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人類精子活化、細胞凋亡與染色質損壞於不孕症診療過程中的角色

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Magnetic-Activated Cell Sorting for Sperm Preparation Reduce Apoptotic Sperm and Improve Acrosome Reaction for Unexplained Infertility

Running title: MACS for unexplained infertility

Abstract

BACKGROUND: This study attempted to identify apoptotic spermatozoa in couples with unexplained infertility (UI) who had twice-failed intrauterine insemination (IUI). In addition, the efficiency and benefit of magnetic- activated cell separation (MACS) for sperm preparation in such patients were also investigated. **METHODS:** Sixty couples with UI and repeated 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 aliquot was further processed by MACS (D+M). The apoptotic status was determined by fluorescence-labeled antibody and flow cytometry, including the externalization of phosphatidylserine (EPS), disrupted mitochondrial membrane potential (MMP), and DNA fragmentation. The fertilization potential of the prepared spermatozoa was analyzed by computer-assisted semen analysis and the induced acrosome reaction test (IART). **RESULTS:** After DGC, spermatozoa had 18.6% EPS, 28.3% disrupted MMP, and 13.5% DNA fragmentation. The apoptotic spermatozoa were significantly reduced by MACS. Although the motility of sperm was slightly decreased after MACS, most semen motion characteristics were not impaired. Interestingly, the IART significantly improved after MACS, especially for the couples with a normal hemizona assay. **CONCLUSIONS:** The spermatozoa prepared by D+M showed a reduced level of apoptosis. The improvement in the IART suggests a high fertilization potential of the processed spermatozoa. The apoptotic markers and MACS may be helpful in directing the management plan for patients with UI and multiple IUI failures.

Key words: acrosome reaction, apoptosis, sperm function, 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).

The conventional semen analysis as recommended by World Health Organization guidelines focuses on the count, motility, and morphology of ejaculated spermatozoa to discriminate infertile men from the fertile population (WHO, 1999). However, the sperm quality necessary for successful IUI is lower than the WHO guidelines for determining male fertility potential (Dickey et al., 1999). Furthermore, the increase in sperm quality parameters above a minimal threshold of sperm concentration ($5x10^6$/ml) and motility (30%) is not associated with elevation corresponding increase in the likelihood of pregnancy by IUI (Dickey et al., 1999). By contrast, DNA fragmentation in spermatozoa has been reported to be relevant to the success of IUI treatment for a cohort of infertile couples comprised of 41% of couples with UI (Duran et al., 2002). In addition, up to 29% of couples with UI have defective zona pellucida (ZP)-induced acrosome reactions subsequent to ZP 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 semen biologic properties, such as apoptosis and DNA fragmentation of the spermatozoa (Aziz and Agarwal, 2008). An alternative approach is performing sperm functional tests, including sperm ZP binding and/or acrosome reaction tests, to direct the treatment plan into standard IVF or ICSI (Liu and Baker, 1994; Liu and Baker, 2003). In our hands, the hemizona assay in addition to semen analysis have 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 ART laboratories (Henkel and Schill, 2003). Some methods are based upon molecular separation, such as attempts to eliminate sperm with apoptotic markers. Annexin V-conjugated magnetic microbeads can be utilized effectively to separate non-apoptotic spermatozoa 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 conceive, were recruited. The goal of this study was in an attempt to survey the incidence of apoptotic or deteriorating spermatozoa in such patients. In addition, the efficiency of the MACS technique for sperm preparation in such patients was also investigated. The fertilizing potential of sperm post-MACS was further evaluated by computer assisted sperm analysis (CASA) and the acrosome reaction test.

