The effect of age on the expression of apoptosis biomarkers in human spermatozoa

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Objective: To evaluate the impact of age on the expression of apoptotic biomarkers in human spermatozoa.

Design: Cross sectional, prospective study.

Setting: Academic centers.

Patient(s): Healthy volunteers with proven fertility, stratified by age (n = 25, range: 20–68 years).

Intervention(s): Examination of serum hormone levels and basic semen parameters, and assessment of early (plasma membrane translocation of phosphatidylserine) and late (DNA fragmentation) sperm apoptotic markers by flow cytometry (using Annexin-V binding and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling).

Main Outcome Measure(s): Apoptosis markers.

Result(s): Advancing male age was significantly and positively correlated with Annexin-V binding results. Although not significant, there was a clear trend for increased DNA fragmentation in the older groups. The age threshold for these observations appears to be 40 years. Advancing male age was positively correlated with FSH and sex hormone-binding globulin (SHBG) levels, and negatively correlated with sperm concentration.

Conclusion(s): Advancing male age is associated with the expression of early apoptotic markers as evidenced by significantly increased plasma membrane translocation of phosphatidylserine, as well as with a more subtle proportion of sperm carrying DNA fragmentation. This study confirmed that male age is also associated with a decline in sperm concentration. (Fertil Steril 2010;94:2609–14. ©2010 by American Society for Reproductive Medicine.)

Key Words: Apoptosis, DNA fragmentation, male age, sperm, translocation of phosphatidylserine

The negative impact of age on reproductive success has been well demonstrated for women, but age-related changes in the male reproductive system appear to be more subtle (1–3). Diminishing testicular function is indicated by a decline in T levels, accompanied by an increase in gonadotropins (4, 5). Some studies have shown increased age-related numerical and structural sperm chromosomal abnormalities, changes in sperm DNA methylation, and a higher mutational load (6–8). Epidemiologic data suggest that increasing male age may be associated with pregnancy loss (9) and with a range of birth defects (10).

In natural conception, time to pregnancy for men has been shown to increase significantly after 50 years of age (11, 12). Among the basic semen parameters studied, semen volume, percentage of motile spermatozoa, and the percentage of normal morphology were more consistently reported to decrease with age (13, 14). Sperm DNA integrity as assessed by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) (15), sperm chromatin structure assay (SCSA) (16, 17), and Comet assay (18), were also shown to be compromised with advancing age. There are many possible causes of sperm DNA damage, including apoptosis, oxidative stress associated with male genital tract infection, exposure to redox cycling chemical agents, and/or defects of spermiogenesis associated with the retention of excess residual cytoplasm (10, 19).

Reports on the impact of male age on the outcome of assisted reproductive technologies (ART) using the donor egg model are conflicting (20–25). This model allows controlling for the effects of variation of oocyte quality by female age. In our program we found that semen volume, sperm motility, and fertilization rate decreased significantly with advanced male age, but there was no correlation between age and mean embryo morphology scores, implantation, pregnancy rate (PR) or miscarriage rate (26).

In the present study we aimed to investigate the impact of male age on the expression of apoptotic biomarkers in ejaculated spermatozoa from healthy men. To accomplish this goal we used fluorescence-activated cell sorter (FACS), which offers the advantage of performing a large number of measurements in a test sample, in an objective, rapid, and reproducible manner, for the assessment of membrane translocation of phosphatidylserine (PS; as a marker of early apoptosis) and DNA fragmentation (a late apoptotic marker). In addition, we examined the basic semen parameters and endocrine profiles of these individuals.

MATERIALS AND METHODS

Subjects

This was a prospectively designed, cross sectional study, with the approval of the Institutional Review Board at the Instituto Nacional de Perinatología, Mexico City, Mexico. Ejaculates were collected after a 3-to 5-day sexual abstinence period from 25 fertile men who volunteered to participate in the study after signed consent was obtained. Men were recruited through written advertisement in visiting lounges in various clinical areas of the Institute, and no compensation was provided. The men ranged in age from 20–68 years and...
were allocated to the following study groups’ according to age stratification: 20–30, 31–40, 41–50, 51–60, and 61–70 years (5 individuals per group). All men had a history of proven fertility (fathered at least one biological child, conceptions occurring within a stable relationship), they had a complete and negative medical history, and a normal physical examination (including absence of varicocele and presence of a normal testicular volume). Cases with evidence of chronic illnesses (including cardiovascular disease and diabetes), use of chronic medications (other than over-the-counter drugs), and chronic exposure to gonadotoxins (alcoholism behavior, cigarette smoking, chemotherapy agents, and no heating exposure related to extreme physical, occupational, and/or environmental activities) were excluded.

