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·*Original Article*·

# Early apoptotic changes in human spermatozoa and their relationships with conventional semen parameters and sperm DNA fragmentation

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**Abstract**

**Aim:** To investigate whether early apoptotic changes in spermatozoa can be significant markers for sperm quality. **Methods:** Two early apoptotic changes in the semen of 56 men were assessed using Annexin V (AN)/propidium iodide (PI) staining for phosphatidylserine externalization and JC-1 staining for mitochondrial membrane potential (MMP). The results were compared with conventional semen parameters and DNA fragmentation identified using the TUNEL assay. **Results:** The different labeling patterns in the bivariate Annexin V/PI analysis identified four distinctive spermatozoa populations. The percentage of $AN^-/PI^-$ spermatozoa positively correlated with conventional semen parameters and MMP, but negatively correlated with TUNEL (+) spermatozoa. As for the $AN^-/PI^+$ fraction, we found an opposite result in comparison to $AN^-/PI^-$ spermatozoa. The level of early apoptotic $AN^+/PI^-$ spermatozoa negatively correlated with MMP and sperm motility. The level of late apoptotic $AN^+/PI^+$ spermatozoa negatively correlated with conventional semen parameters and MMP, and positively correlated with TUNEL (+) spermatozoa. MMP positively correlated with conventional semen parameters, but negatively correlated with TUNEL (+) spermatozoa. **Conclusion:** Although early apoptotic $AN^+/PI^-$ spermatozoa only negatively correlates with sperm motility, the differences in proportion of each subpopulation of spermatozoa (especially, the percentage of $AN^-/PI^-$ spermatozoa), and decreased MMP might be significant markers for diagnosing male infertility. They possibly bring additional information to predict the outcome of *in vitro* fertilization. *(Asian J Androl 2008 Mar; 10: 227–235)*

**Keywords:** Annexin V; apoptosis; DNA fragmentation; infertility; mitochondria; sperm

## 1 Introduction

Most *in vitro* fertilization (IVF) cases result from male factor deficiencies. The quality of sperm is one of the factors determining the success rate of IVF. Currently, sperm quality is evaluated by conventional semen analysis using a light microscope to determine sperm concen-

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tration, motility and morphology. These parameters comply with World Health Organization criteria [1]. However, conventional semen analysis has limited clinical value for predicting the success rate of IVF as 50% of couples with failed fertilization have normal pre-IVF semen analysis [2]. Furthermore, conventional semen analysis does not assess the presence of apoptotic spermatozoa, which might be partially responsible for the low fertilization and implantation rates in assisted reproduction. Characteristics that are typical of ejaculated apoptotic human spermatozoa include phosphatidylserine (PS) externalization, decreased mitochondrial membrane potential (MMP), caspase activation and DNA fragmentation [3, 4].

The plasma membrane is a key organelle for sperm function. Successful fertilization requires a sperm plasma membrane with normal integrity and function. An early apoptotic event in somatic cells is characterized by the loss of phospholipid asymmetry, such as translocation of PS from the inner to the outer leaflet of the membrane [3]. Exposure of PS on the outside of the cell surface, referred to as PS externalization, provides an opportunity to detect cells that are in the early stage of apoptosis. In sperm, such cells can be identified by the calcium-dependent binding of fluorescence-conjugated Annexin-V (AN) to externalized PS in combination with staining by fluorescent dyes, such as propidium iodide (PI), which make it possible to simultaneously distinguish live and dead spermatozoa. Mitochondria are also a key organelle for sperm function. In humans, a correlation exists between poor sperm mitochondrial function detected by reduced MMP, diminished motility and reduced fertility [5]. Analysis of mitochondrial function might offer a means to assess the motility of sperm. Mitochondria are the coordinators of apoptosis in various cellular systems because they are involved in many apoptotic processes, including caspase activation, decreased MMP and alterations of the intracellular reduction-oxidation potential [6].