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 inform consent for study participation was obtained from all study 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 serum hormone levels of prolactin, estradiol, testosterone, FSH, LH, and TSH. The infertile couples with the following characteristics were included for analysis: ovulating women with bilateral patent oviducts, the presence of bilateral ovaries, no endocrine disorders (polycystic ovarian 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 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 consistent with normal (sperm concentration exceeding 20×10^6/ml, percentage of motile sperm > 50%, and normal sperm morphology was 14% or greater) for at least 2 of 3 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 cycle days 6, 8, and 10, and then daily until the administration of hCG. Further, 250 mg of hCG (Ovitrelle; Serono) was administered to all patients in whom two leading follicles reached ≥18 mm in diameter, and IUI was arranged 36 hours subsequent to hCG administration.

Semen samples 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 twice-failed IUI. Following a period of 3–5 days of sexual abstinence, fresh semen samples were collected by masturbation into sterile plastic jars from

the male partners on the day of the hemizona assay. After liquefaction, the ejaculated sperm were prepared by discontinuous gradient centrifugation (DGC) at 300g 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 magnetic cell sorting (MACS; Figure 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 sperm/microbeads suspension was loaded in a separation column containing coated cell-friendly matrix containing iron balls, which was fitted in a magnet (MiniMACS; Miltenyi Biotec). The fraction composed of apoptotic spermatozoa 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 tesla between the poles of the magnet and up to 1.5 tesla 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 procedures eluted through the column labeled as annexin-negative (D+M group). In addition, sperm motion characteristics by CASA and the induced acrosome reaction assay were also performed for both groups.

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 internal membrane to the external membrane is reflective of early apoptotic events. Binding of annexin-V to EPS 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 sperm) and with (live, but apoptotic) PS translocation.

The technique used for the annexin V assay was perfumed according to a previous report with minor modifications (Oosterhuis et al., 2000). The sperm were washed twice with annexin V binding buffer. Then, the sperm cells were incubated with 0.1 mg/ml fluorescein isothiocyanate (FITC)–labeled annexin V at room temperature for 30 min in the dark, followed by adding 50 mg/ml PI (Sigma, St. Louis, MO, USA). The sperm were then analyzed in a FACS caliber flow cytometer (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: 1) live, intact spermatozoa with no EPS (no fluorescence), namely annexin V-negative and PI-negative (AV-/PI-), 2) live spermatozoa with EPS (green), namely AV+/PI-, corresponding to early apoptotic spermatozoa, 3) dead spermatozoa with no EPS, but positive for PI (red), namely AV-/PI+, and 4) spermatozoa with EPS and positive for PI (red and green), namely annexin AV+/PI+, corresponding to late apoptotic or necrotic spermatozoa.

Determination of sperm mitochondrial functional integrity

Sperm mitochondria functional integrity 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 200g for 5 min to pellet the cells. The cells were then resuspended in 1mL 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 FACScalibur flow cytometer (Becton Dickinson). 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 due 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 deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end labeling (TUNEL) assay

DNA fragmentation was evaluated using the TUNEL assay (Boehringer Mannheim, Mannheim, Germany), as in our previous report (Huang et al., 2005) with minor modifications. Briefly, the semen 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 caliber flow cytometer (Becton Dickinson). 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 hour (range, 0.5-1.5 h) prior to analyses for motion characteristics. Sperm motion characteristics were analyzed by the assistance of the Copenhagen Rigshospitalet Image House Sperm Motility Analysis System (CRISMAS; ImageHouseA/S, Copenhagen, Denmark), which is described elsewhere (Lee et al., 2008). In brief, the setting parameters for analysis included the following: 80 Hz, image-acquisition rate; at least 200 sperms sampled; and at least one microscopic field sampled microscopically with 10×20 magnification. The chamber utilized for sperm analysis was 0.01 mm in surface area and 0.01 mm in depth (Sefi-medical Instruments, Haifa, Israel).

CRISMAS was used to determine various sperm parameters, including concentration, motility, average path velocity (VAP), straightness of sperm motion (STR), and the amplitude of lateral head displacement (ALH). Other semen parameters measured included the ratio of progressive motile sperm, featuring a VAP > 25 μm/s and a STR > 80%. The definition of progressive motile sperm is similar to the "grade a" sperm motility determined according to WHO guidelines (WHO, 1999).

