Kit (MitoSensor; Clontech Laboratories Inc., Palo Alto, CA, USA) that allowed detection of changes in the mitochondrial transmembrane potential during the early stages of apoptosis. Briefly, aliquots of spermatozoa samples were centrifuged at 200*g* for 5 min to pellet the cells. The cells were then resuspended in 1 ml of MitoSensor incubation buffer, gently mixed and incubated under 5% CO_2 at 37°C for 40 min in darkness. MitoSensor (1 μl) was then added and the preparation was incubated at 37°C for an additional 10 min before analysis in a FACS.

In healthy cells, MitoSensor is taken up by the mitochondria, where it forms aggregates and emits intense red/orange fluorescence. In dysfunctional (including apoptotic) cells, the MitoSensor cannot aggregate in the mitochondria owing to alterations in the membrane potential. The stain remains as a monomer in the cytoplasm and emits green fluorescence. For each sample, at least 10 000 spermatozoa were counted.

Terminal deoxynucleotidyltransferase-mediated deoxyuridine triphosphate-biotin nick-end labeling assay

DNA fragmentation was evaluated using the TUNEL (terminal deoxynucleotidyltransferase-mediated deoxyuridine triphosphate-biotin nick-end labeling) assay (Boehringer Mannheim, Mannheim, Germany) as in our previous report (Huang *et al*., 2005), with minor modifications. Briefly, the sperm samples were washed twice in phosphate-buffered saline (PBS) for 5 min, followed by centrifugation for collecting spermatozoa at 200*g*. The spermatozoa were then treated with a solution containing 0.1% Triton X-100 (Sigma) and 0.1% sodium citrate (Sigma) for 2 min on ice. A 30-μl TUNEL mixture consisting of terminal deoxynucleotidyl transferase (TdT) and fluorescein dUTP was added to the same volume of each sample. The samples were incubated for 60 min at 37°C in a moist chamber in darkness, washed three times with PBS and then analyzed in a FACS. At least 10 000 cells were counted. The presence of green fluorescent signals was regarded as positive. For positive controls, spermatozoa were processed in the same way, except for a prior incubation with DNase I (1 μg/ml, D-4263; Sigma) for 10 min at room temperature to induce DNA fragmentation before the addition of the TUNEL mixture. For negative controls, the spermatozoa were similarly processed, but the TUNEL mixtures were added without the presence of TdT.

Sperm motion characteristics

The semen samples were kept at 37°C for liquefaction for an average of 1 h (range 0.5–1.5 h) prior to analyses for motion characteristics. Sperm motion characteristics were analyzed using the Copenhagen Rigshospitalet Image House Sperm Motility Analysis System (CRISMAS; ImageHouseA/S, Copenhagen, Denmark), which is described elsewhere (Lee *et al*., 2008) and is performed following the guidelines of the European Society of Human Reproduction and Embryology in 1998 (ESHRE Andrology Special Interest Group, 1998). The results of CRISMAS have been shown to be more reliable and reproducible than previous versions of CASA and the results reported by experienced technicians show a median difference of <5% (Larsen *et al*., 2000). In brief, the setting parameters for analysis included the following: 80 Hz, image-acquisition rate; at least 200 spermatozoa sampled; and at least one microscopic field sampled at ×200 magnification. The chamber utilized for sperm analysis was 0.01 mm^2 in surface area and 0.01 mm deep (Sefi-medical Instruments, Haifa, Israel).

CRISMAS was used to determine various sperm parameters, including concentration, motility, average path velocity (VAP), straight line velocity (VSL), straightness of sperm motion (STR) and the amplitude of lateral head (ALH) displacement . Other sperm parameters measured included the ratio of progressive motile spermatozoa, featuring a VAP > 25 μm/s

and an STR > 80%. The definition of progressive motile spermatozoa is similar to the 'Grade A' sperm motility determined according to WHO guidelines (WHO, 1999).