PS externalization and decreased MMP are two characteristics of early stages of apoptosis in somatic cells. These changes precede other manifestations of programmed cell death, such as DNA fragmentation. TUNEL assay is commonly used for measuring DNA fragmentation, which is correlated to the outcome of IVF in humans [7]. The extent of DNA fragmentation is closely related to sperm function and male infertility [8, 9]; however, the hypothesis about the origin of such damage is still controversial. The significance of the two early apoptotic changes encountered in infertile sperm remains unknown. Hence, we investigated the possible correlations of the two early apoptotic changes with conventional semen parameters and DNA fragmentation identified by TUNEL assay. Our research question is "Can early apoptotic changes be significant markers of sperm quality?"

## 2 Materials and methods

### *2.1 Semen collection and processing*

We studied 56 men who underwent seminal fluid evaluation for various reasons at the Reproductive Medical Center of Shandong University, Shandong Provincial Hospital (Jinan, China). All subjects were partners of women who had failed to conceive after 2 years of unprotected intercourse. The ethics committee of the hospital approved the study. Informed consent for participation in the study was obtained from all participants. The samples were collected by masturbation into sterile plastic jars after 3–5 days of sexual abstinence. Within 1 h of collection, routine semen analysis was performed using a light microscope to determine sperm quality according to World Health Organization criteria [10].

### *2.2 Evaluation of conventional sperm parameters: concentration, motility and morphology*

Sperm concentration, motility and morphology were evaluated in the original raw sample. After liquefaction, 10 μL of each sample were placed into a Makler Chamber and read under a light microscope. Sperm morphology was assessed according to strict criteria at 1 000 × magnification. Each sample (10 μL) was spread along the slide and allowed to dry for 20 min before staining with Diff-Quik staining. An average of 100 spermatozoa per slide was read under microscope with a 100 × oil immersion objective lens. Coefficients of inter-observer and intra-observer variability were 5%.

### *2.3 Evaluation of externalization of PS with AN*

An AN-fluorescein isothiocyanate (FITC) Apoptosis Detection Kit (BMS306FI, Bender, Grünberg, Germany) was used to detect the translocation of PS from the inner to the outer leaflet of the plasma membrane of spermatozoa as recommended by the manufacturer with slight modifications. Generally, an aliquot of semen specimen containing $1 \times 10^6$ spermatozoa were washed twice (300 × *g*, 10 min, 4ºC) in phosphate buffered saline (PBS). The sperm pellet was re-suspended in

ANBinding Buffer (10 mmol/L Hepes/NaOH, pH 7.4, 140 mmol/L NaCl, 2.5 mmol/L $CaCl_2$) at room temperature to a concentration of $2 \times 10^6$ sperm/mL. Aliquots (100 μL each, $2 \times 10^5$ cells) of sperm were transferred into culture tubes. 5 μL of AN-FITC and 10 μL of PI (20 μg/mL), or nothing were added to the samples (nothing was added in the case of the negative control). Single color staining with either AN-FITC or PI alone was applied to adjust the compensation. The tubes were gently mixed and incubated for 15 min at room temperature in the dark, and additional Binding Buffer (400 μL) (BMS306FI, Bender, Grünberg, Germany) was added to each tube. Flow cytometric evaluation was conducted within 5 min.

*2.4 Determination of the integrity of MMP*

A lipophilic cationic dye JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenimidazolyl carbocyanine iodide) was used to detect intact trans-membrane potential of mitochondria in spermatozoa (MitoCapture Apoptosis Detection Kit: Catalogue No. 475866; Calbiochem, La Jolla, CA, USA). Spermatozoa with intact mitochondria excite an intense red fluorescence due to the formation of the dye aggregates, whereas the monomer dye fluoresces green in the presence of spermatozoa with a disrupted mitochondrial membrane. Compensation between FL1 and FL2 was carefully adjusted according to the manufacturer's instructions. All aliquots were incubated at 37ºC for 20 min in 1 μg of the lipophilic cation diluted in 1 mL PBS. Negative controls were identically processed for each fraction except that the stain was replaced with 10 μL PBS. Flow cytometric evaluation was conducted within 30 min.