Hemizona assays

Fresh unfertilized oocytes from our assisted reproduction program were utilized as the source of ZP. Signed consent was obtained prior to donation of unfertilized oocytes. The sperm concentration was diluted to 10 ~ 20×10^6 /ml and a total of 20,000 sperm were put into a droplet of medium. The pair of hemizona was co-incubated at 37°C in an atmosphere of 5% CO_2 in air for 4 h, with spermatozoa from the patient (test) or a fertile man (control). 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 test droplet vs. the number of spermatozoa bound in the control droplet). A HZI level > 36% was considered to indicate effective fertilization potential (Oehninger et al., 1989; Lee et al., 2008).

Induced acrosome reaction test (IART)

Peanut agglutinin (PNA) from *Arachis hypogea* is specific for β-D-galactose residues, and hence binds to and labels the outer acrosomal membrane. The semen sample was mixed with an equal volume of 2 mg/ml of pentoxifylline (final concentration of 1 mg/ml; Hoechst, UK) . Subsequent to incubation of sperm samples for 1 h at 37°C and in5% CO_2, sperm acrosome status was assessed by FITC-PNA staining (Sigma; Gearon et al., 1994). Briefly, 20 μL of sperm suspension were spread over a clean microscope slide and allowed to air-dry. The smear was then fixed in 95% ethanol for 5 minutes 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 minutes at ambient temperature. Slides were rinsed by dipping in PBS twice before fixing for 15 minutes in paraformaldehyde at ambient temperature. Slides were air-dried, mounted, and stored in the dark until scoring.

Between 100 and 250 sperm were counted per slide and scored into three classes for PNA labeling: 1) acrosome intact, where whole acrosome labeling denotes an intact outer acrosomal membrane; 2) partially acrosome reacted, where patchy acrosome labeling is suggestive of a transition stage in which the outer acrosomal membrane is fenestrated; and 3) 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 the class 3 spermatozoa were considered to have undergone an induced acrosome reaction.

Statistical analysis

Data were analyzed by using inbuilt functions within the Statistical Package for Social Science, version 14 (SPSS UK Ltd., Chertsey, Surrey, UK). Student's pair t-test or the Wilcoxon signed rank test were 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 semen 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 twice-failed IUI were recruited for this study. The basic sperm analysis revealed an average semen volume of 3.6±1.8 ml (range, 2~11 ml), a count of 110.6±81.7x 10^6/ml (range, 20 ~436 x10^6/ml), 78.6±15.7% sperm motility (range, 50%-100%), and 19.7±1.7% normal sperm morphology (range, 14%-31%).

The sperm prepared by DGC alone or DGC + MACS were compared for the percentage of apoptotic markers, such as EPS, disrupted MMP, and DNA fragmentation, by a flow cytometry study (Table 1). The percentage of early apoptosis markers (AV^+/ $PI^-$and MMP) in sperm prepared by DGC alone or DGC + MACS was significantly reduced (8.5±11.5% vs. 5.6±7.3% for AV^+/ PI^- and 28.3±17.6% vs. 19.2±10.8% for disrupted MMP; both $p<0.001$). About 30% of the sperm with early apoptotic markers were removed by MACS preparation. Furthermore, sperm with DNA fragmentation by TUNEL stain (a late apoptosis marker) were also significantly reduced by MACS sorting from 13.5±5.6% to 9.9±3.6% ($p<0.001$). Approximately one-fourth of sperm with sperm DNA fragmentation were removed.

The motion characteristics of the initial sperm samples, and the motion characteristics subsequent to DGC and MACS preparation, are shown in Table 2. The sperm motility percentage did not change subsequent to DGC (78.6±15.7% vs. 79.3±13.6%, $p>0.05$). However, the motility after MACS significantly decreased from 79.3±13.6% to 67.0±22.4% ($p<0.05$). The sperm motion characteristics were significantly improved by DGC preparation, including average path velocity (VAP), straightness of sperm motion (STR), amplitude of lateral head displacement (ALH), and percentage of progressive motile sperm (grade a). Nevertheless, all of the previously described motion characteristics were not significantly

changed after MACS (Table 2).