Hemizona assays

Fresh unfertilized oocytes from our ART program were utilized as the source of zona pellucida. Signed consent was obtained prior to donation of unfertilized oocytes. The sperm concentration was diluted to 10–20 $\times 10^6$/ml, and a total of 20 000 spermatozoa were put into a droplet of medium. The pair of hemizona was co-incubated with spermatozoa from the patient (test) or a fertile man (control) at 37°C in an atmosphere of 5% CO_2 in air for 4 h. The number of spermatozoa tightly bound to the zona was counted and the results of the hemizona assay were expressed as a hemizona index (HZI; the ratio of the number of spermatozoa bound in the test droplet to the number of spermatozoa bound in the control droplet). An HZI level >36% was considered to indicate effective fertilization potential (Oehninger *et al.*, 1989; Lee *et al.*, 2008).

Induced acrosome reaction test

Peanut agglutinin (PNA) from *Arachis hypogea* is specific for β-D-galactose residues, and hence binds to, and labels, the outer acrosomal membrane. The sperm sample was mixed with an equal volume of 2 mg/ml of pentoxiphylline (final concentration of 1 mg/ml; Hoechst, Telford, UK). Subsequent to incubation of sperm samples for 1 h at 37°C and 5% CO_2, sperm acrosome status was assessed by FITC-PNA staining (Sigma; Gearon *et al.*, 1994). Briefly, 20 µl of sperm suspension was spread over a clean microscope slide and allowed to air-dry. The smear was then fixed in 95% ethanol for 5 min and again allowed to air-dry. Fixed slides were stained in FITC-PNA (600 µl aliquot of FITC-PNA in 15.4 ml of reagent water in a foil-covered Coplin jar) for 15 min at ambient temperature. Slides were rinsed by dipping in PBS twice before fixing for 15 min in paraformaldehyde at ambient temperature. Slides were air-dried, mounted and stored in the dark until scoring.

Between 100 and 250 spermatozoa were counted per slide and scored into three classes for PNA labeling: (i) acrosome intact, where whole acrosome labeling denotes an intact outer acrosomal membrane; (ii) partially acrosome reacted, where patchy acrosome labeling is suggestive of a transition stage in which the outer acrosomal membrane is fenestrated; and (iii) acrosome reacted, where the equatorial segment only is labeled, thus denoting a normally acrosome-reacted spermatozoa that has lost the outer acrosomal membrane over the anterior cap of the acrosome, but has retained the equatorial segment of the acrosome intact. Only Class 3 spermatozoa were considered to have undergone an induced acrosome reaction.

Statistical analysis

Data were analyzed using inbuilt functions within the Statistical Package for the Social Sciences, version 14 (SPSS UK Ltd, Chertsey, Surrey, UK). The Wilcoxon signed rank test was used to assess the statistical differences between the two groups, as the study variables were not normally distributed. The correlation between apoptotic markers, acrosome reaction rate and sperm parameters was determined by the Spearman correlation test. A level of $P < 0.05$ was considered significant.

Results

From June 2008 to June 2009, a total of 60 patients with UI who had failed IUI on two attempts were recruited for this study. The basic semen analysis revealed an average (±SD) semen volume of 3.6 ± 1.8 ml (range 2–11 ml), a sperm count of 110.6 ± 81.7 $\times 10^6$/ml (range 20–436 $\times 10^6$/ml), 78.6 ± 15.7% sperm motility (range 50–100%) and 19.7 ± 1.7% normal sperm morphology (range 14–31%).