*2.5 TUNEL assay*

TUNEL assay in human sperm was conducted in our laboratory using Fluorescein FragEL DNA Fragmentation Detection kit (Catalogue No. QIA39; Calbiochem, La Jolla, CA, USA). Approximately $2 \times 10^6$ sperm were first washed with PBS, and then fixed in 4% paraformaldehyde for 10 min at room temperature. The fixed cells were washed in TBS (2 mmol/L Tris pH 7.6, 140 mmol/L NaCl ), and then re-suspended in 20 μg/mL proteinase K for 10 min at room temperature. After washing with terminal deoxynucleotidyl transferase (Tdt) equilibration buffer, the cells were gently re-suspended in Tdt reaction solution containing the Tdt enzyme and FITC-labeled nucleotides. For each batch, a negative control without the addition of Tdt enzyme and a positive control with DNase I treatment were always included to ensure the reproducibility of the assay. After incubation in a humidified chamber for 60 min at 37ºC in the dark, the sample was analyzed using flow cytometry.

*2.7 Flow cytometric analysis*

The extent of externalized PS, MMP and TUNEL were evaluated by flow cytometer analyses. All of the fluorescence signals of labeled spermatozoa were analyzed by the flow cytometer (Beckman-Coulter EpicsXL-4; Beckman-Coulter, Inc., Fullerton, CA, USA). A minimum of 10 000 spermatozoa were examined for each assay at a flow rate of < 100 cells/s. The sperm population was gated using 90° and forward-angle light scatter to exclude debris and aggregates. The excitation wavelength was 488 nm supplied by an argon laser at 15 mW. Green fluorescence (480–530 nm) was measured in the FL-1 channel and red fluorescence (580–630 nm) in the FL-2 channel. The percentage of positive cells and the mean fluorescence were evaluated on a 1023-channel scale using the flow cytometer System II Version 3.0 software (Beckmann Coulter, Miami, FL, USA).

*2.8 Statistical analysis*

Data are presented as mean ± SEM. Results were analyzed using SPSS version 10.0 software for Windows (SPSS, Chicago, IL, USA). The Pearson rank correlation test was used to calculate the correlation coefficient between cytofluorometric analyses. The Spearman rank correlation test was used to evaluate the relationship between conventional semen parameters and cytofluorometric examination. The statistical significance level was set at $P < 0.05$.

## 3 Results

A total of 56 men who visited the reproductive medical center during the study period (April 2006–July 2006) were recruited. Their ages ranged from 24 to 42 years (31 ± 4 years). The results of the routine semen analyses are shown in Table 1. The semen samples had a sperm concentration of (42 ± 30) × $10^6$/mL, 48% ± 15% of the spermatozoa appeared to be motile, and 29% ± 3% of the spermatozoa had normal forms.

In the present study, three assays were applied to examine the apoptotic changes in ejaculated sperm (Figures 1–3). These assays are based on flow cytometry

and are more accurate, rapid and can provide less subjective and statistically more reliable results than microscopic examination. The different labeling patterns in the bivariate AN/PI analysis identified four distinctive spermatozoa populations: live spermatozoa with no translocation of membrane PS, namely 'AN$^-$/ PI$^-$'; live spermatozoa with translocation of membrane PS, namely 'AN$^+$/ PI$^-$'; dead spermatozoa with translocation of membrane PS, namely 'AN$^+$/ PI$^+$'; and dead spermatozoa with no binding of AN, namely 'AN$^-$/ PI$^+$'. The distributions of

Table 1. Characteristics of seminal parameters according to World Health Organization [1, 10].

| Seminal parameters | *n* | Mean + SD | Minimum | Median | Maximum |
|---|---|---|---|---|---|
| Volume (mL) | 56 | 4 ± 1 | 2 | 4 | 6 |
| Concentration (× $10^6$/mL) | 56 | 42 ± 30 | 4 | 36 | 156 |
| Progressive motility (%) | 56 | 34 ± 12 | 8 | 36 | 56 |
| Motile (%) | 56 | 48 ± 15 | 16 | 52 | 70 |
| Vitality (%) | 56 | 78 ± 9 | 55 | 78 | 93 |
| % of normal forms | 56 | 29 ± 3 | 20 | 30 | 33 |