The IART was also performed for sperm prepared by DGC alone or by DGC + MACS. The proportion of induced acrosome reaction increased subsequent to DGC + MACS (22.8±15.7% vs. 32.3±21.9%, p<0.001). When the studied population was divided into two groups by a HZI level of 36%, we found an interesting result. The acrosome reaction percentage (21.9±12.1% vs. 24.1±11.9%, 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 sorting (23.4±20.7% vs. 34.0±24.5%, p<0.001, Figure 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 and 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; and 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.

Sperm motility was intimately relevant to EPS and DNA fragmentation (Table III). Therefore, the correlation between apoptotic markers, IART, and semen motion characteristics was further determined, part of the results of which 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; and r= -0.447, p=0.006, respectively) and disrupted MMP (r= -0.375, p=0.016; and 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 relevant to the early apoptosis (AV+/PI-; r= -0.389, p=0.012; and r= -0.313, p=0.046, respectively). Nevertheless, the grade a spermatozoa only correlated with early apoptosis (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 DGC + MACS (r=-0.391, p=0.011).

Discussion

It has been reported that ejaculated spermatozoa do exhibit changes consistent with apoptosis, such as EPS, disrupted MMP, and/or DNA fragmentation (Said et al., 2005; Barroso et al., 2006; de Vantery et al., 2009). In raw sperm 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 apoptotic spermatozoa (Henkel and Schill, 2003). After DGC using semen samples obtained from healthy donor, 4.56% EPS was reported (Said et al., 2005). In a population seeking

infertility treatment with normal sperm concentrations and motilities, a 15.2% EPS, comprised of 6.1% early apoptosis and 9.1% late apoptosis 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 a 14% EPS, consisting of 3% early apoptosis and 11% late apoptosis or necrosis (de Vantery et al., 2009). In the current study, spermatozoa after DGC was still characterized by an18.6% EPS, including an 8.5% early apoptosis and a 10.1% late apoptosis or necrosis. The data confirmed the presence of apoptotic markers (EPS) in spermatozoa prepared by DGC for either heath donors or subfertile couples.

The proportion of EPS after DGC in the present study was similar or slightly higher to 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 gonadotropin stimulation cycles (Duran et al., 2002). A significant correlation between DNA fragmentation (TUNEL stain) and late apoptosis (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 apoptosis, either after DGC or after MACS, 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 couples who twice-failed IUI 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 combing DGC and MACS is the elimination of the poor quality sperm (both immature and apoptotic cells; 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 sperm from the non-apoptotic portion for patients with UI. Previous studies regarding the removal of apoptotic spermatozoa from healthy donors indicated that DGC + MACS obtained optimal results (Paasch et al., 2004; Said et al., 2005) Apoptotic sperm 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.

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 contain a higher rate of apoptotic spermatozoa compared to healthy donors (Gandini et al., 2000; Shen et al., 2002). In the present study involving patients with UI and normal semen parameters, a relatively high rate

of EPS after DGC was noted. The high rate of EPS may be responsible for IUI failure in such patients. Although some reports have demonstrated that EPS is a physiologic 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 were 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 is a phenotype of early apoptosis, similar to that described in somatic cells.

It has been reported that sperm motility decreased after MACS compared to spermatozoa after DGC because of 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 2). 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 by MACS.