The spermatozoa prepared by DGC alone or D + M were compared for the percentage of apoptotic markers, such as EPS, disrupted MMP and DNA fragmentation, by a flow cytometry study (Table I). The percentage of early apoptosis markers (AV^+/PI^- and disrupted MMP) in sperm prepared by D + M compared with DGC alone was significantly reduced (8.5 ± 11.5 versus 5.6 ± 7.3% for AV^+/PI^- and 28.3 ± 17.6 versus 19.2 ± 10.8% for disrupted MMP; both $P < 0.001$). About 30% of the spermatozoa with early apoptotic markers were removed by MACS preparation. Furthermore, numbers of spermatozoa with DNA fragmentation, as assessed by TUNEL stain (a late apoptosis marker), were also significantly reduced by including MACS, from 13.5 ± 5.6 to 9.9 ± 3.6% ($P < 0.001$). Approximately one-fourth of spermatozoa with sperm DNA fragmentation were removed.

The motion characteristics of the initial sperm samples and the motion characteristics subsequent to DGC and MACS preparation

Table I Analysis of apoptotic markers in sperm from patients with UI.

Group	DGC	DGC + MACS	*P*-value
Annexin V^+, PI^- (%)	8.5 ± 11.5	5.6 ± 7.3	<0.001
Annexin V^+, PI^+ (%)	10.1 ± 4.8	8.3 ± 3.9	<0.001
Annexin V^-, PI^+ (%)	11.9 ± 9.7	7.3 ± 5.8	<0.001
Disrupted MMP (%)	28.3 ± 17.6	19.2 ± 10.8	<0.001
Sperm DNA fragmentation (%)	13.5 ± 5.6	9.9 ± 3.6	<0.001

Sperm were prepared by DGC and subsequent MACS methods with flow cytometry (data are mean ± SD, $n = 60$).
Analysis by the Wilcoxon signed rank test. PI, propidium iodide; MMP, mitochondrial membrane potential.

Table II Characteristics of sperm from patients with UI, prepared by DGC and subsequent MACS (data are mean ± SD, *n* = 60).

	Original	DGC	DGC + MACS
Motility (percentage of active sperm)	78.6 ± 15.7	79.3 ± 13.6	67.0 ± 22.4*
VAP (µm/s)	36.7 ± 9.8*	50.1 ± 13.3	49.3 ± 10.7
Straightness of sperm motion (%)	71.8 ± 7.5*	62.3 ± 13.3	59.8 ± 12.1
ALH displacement (µm)	3.4 ± 3.9*	3.1 ± 1.1	3.2 ± 1.3
Percentage of progressive motile sperm (%)	24.5 ± 11.9*	28.9 ± 11.2	26.1 ± 11.8

*$P < 0.05$ compared with the other two groups by the Wilcoxon signed rank test.

are shown in Table II. The sperm motility percentage did not change subsequent to DGC (78.6 ± 15.7 versus 79.3 ± 13.6%; $P > 0.05$). However, the motility after MACS significantly decreased from 79.3 ± 13.6 to 67.0 ± 22.4% ($P < 0.05$). Some sperm motion characteristics were significantly improved by DGC preparation, including VAP and percentage of progressive motile sperm (Grade A), but not STR and ALH. Nevertheless, all of the previously described motion characteristics were not changed after MACS (Table II).

The IART was also performed for spermatozoa prepared by DGC alone or by D + M. The proportion of induced acrosome reaction increased after D + M (24.7 ± 19.4 versus 33.5 ± 22.8%; $P < 0.001$). When the population studied was divided into two groups by an HZI level of 36%, we found an interesting result: the acrosome reaction percentage (23.9 ± 11.9 versus 25.2 ± 11.7%; $P > 0.05$) did not change for couples with an abnormal hemizona assay result (HZI < 36%, $n = 8$); however, the patients with an acceptable hemizona assay result (HZI > 36%, $n = 52$) exhibited a significant increase in the acrosome reaction after MACS (25.6 ± 19.6 versus 34.5 ± 24.5%; $P < 0.001$; Fig. 2).