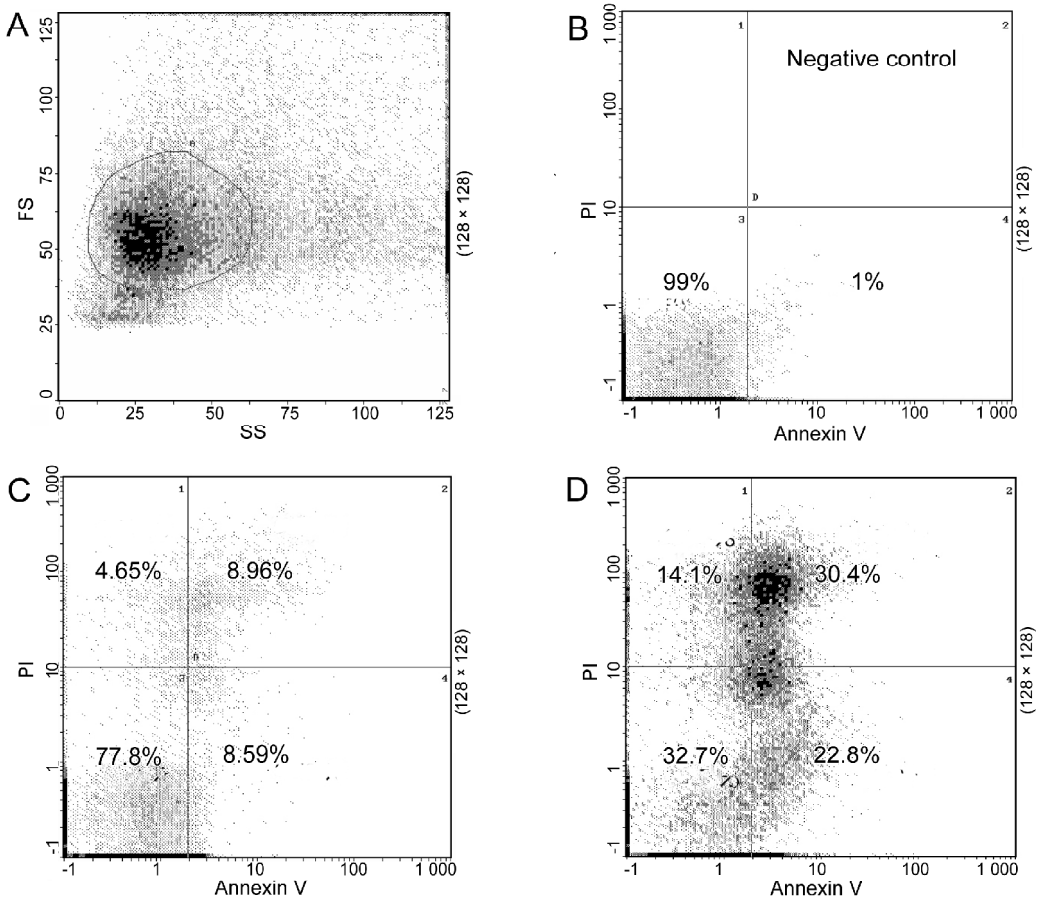


Figure 1. Annexin V (AN)/propidium iodide (PI) bivariate analysis was used to detect apoptosis in spermatozoa. The lower-left quadrant of each graph contains AN$^-$/PI$^-$, viable, non-apoptotic sperm. The lower-right quadrant shows AN$^+$/PI$^-$ early apoptotic sperm. The upper-right quadrant represents AN$^+$/PI$^+$ apoptotic sperm. The upper-left quadrant contains AN$^-$/PI$^+$ necrotic sperm. Data from flow cytometry: Forward-angle light scatter/side-angle light scatter dotplot (A), negative control in which virtually no cells stained positive (B), one semen sample with high quality sperm (C), and one semen sample with bad quality sperm (D). FS, forward scatter; SS, side scatter.