In the present study, the hemizona assay and acrosome reaction test 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-ZP binding (Franken et al., 1993). The poor HZI results in the present study may be relevant to 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 ZP (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-ZP binding assay undergoing conventional IVF have defective ZP-induced acrosome reaction (Liu et al., 2001; Liu and Baker, 2003). The ZP-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 separation in the current study suggested that the D+M prepared spermatozoa might be able to improve the outcome of IUI and the fertilization rate in the IVF program for couples with UI. Therefore, couples with UI and a normal hemizona assay may

benefit from the MACS preparation technique based upon the results of current study. 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 either after DGC or DGC plus MACS (data about 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 Kruger strict criteria utililzed for sperm morphology; nevertheless, the current study only included subfertile couples with normal sperm parameters and the WHO criteria were adopted for sperm mophology. Said et al. (2005) reported that sperm apoptosis markers (EPS, caspase-3, and disrupted MMP) were significantly correlated with sperm morphology by Kruger criteria, but not by the WHO criteria. The lack of correlation between sperm morphology and apoptotic markers in the present study may result from the difference in population and/or different methods for sperm morphology.

The IART by pentoxyphylline after D+M, but not after DGC, was relevant to sperm concentration (Table III). The ZP-induced acrosome reaction rate obtained from patients with UI also exhibited a significant correlation with sperm concentration (Liu and Baker, 2003; Liu and Baker, 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 apoptotic spermatozoa may facilitate developing the optimal treatment plan for patients with UI who twice-failed IUI. Furthermore, elimination of apoptotic spermatozoa 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 twice-failed IUI failure. 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 beneficial potential for further ART management of such patients.

References

Arslan M, Morshedi M, Arslan EO, Taylor S, Kanik A, Duran HE, Oehninger S. Predictive value of the hemizona assay for pregnancy outcome in patients undergoing controlled ovarian hyperstimulation with intrauterine insemination. *Fertil Steril* 2006**;85**:1697-1707.

Aziz N and Agarwal A. Evaluation of sperm damage: beyond the World Health Organization criteria. *Fertil Steril* 2008**;90**:484-485.

Barroso G, Taylor S, Morshedi M, Manzur F, Gavino F, Oehninger S. Mitochondrial membrane potential integrity and plasma membrane translocation of phosphatidylserine as early apoptotic markers: a comparison of two different sperm subpopulations. *Fertil Steril* 2006**;85**:149-154.

Crosignani PG, Collins J, Cooke ID, Diczfalusy E, Rubin B. Recommendations of the ESHRE workshop on 'Unexplained Infertility'. Anacapri, August 28-9, 1992. *Hum Reprod* 1993**;8**:977-980.

de Vantery AC, Lucas H, Chardonnens D, de Agostini A. Removal of spermatozoa with externalized phosphatidylserine from sperm preparation in human assisted medical procreation: effects on viability, motility and mitochondrial membrane potential. *Reprod Biol Endocrinol* 2009**;7**:1-

Dickey RP, Pyrzak R, Lu PY, Taylor SN, Rye PH. Comparison of the sperm quality necessary for successful intrauterine insemination with World Health Organization threshold values for normal sperm. *Fertil Steril* 1999**;71**:684-689.

Duran EH, Morshedi M, Taylor S, Oehninger S. Sperm DNA quality predicts intrauterine insemination outcome: a prospective cohort study. *Hum Reprod* 2002**;17**:3122-3128.

Franken DR, Kruger TF, Oehninger S, Coddington CC, Lombard C, Smith K, Hodgen GD. The ability of the hemizona assay to predict human fertilization in different and consecutive in-vitro fertilization cycles. *Hum Reprod* 1993**;8**:1240-1244.

Gadella BM and Harrison RA. Capacitation induces cyclic adenosine 3',5'-monophosphate-dependent, but apoptosis-unrelated, exposure of aminophospholipids at the apical head plasma membrane of boar sperm cells. *Biol Reprod* 2002**;67**:340-350.

Gandini L, Lombardo F, Paoli D, Caponecchia L, Familiari G, Verlengia C, Dondero F, Lenzi A. Study of apoptotic DNA fragmentation in human spermatozoa. *Hum Reprod* 2000**;15**:830-839.

Gearon CM, Mortimer D, Chapman MG, Forman RG. Artificial induction of the acrosome reaction in human spermatozoa. *Hum Reprod* 1994**;9**:77-82.