The correlation between apoptotic markers, acrosome reaction rates and basic semen parameters is shown in Table III. The proportion of early apoptosis markers (AV^+/PI^-) and DNA fragmentation were inversely correlated to sperm motility after DGC preparation ($r = -0.422$, $P = 0.006$; $r = -0.397$, $P = 0.010$, respectively). Furthermore, the early apoptosis markers and DNA fragmentation were still negatively related to sperm motility ($r = -0.437$, $P = 0.004$; $r = -0.296$, $P = 0.048$, respectively) after subsequent MACS preparation. The IART was positively correlated with sperm concentration ($r = 0.366$, $P = 0.033$) after DGC + MACS; however, the correlation was not observed for spermatozoa prepared by DGC alone. None of the apoptotic markers and IART correlated with the morphology of spermatozoa.

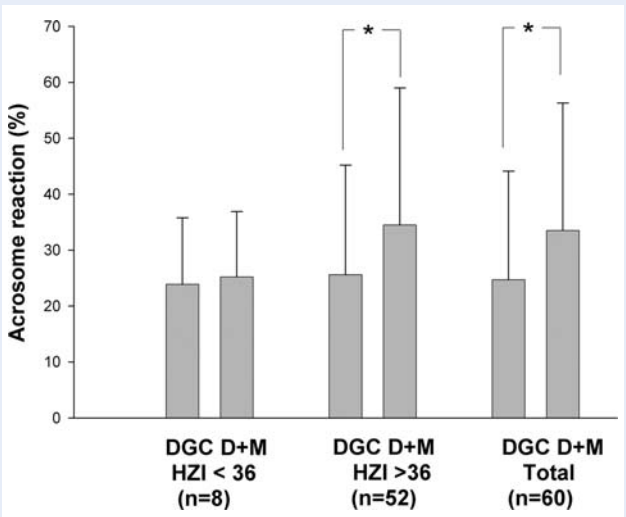

Figure 2 The rate of induced acrosome reaction after sperm preparation by DGC compared with DGC plus MACS (D + M). The study population was divided into two groups by an HZI of 36%. Mean values and the SD bar are shown. *$P < 0.001$ by Wilcoxon signed rank test.

Sperm motility was closely associated with EPS and DNA fragmentation (Table III). Therefore, the correlation between apoptotic markers, IART and sperm motion characteristics was further determined, and some results are shown in Table IV. The VAP and Grade A spermatozoa were negatively correlated with early apoptosis (AV^+/PI^-; $r = -0.398$, $P = 0.012$; $r = -0.447$, $P = 0.006$, respectively) and disrupted MMP ($r = -0.375$, $P = 0.016$; $r = -0.379$, $P = 0.002$, respectively) for spermatozoa prepared by DGC. Similar results were noted for spermatozoa prepared by DGC + MACS. After MACS preparation, the VAP was also inversely correlated to the early apoptotic markers (AV^+/PI^-; $r = -0.389$, $P = 0.012$; $r = -0.313$, $P = 0.046$, respectively). Nevertheless, Grade A spermatozoa only correlated with early apoptotic markers (AV^+/PI^-; $r = -0.421$, $P = 0.010$), but not disrupted MMP ($r = -0.266$, $P = 0.117$). The levels of DNA fragmentation were significantly related to VAP after DGC ($r = -0.363$, $P = 0.020$) and after D + M ($r = -0.391$, $P = 0.011$).

Discussion

It has been reported that ejaculated spermatozoa do exhibit changes consistent with apoptosis in somatic cells, such as EPS, disrupted MMP and/or DNA fragmentation (Said *et al*., 2005; Barroso *et al*., 2006; de Vantery *et al*., 2009). In raw semen of subfertile couples, a mean of 20% (range 5–68%) of spermatozoa have EPS (de Vantery *et al*., 2009). However, DGC, the commonly utilized sperm preparation technique in ART laboratories, does not completely eliminate spermatozoa with apoptotic markers (Henkel and Schill, 2003). After DGC using semen samples obtained from healthy donors, 4.56% EPS was reported (Said *et al*., 2005). In a population with normal sperm concentrations and motilities which was seeking infertility treatment, 15.2% EPS, comprising 6.1% early apoptotic markers and 9.1% late apoptotic markers or necrosis, was noted in the high motility fraction of spermatozoa, following DGC (Barroso *et al*., 2006). The spermatozoa after DGC from subfertile couples featured 14% EPS, consisting of 3% early apoptotic markers and 11% late apoptotic markers or necrosis (de Vantery *et al*., 2009). In the current study, spermatozoa after DGC were still characterized by 18.6% EPS, including 8.5% early apoptotic markers and 10.1% late apoptotic markers or necrosis. The data confirmed the presence of apoptotic markers (EPS) in spermatozoa prepared by DGC for either healthy donors or subfertile couples.