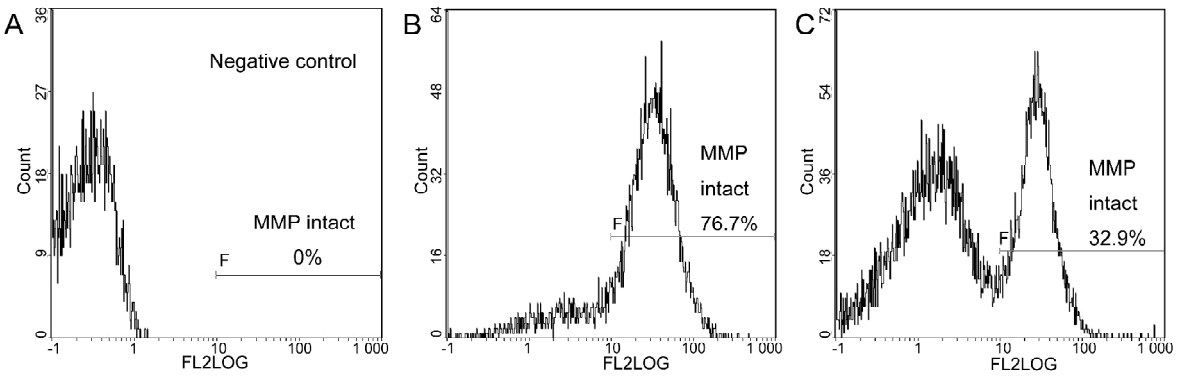


Figure 2. JC-1 staining for mitochondrial membrane potential (MMP). JC-1 staining produces two fluorescence emission peaks that reflect the existence of two forms of the dye. The JC-1 monomers which emit green fluorescence are predominant at low MMP, while the JC-1 aggregates (red-orange fluorescence) are predominant at high MMP. We have relied on the red-orange fluorescence emission of JC-1 aggregates to monitor changes in MMP. Histogram data from flow cytometry of a negative control (A), one semen sample of high quality with high MMP spermatozoa (B), and one semen sample of bad quality with low MMP spermatozoa (C). FL2LOG, fluorescence detector 2 log.