Gleicher N and Barad D. Unexplained infertility: does it really exist? *Hum Reprod* 2006**;21**:1951-1955.

Grunewald S, Reinhardt M, Blumenauer V, Said TM, Agarwal A, Abu HF, Glander HJ, Paasch U. Increased sperm chromatin decondensation in selected nonapoptotic spermatozoa of patients with male infertility. *Fertil Steril* 2008**;**

Guzick DS and others. Efficacy of superovulation and intrauterine insemination in the treatment of infertility. National Cooperative Reproductive Medicine Network. *N Engl J Med* 1999**;340**:177-183.

Henkel R, Hajimohammad M, Stalf T, Hoogendijk C, Mehnert C, Menkveld R, Gips H, Schill WB, Kruger TF. Influence of deoxyribonucleic acid damage on fertilization and pregnancy. *Fertil Steril* 2004**;81**:965-972.

Henkel RR and Schill WB. Sperm preparation for ART. *Reprod Biol Endocrinol* 2003**;1**:108-
Huang CC, Lin DP, Tsao HM, Cheng TC, Liu CH, Lee MS. Sperm DNA fragmentation negatively correlates with velocity and fertilization rates but might not affect pregnancy rates. *Fertil Steril* 2005**;84**:130-140.
Lee TH, Liu CH, Huang CC, Chen HH, Chen SU, Lee MS. The association between polypronucleate zygote formation with certain motion characteristics of sperm and IVF outcome. *J Assist Reprod Genet* 2008**;25**:35-41.
Liu DY and Baker HW. Disordered acrosome reaction of spermatozoa bound to the zona pellucida: a newly discovered sperm defect causing infertility with reduced sperm-zona pellucida penetration and reduced fertilization in vitro. *Hum Reprod* 1994**;9**:1694-1700.
Liu DY and Baker HW. Disordered zona pellucida-induced acrosome reaction and failure of in vitro fertilization in patients with unexplained infertility. *Fertil Steril* 2003**;79**:74-80.
Liu DY and Baker HW. Human sperm bound to the zona pellucida have normal nuclear chromatin as assessed by acridine orange fluorescence. *Hum Reprod* 2007**;22**:1597-1602.
Liu DY, Clarke GN, Martic M, Garrett C, Baker HW. Frequency of disordered zona pellucida (ZP)-induced acrosome reaction in infertile men with normal semen analysis and normal spermatozoa-ZP binding. *Hum Reprod* 2001**;16**:1185-1190.
Oehninger S, Coddington CC, Scott R, Franken DA, Burkman LJ, Acosta AA, Hodgen GD. Hemizona assay: assessment of sperm dysfunction and prediction of in vitro fertilization outcome. *Fertil Steril* 1989**;51**:665-670.
Oehninger S, Franken DR, Sayed E, Barroso G, Kolm P. Sperm function assays and their predictive value for fertilization outcome in IVF therapy: a meta-analysis. *Hum Reprod Update* 2000**;6**:160-168.
Oosterhuis GJ, Mulder AB, Kalsbeek-Batenburg E, Lambalk CB, Schoemaker J, Vermes I. Measuring apoptosis in human spermatozoa: a biological assay for semen quality? *Fertil Steril* 2000**;74**:245-250.
Paasch U, Grunewald S, Agarwal A, Glandera HJ. Activation pattern of caspases in human spermatozoa. *Fertil Steril* 2004**;81 Suppl 1**:802-809.
Said TM, Grunewald S, Paasch U, Glander HJ, Baumann T, Kriegel C, Li L, Agarwal A. Advantage of combining magnetic cell separation with sperm preparation techniques. *Reprod Biomed Online* 2005**;10**:740-746.
Shen HM, Dai J, Chia SE, Lim A, Ong CN. Detection of apoptotic alterations in sperm in subfertile patients and their correlations with sperm quality. *Hum Reprod* 2002**;17**:1266-1273.
Siristatidis C and Bhattacharya S. Unexplained infertility: does it really exist? Does it matter? *Hum Reprod* 2007**;22**:2084-2087.
World Health Organization. WHO Laboratory Manual for the Examination of Human Semen and Semen-Cervical Mucus Interactions. Cambridge University Press, Cambridge. 1999

Tables

Table I. Analysis of apoptotic markers after sperm preparation by density gradient centrification (DGC) and subsequent magnetic-activated cell sorting (MACS) methods (data are presented as mean±S.D. for percentage) with flow cytometry.