The proportion of EPS after DGC in the present study was similar or slightly higher than in the study for subfertile couples (de Vantery *et al*., 2009) or infertile couples with normal semen parameters (Barroso *et al*., 2006). Furthermore, the current report and the two prior studies (Barroso *et al*., 2006; de Vantery *et al*., 2009) all exhibited a higher rate of EPS than the report for healthy donors (Said *et al*., 2005). A previous study reported that the level of DNA fragmentation and the age of the man were the sole predictors of successful IUI with gonadotrophin-stimulation cycles (Duran *et al*., 2002). A significant correlation between DNA fragmentation (TUNEL stain) and early apoptotic markers (AV^+, PI^+) in raw spermatozoa has been reported (Shen *et al*., 2002). In the present study, a significant positive correlation between DNA fragmentation and early apoptotic markers, after DGC or D + M, was also noted (data not shown). Therefore, the difference in EPS between the present study and other reports may have resulted from population selection. The sperm of UI

Table III The correlation between basic semen analysis parameters of raw semen samples with apoptosis markers and induced acrosome reaction rate.

Sperm parameters	Concentration (10^6/ml)	Motility	Morphology
After DGC (%)			
Annexin V^+, PI^-	−0.172 (0.281)	−0.422 (0.006)*	−0.247 (0.124)
Annexin V^+, PI^+	−0.165 (0.304)	−0.154 (0.338)	−0.267 (0.096)
Disrupted MMP	−0.180 (0.259)	−0.225 (0.156)	−0.220 (0.172)
DNA fragmentation	−0.129 (0.422)	−0.397 (0.010)*	−0.185 (0.254)
Acrosome reaction rate	0.162 (0.359)	−0.001 (0.996)	0.193 (0.246)
After DGC + MACS (%)			
Annexin V^+, PI^-	−0.230 (0.148)	−0.437 (0.004)*	−0.222 (0.170)
Annexin V^+, PI^+	−0.172 (0.283)	−0.224 (0.159)	−0.152 (0.349)
Disrupted MMP	−0.180 (0.260)	−0.244 (0.124)	−0.242 (0.132)
DNA fragmentation	−0.111 (0.490)	−0.269 (0.048)*	−0.045 (0.785)
Acrosome reaction rate	0.366 (0.033)*	0.229 (0.192)	0.265 (0.102)

The data are Spearman correlation coefficients (*P*-value).
*$P < 0.05$.

Table IV The correlation between sperm motion characteristics of raw semen samples with apoptosis markers and induced acrosome reaction rate.

Sperm motion characteristics	VAP (μm/s)	Straightness of motion	Progressive motile sperm
After DGC (%)			
Annexin V^+, PI^-	−0.389 (0.012)*	−0.287 (0.069)	−0.447 (0.006)*
Annexin V^+, PI^+	−0.093 (0.562)	−0.151 (0.345)	−0.008 (0.961)
Disrupted MMP	−0.375 (0.016)*	−0.092 (0.567)	−0.379 (0.002)*
DNA fragmentation	−0.363 (0.020)*	−0.085 (0.596)	−0.172 (0.317)
Acrosome reaction rate	0.062 (0.728)	0.005 (0.976)	0.149 (0.399)
After DGC + MACS (%)			
Annexin V^+, PI−	−0.389 (0.012)*	−0.187 (0.242)	−0.421 (0.010)*
Annexin V^+, PI^+	−0.024 (0.881)	−0.044 (0.785)	−0.033 (0.850)
Disrupted MMP	−0.313 (0.046)*	−0.125 (0.437)	−0.266 (0.117)
DNA fragmentation	−0.391 (0.011)*	−0.083 (0.607)	−0.223 (0.191)
Acrosome reaction rate	0.006 (0.973)	0.121 (0.497)	0.160 (0.367)