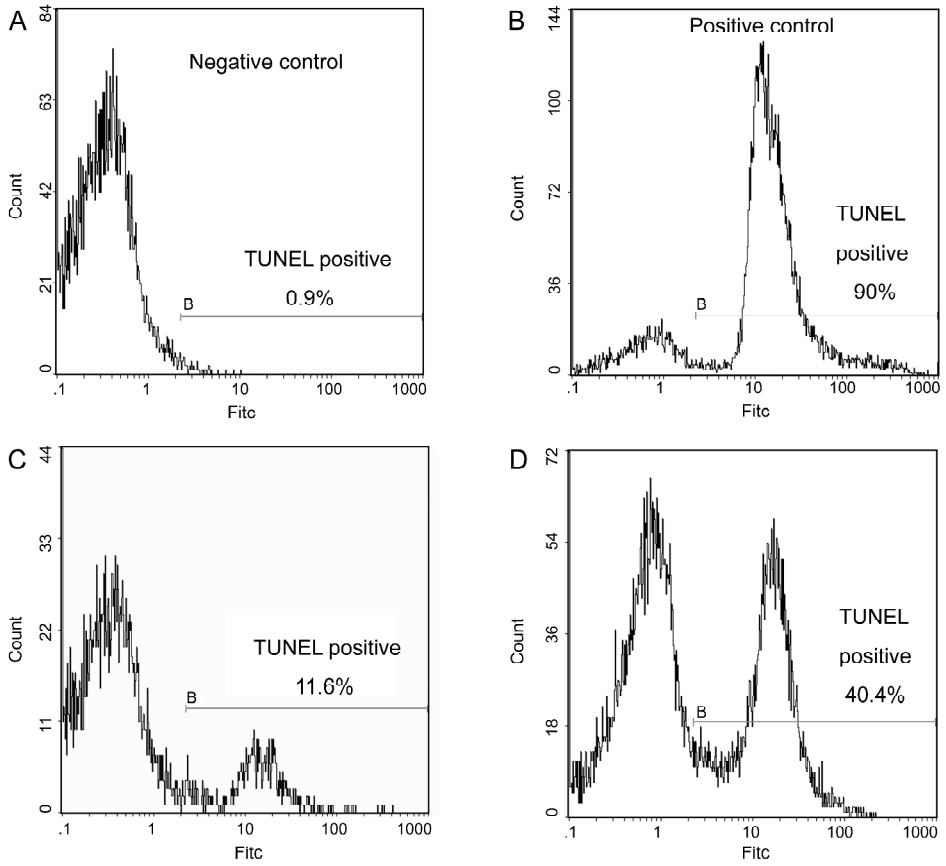


Figure 3. TUNEL assay used to detect apoptosis in spermatozoa. Histogram data from flow cytometry of a TUNEL-negative control (A), one sample with 90% cells labeled with high fluorescein isothiocyanate (FITC) fluorescence as a TUNEL-positive control (B), one semen sample of high quality with 11.6% TUNEL-positive cells (C), and one semen sample of bad quality with 40.4% TUNEL-positive cells (D).

Table 2. PS expression (Annexin V-binding), MMP and DNA fragmentation (TUNEL assay) of the semen sample. MMP, mitochondrial membrane potential.

| | *n* | Mean + SD | Minimum | Median | Maximum |
|---|---|---|---|---|---|
| Annexin $V^-/PI^-$ (%) | 56 | 55 ± 16 | 26 | 57 | 84 |
| Annexin $V^+/PI^-$ (%) | 56 | 10 ± 6 | 1 | 9 | 27 |
| Annexin $V^+/PI^+$ (%) | 56 | 23 ± 10 | 7 | 21 | 52 |
| Annexin $V^-/PI^+$ (%) | 56 | 11 ± 7 | 2 | 10 | 36 |
| MMP (%) | 56 | 55 ± 22 | 9 | 61 | 86 |
| TUNEL (%) | 56 | 23 ± 14 | 5 | 19 | 71 |

Table 3. Correlation between semen parameters and results of the flow cytometry assay. $^bP < 0.05$, $^cP < 0.01$. MMP, mitochondrial membrane potential.

| | $AN^-/PI^-$ | $AN^+/PI^-$ | $AN^+/PI^+$ | $AN^-/PI^+$ | MMP | TUNEL |
|---|---|---|---|---|---|---|
| Sperm concentration | $0.472^c$ | –0.152 | $–0.408^c$ | $–0.297^b$ | $0.480^c$ | $–0.466^c$ |
| Progressive motility | $0.545^c$ | –0.261 | $–0.435^c$ | $–0.347^c$ | $0.732^c$ | $–0.492^c$ |
| Motility | $0.563^c$ | $–0.341^b$ | $–0.453^c$ | $–0.322^b$ | $0.701^c$ | $–0.524^c$ |
| Percentage of normal forms | $0.402^c$ | –0.152 | $–0.375^c$ | $–0.394^c$ | $0.477^c$ | $–0.352^c$ |

Table 4. Correlation between apoptotic markers. $^bP < 0.05$, $^cP < 0.01$. MMP, mitochondrial membrane potential.

| | $AN^-/PI^-$ | $AN^+/PI^-$ | $AN^+/PI^+$ | $AN^-/PI^+$ | MMP |
|---|---|---|---|---|---|
| TUNEL | $–0.55^c$ | 0.216 | $0.498^c$ | $0.306^b$ | $–0.464^c$ |
| MMP | $0.779^c$ | $–0.307^b$ | $–0.612^c$ | $–0.569^c$ | |

the populations are as follows: $AN^-/PI^-$, (55 ± 16)%; $AN^+/PI^-$, (10 ± 6)%; $AN^+/PI^+$, (23 ± 10)%; $AN^-/PI^+$, (11 ± 7)%. The MMP and DNA fragmentation (TUNEL assay) of the semen samples are as follows: (55 ± 22)% and (23 ±14)%, respectively (Table 2).