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 mitochondrial membrane potential (%)	28.3±17.6	19.2±10.8*	<0.001
Sperm DNA fragmemtation (%)	13.5±5.6	9.9±3.6*	<0.001

Analysis by Wilcoxon Signed Ranks Test

Table II. Semen characteristics of patients with unexplained infertility participating in the sperm preparation study by density gradient centrification (DGC) and subsequent magnetic-activated cell sorting (MACS) methods (data are presented as mean±S.D.).

	Original	DGC	DGC+MACS
Motility (% of active sperm)	78.6±15.7	79.3±13.6	67.0±22.4*
Average path velocity (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
Amplitude of lateral head 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 to the other two groups by Wilcoxon Signed Ranks Test

Table III. The correlation between basic semen analysis parameters of raw semen samples with apoptosis markers and induced acrosome reaction rate. The data are presented as Spearman correlation coefficient and the pvalue within the parenthesis.

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 fragmemtation (%)	-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 plus 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 fragmemtation (%)	-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)

* $p<0.05$

Table IV. The correlation between sperm motion characteristics of raw semen samples with apoptosis markers and induced acrosome reaction rate. The data are presented as Spearman correlation coefficient and the pvalue within the parenthesis.

Sperm motion characterisitics	Average path velocity (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(0.022)*
DNA fragmemtation (%)	-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 plus 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 fragmemtation (%)	-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)

* $p<0.05$

Figure legends

Figure 1. Flow chart of the study. The sperm sample prepared by density gradient centrifugation was divided into two aliquots: a control and the other underwent magnetic-activated cell sorting. EPS and MMP denote externalization of phosphatidylserine and mitochondrial membrane potential, respectively.

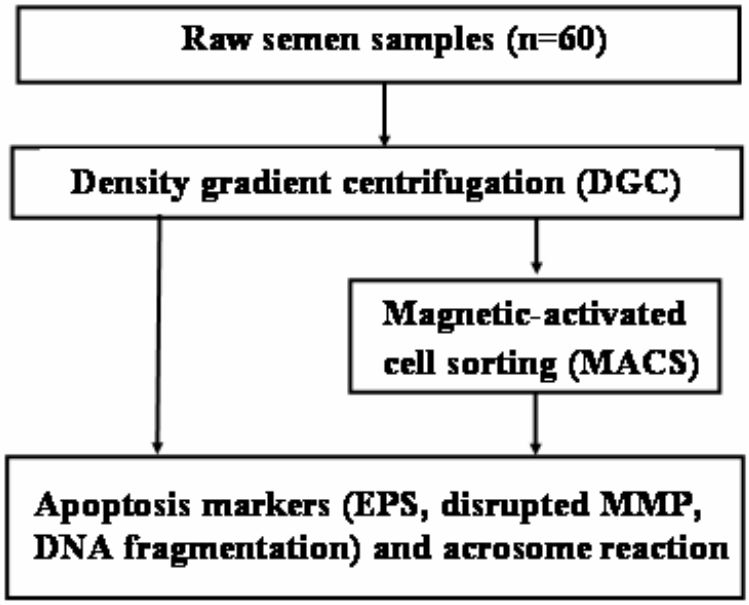

Figure 2. The rate of induced acrosome reaction after sperm preparation by density gradient centrifugation (DGC) compared to by DGC plus magnetic-activated cell sorting (D+M). The studied population was divided into two groups by a hemizona assay index level (HZI) of 36%. The column is mean and the error bar is standard deviation (S.D.)