The data are Spearman correlation coefficients (*P*-value).
*$P < 0.05$.

couples who failed IUI on two attempts feature a higher proportion of DNA fragmentation and other markers of apoptosis.

The magnetic separation process is based upon EPS to the outer layer of the sperm membrane. The benefit of combining DGC and MACS is the elimination of the poor quality spermatozoa (both immature and featuring apoptotic markers; Said *et al*., 2005). Similar to the previous reports involving healthy donors (Said *et al*., 2005) and infertile couples (Barroso *et al*., 2006; de Vantery *et al*., 2009), in the present study, MACS separated deteriorating spermatozoa from the portion without apoptotic markers for patients with UI. Previous studies regarding the removal of spermatozoa with apoptotic markers from healthy donors indicated that D + M obtained optimal results (Paasch *et al*., 2004; Said *et al*., 2005). Spermatozoa with apoptotic markers significantly decreased after MACS (70% EPS and 60% disrupted MMP; de Vantery *et al*., 2009). In the present study, MACS decreased the EPS by 25% and the disrupted MMP by 32%. The efficiency of MACS for subgroups of infertile couples needs further investigation and elucidation.

Several reports have established the connection between fertilization failure and apoptotic markers in spermatozoa (Gandini *et al*., 2000; Duran *et al*., 2002; Henkel *et al*., 2004; Huang *et al*., 2005). Furthermore, patients with male infertility show a higher rate of spermatozoa with apoptotic markers compared with healthy donors (Gandini *et al*., 2000; Shen *et al*., 2002). In the present study involving patients

with UI and normal sperm parameters, a relatively high rate of EPS after DGC was noted and this high rate of EPS may be responsible for IUI failure in such patients. Although some reports have demonstrated that EPS is a physiological sign of capacitation (Gadella and Harrison, 2002), the strong correlation between EPS and disrupted MMP in spermatozoa with high- and low-motility fractions after DGC indicated that both EPS and disrupted MMP were probably signs of early apoptosis and were indeed present in ejaculated spermatozoa (Barroso *et al*., 2006). In addition, Paasch *et al*. (2004) showed that deterioration of the sperm plasma membrane, as characterized by EPS, is associated with activated caspases. Taken together, EPS in spermatozoa may be a phenotype of early apoptosis, similar to that described in somatic cells. However, owing to the short life of spermatozoa, apoptotic markers may be relevant to defective spermatogenesis, and the significance of this still needs further investigation.

It has been reported that sperm motility decreased after MACS, compared with spermatozoa after DGC, because of the further centrifugation steps in patients with oligoathenoteratozoospermia and asthenoteratozoospermia (Grunewald *et al*., 2008). However, the sperm chromatin decondensation rate after MACS still increased (a surrogate marker for fertilization potential; Grunewald *et al*., 2008). The data in the present study also revealed that D + M was associated with a decrease in sperm motility (Table II). However, most motion characteristics, such as VAP, ALH and straightness, were not significantly different for spermatozoa after DGC and D + M. The results suggest that the sperm function may not be affected by further manipulation during MACS.