To assess the possible impact of sperm apoptosis on sperm quality, we further analyzed the correlations between the apoptotic indices and various semen parameters, and the results are summarized in Table 3. We found that the amounts of both $AN^+/PI^+$ (late apoptosis) spermatozoa and TUNEL (+) spermatozoa were inversely correlated to concentration, motility and sperm normal morphology. In contrast, no correlation was observed between the amount of $AN^+/PI^-$ (early apoptosis) spermatozoa and sperm concentration or sperm normal morphology. However, the amount of $AN^+/PI^-$ (early apoptosis) spermatozoa is inversely correlated with motility. We also found that MMP and the amount of $AN^-/PI^-$ spermatozoa positively correlated with concentration, motility and sperm normal morphology.

The correlations of the two early apoptotic alterations with the TUNEL assay are summarized in Table 4. The amount of TUNEL (+) spermatozoa positively correlated with the levels of both $AN^+/PI^+$ (late apoptosis) and $AN^-/PI^+$ spermatozoa, but negatively correlated with both the level of $AN^-/PI^-$ spermatozoa and MMP. In contrast, MMP positively correlated with the level of $AN^-/PI^-$ spermatozoa, but negatively correlated with the levels of $AN^+/PI^+$ (late apoptosis), $AN^+/PI^-$ (early apoptosis) and $AN^-/PI^+$ spermatozoa.

## 4 Discussion

Apoptosis is an important process involved in normal spermatogenesis [11]. However, deregulations of this biological process involve abnormalities in the production of male gametes, and male infertility. In recent years, much attention has been given to the role of apoptosis in ejaculated sperm. To date, however, whether defective apoptosis accounts for a significant proportion of DNA damage in the spermatozoa of infertile men is still questionable. In this regard, we hypothesize that the differences in proportion of each subpopulation of spermatozoa (according to the AN/PI staining) and decreased MMP of the semen sample might correlate with the quality of sperm.

### *4.1 $AN^-/PI^-$ and $AN^-/PI^+$ spermatozoa*

The percentage of $AN^-/PI^-$ spermatozoa positively

correlated with conventional semen parameters and MMP, but negatively correlated with TUNEL (+). $AN^-/PI^-$ spermatozoa displayed superior quality in terms of high motility, low activated caspases, high MMP and small extent of DNA fragmentation [12, 13]. Moreover, $AN^-/PI^-$ spermatozoa might be a sperm subpopulation of good functional competence that is able to adapt to freeze/thaw-induced stress. These findings lead us to postulate that the $AN^-/PI^-$ spermatozoa represent high quality sperm so as to serve as an objective indicator for the fertility potential of an individual. As for the $AN^-/PI^+$ fraction, we found an opposite result in comparison to $AN^-/PI^-$ spermatozoa. Such spermatozoa exist, but have not been described by some of other researches [14, 15]. It has been suggested these spermatozoa are either necrotic cells characterized by a high degree of membrane disorganization, which might prevent binding of AN, or spermatozoa in the later stage of apoptosis [16]. However, the significance of this subpopulation needs to be investigated further.

*4.2 $AN^+/PI^-$ and $AN^+/PI^+$ spermatozoa*

Controversy exists as to the correlation of the amount of $AN^+/PI^-$ group with motility, i.e. some studies report negative correlations [13, 16] or a positive correlation [3], whereas other reports no correlation [16]. Our study demonstrated that the levels of both early apoptotic $AN^+/PI^-$ spermatozoa and late apoptotic $AN^+/PI^+$ spermatozoa negatively correlated with sperm motility. There are two major hypotheses explaining the externalization of PS as visualized by AN-staining. It might be an early event in spermatozoa apoptosis and/or a reflection of sperm capacitation. Capacitation is associated with an increase in the motion parameters, wheras motility is reduced in apoptotic sperm. Our results support the apoptosis hypothesis. Moreover, we demonstrated that the late apoptotic alterations in sperm were positively associated with abnormal sperm morphology. Our results are, at least in part, consistent with previous findings [15]. The results can be explained by the abortive apoptosis theory proposed by Sakkas *et al*. [8, 17]. With respect to the relationship between apoptotic changes and the sperm concentration, available data are still controversial. Oosterhuis *et al*. [18] found that the level of spermatozoa that express PS ($AN^+$) or the percent of TUNEL (+) sperm negatively correlated with sperm concentration. In contrast, Shen *et al*. [15] found that late apoptotic alterations in sperm positively correlated with sperm concentration. Our results contradict those of Shen *et al*. [15] and can also be explained by the abortive apoptosis theory. One possible explanation for the finding of Shen *et al.* [15] is that the sperm samples used in their study had two distinctive features: (i) most of them were within the normal range of sperm concentration; and (ii) the percentage of sperm defects was unusually high. Additionally, we found that the percentage of early apoptotic $AN^+/PI^-$ spermatozoa was not correlated with the percentage of TUNEL (+) spermatozoa but the percentage of late apoptotic $AN^+/PI^+$ spermatozoa was positively correlated with the percentage of TUNEL (+) spermatozoa. This can be explained by the common understanding that DNA fragmentation usually occurs at the late stage of apoptosis caused by endonuclease activation. Such correlations also suggest that the $AN^+/PI^+$ spermatozoa are more likely to be late apoptotic cells, rather than necrotic cells. These results pointed to an abortive apoptosis that takes place in many men who have sperm parameters that are below normal. Apoptosis in mature sperm is initiated during spermatogenesis in which some cells, earmarked for elimination, might escape the removal mechanism and contribute to poor sperm quality [15, 19]. In certain men, abortive apoptosis might fail in the total clearance of spermatozoa earmarked for elimination by apoptosis. In our study, significant correlations are reported between apoptotic changes (according to the AN/PI staining) in sperm with some conventional sperm parameters (Table 3). However, such results appear in contrast with previous similar works [16]. This difference could be a result of the different method used and/or to the different patient population studied.

*4.3 MMP versus conventional sperm parameters and apoptotic changes*

At present, MMP is considered a good indicator of sperm motility. Our results, together with other observations, indicates that mitochondrial damage might result in a reduction of sperm motility [5, 19]. It might also provide a possible explanation for poor sperm motility in asthenozoospermic samples. In addition, we found that MMP positively correlated with sperm concentration and sperm morphology. This result is in line with the data reported by other researchers [5, 20]. Marcheeti *et al.* [21] found that determination of MMP represents the most sensitive test by which to evaluate sperm quality. Furthermore, our study also showed that MMP nega-

tively correlated with the percentage of TUNEL (+) spermatozoa. This finding strongly implied an association of male infertility with mitochondrial alterations observed during apoptosis. However, it is not known if apoptosis hypotheses could explain the overall DNA damage or if there are others factors involved in the final DNA damage. Sakkas *et al.* [22] showed that DNA fragmentation is not always concomitant with the expression of the apoptotic markers (such as Fas, p53 and Bcl-x) and that problems in nuclear remodeling of spermiogenesis might account for a fraction of DNA fragmentation. Wang *et al.* [20] reported that increased oxidative stress is associated with alterations in MMP and increased spermatozoa DNA damage. This can also explain the negative relationship of our result. In addition, Moustafa *et al.* [23] observe a strong correlation between reactive oxygen species (ROS) and the level of apoptosis. This might reflect a causal relationship between ROS and apoptosis. As mentioned above, the significance of the decreased MMP encountered in infertile sperm remains unknown but the assumption that it manifests an early stage of cell death might explain why detection of mitochondrial changes represents a sensitive test in our study.

In conclusion, although early apoptotic $AN^+/PI^-$ spermatozoa only negatively correlates with sperm motility, the differences in proportion of each subpopulation of spermatozoa (especially the percentage of $AN^-/PI^-$ spermatozoa), and decreased MMP might be significant markers for diagnosing male infertility. They possibly bring additional information to predict the outcome of IVF. Further studies are needed to determine their relationships with the occurrence of pregnancy or fertilization *in vitro*.

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