In the present study, the hemizona assay and IART were utilized to further evaluate the fertilization potential of spermatozoa after D + M. It has been reported that the hemizona assay is highly predictive of pregnancy outcomes in couples with UI or male factor undergoing IUI management (Arslan *et al*., 2006). Poor HZI usually indicates severe sperm abnormalities, and the defects in sperm motility and morphology or sperm antibody are the main reasons for reduced sperm–zona pellucida binding (Franken *et al*., 1993). The poor HZI results in the present study may be relevant to the failure of IUI. Furthermore, even in patients with a high percentage (>70%) of denatured DNA in DGC-prepared spermatozoa, very few spermatozoa with intact DNA are able to bind to the zona pellucida (Liu and Baker, 2007). A low level of HZI might indicate a low level of spermatozoa with intact DNA. The low proportion of spermatozoa with intact DNA might explain why the MACS technique failed to improve the IART in couples with UI and poor HZI in the current study. For such patients, ICSI is the choice of treatment modality instead of additional IUI attempts or conventional IVF.

Some patients with UI and a normal sperm–zona pellucida binding assay who undergo conventional IVF have a defective zona pellucida-induced acrosome reaction (Liu *et al*., 2001; Liu and Baker, 2003). The zona pellucida-induced acrosome reaction is the sole sperm characteristic associated with the fertilization rate in an IVF program for UI (Liu and Baker, 2003). An improvement in the acrosome reaction after MACS in the current study suggests that using spermatozoa prepared by D + M might improve the outcome of IUI and the fertilization rate in the IVF program for couples with UI. Therefore, our study suggests that couples with UI and a normal hemizona assay may benefit from the MACS preparation technique.

Sperm motility, but not sperm morphology or sperm concentration, is the sole basic sperm parameter correlated with early apoptosis markers (AV^+, PI^-) and DNA fragmentation (TUNEL). Our previous study (Huang *et al*., 2005) reported that DNA fragmentation (TUNEL) was negatively associated with sperm velocity (VAP and VSL) and sperm morphology. In Table IV, the sperm velocity (VAP) was also negatively associated with early apoptosis (AV^+, P^-), disrupted MMP and TUNEL after DGC or D + M (data for VSL were similar, but not shown). Our previous report (Huang *et al*., 2005) recruited a cohort of infertile couples with normal and abnormal sperm parameters and Tygerberg strict criteria utilized for sperm morphology; nevertheless, the current study only included subfertile couples with normal sperm parameters, and the rapid stain process in WHO Guidelines 1999 was used for sperm morphology. Said *et al*. (2005) reported that spermatozoa with apoptotic markers (EPS, caspase-3 and disrupted MMP) were significantly correlated with sperm morphology by Tygerberg criteria, but not by the WHO rapid stain process. The lack of correlation between sperm morphology and apoptotic markers in the present study may result from the difference in study population and/or different methods used to assess for sperm morphology.

The IART after D + M, but not after DGC, was correlated to sperm concentration (Table III). The zona pellucida-induced acrosome reaction rate obtained from patients with UI also exhibited a significant correlation with sperm concentration (Liu and Baker, 2003, 2007). Furthermore, the induced acrosome reaction rate is considered to be one of the efficient methods to evaluate sperm function for predicting IVF outcomes (Oehninger *et al*., 2000). The results of the current study suggest that determining spermatozoa with apoptotic markers may facilitate developing the optimal treatment plan for patients with UI who had failed IUI on two attempts. Furthermore, elimination of spermatozoa with apoptotic markers may improve the fertilization potential of sperm and possibly the outcome of further ART cycles.

In conclusion, EPS and disrupted MMP existed in spermatozoa after DGC for patients with UI who has failed IUI on two attempts. Spermatozoa with such apoptotic markers can be eliminated by MACS techniques. Although sperm motility may be impaired, most sperm motion characteristics were not affected by MACS preparation. Furthermore, the induced acrosome-reaction rate improved after MACS, especially for the population with normal hemizona assay results. The MACS technique has the potential to further benefit the ART management of patients with UI and unsuccessful IUI.

Funding

Grant NSC 97-2314-B-040-001 from National Science Council, Taiwan.

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