**Semen Preparation**

Semen samples were collected by masturbation and semen analysis was performed after semen liquefaction for 30 minutes at room temperature. Basic semen parameters were assessed according to the World Health Organization criteria (27), and sperm morphology was examined according to strict criteria (28). Sperm concentration and progressive motility were assessed using a Makler counting chamber (Irvine Scientific, Santa Ana, CA) and sperm morphology was examined using strict criteria (x1,000 magnification) after smear staining with Diff-Quik dye (Dade Diagnostics AG, Dudingen, Switzerland). Any contaminating leukocytes were removed using paramagnetic beads coated with CD45 antibodies (Dynabeads M-450 CD45 Pan leukocyte; Dynal, Oslo, Norway), as previously described (29, 30). The absence of beads coated with CD45 antibodies (Dynabeads M-450 CD45 Pan leukocyte; Dynal, Oslo, Norway). Any contaminating leukocytes were removed using paramagnetic smear staining with Diff-Quik dye (Dade Diagnostics AG, Dudingen, Switzerland). Any contaminating leukocytes were removed using paramagnetic beads coated with CD45 antibodies (Dynabeads M-450 CD45 Pan leukocyte; Dynal, Oslo, Norway), as previously described (29, 30). The absence of leukocytes was further confirmed by methodical examination of peroxidase-stained slides. Individuals were excluded if any of the following were found: antisperm antibodies (by direct immunobead testing), round cell concentration >1 × 10^7/mL, positive cultures for microorganisms, and/or viability <50% (eosin-Y).

**Annexin-V Binding and TUNEL Assays**

Immediately after liquefaction, samples were washed two times with 1 mL of phosphate-buffered saline (PBS) and centrifuged at 200 g for 20 minutes. Washed samples were then divided into two aliquots, one to be used for Annexin-V binding and the other one for TUNEL.

For Annexin-V binding, cells were stained with Annexin-V-Fluos Staining Kit (Roche Diagnostics GmbH, Mannheim, Germany) following the manufacturer’s instructions. Cells were adjusted to a concentration of 1 × 10^6 spermatozoa/mL, and then resuspended in 100 µL of Annexin-V-Fluos labeling solution containing 2 µL of Annexin-V-Fluos labeling reagent and 2 µL of propidium iodide (PI) solution, diluted in 100 µL of incubation buffer. Cells were incubated for 15 minutes at 25°C and resuspended in 500 µL of incubation buffer. For each experimental set, a negative control was prepared without Annexin-V-Fluos labeling solution. Finally, cells were analyzed by flow cytometry on a Cytomics FC 500 Series Flow Cytometry System (Beckman Coulter, Brea, CA) through excitation with a 15-nm 488-nm air-cooled argon ion laser and 515-nm bandpass filter for FLUOS detection. For each suspension, 10,000 sperm were analyzed for Annexin-V binding and/or PI uptake. Emission data were collected and analyzed using CXP software (Beckman Coulter).

For TUNEL, we used the APO-DIRECT kit (Becton–Dickinson, Franklin Lakes, NJ) that detects DNA strand breaks by adding the terminal enzyme deoxyribonuclease I (TdT) and fluorescein isothiocyanate conjugate (FITC). Briefly, sperm fractions were resuspended in 1% (vol/vol) paraformaldehyde in PBS with a 5 × 10^6 cells/mL concentration. The suspension was placed on ice for 120 minutes, centrifuged at 300 × g for 5 minutes, and washed twice in 5 mL of PBS and centrifuged for 20 minutes at 300 × g. The obtained pellet was then resuspended in PBS by moderate agitation with vortex, adjusted to 1 × 10^6 cells/mL, and maintained in 70% cold ethanol for 30 minutes. Ethanol was removed and the cells were washed with PBS.

The elongation reaction was performed by incubating the sperm cells in 50 µL of labeling solution containing the deoxyribonuclease I (TdT) and dUTP for 1 hour at 37°C. For each experimental set, a negative control was prepared by omitting deoxyribonuclease I (TdT) from the reaction mixture. Two subsequent washes were performed to stop the reaction. For the labeling reaction, the highly dUTP-specific fluorescein-labeled MrBM antibody (Becton–Dickinson) was incubated with sperm for 30 minutes at room temperature in the dark. Before flow cytometry analysis, the sperm were washed twice with PBS, labeled with 150 µM PI, and filtered. Positive controls were prepared as described previously with an additional treatment with 10 IU DNAse I for 1 hour at 37°C before the elongation reaction. At the end of the incubation time, 1.0 mL of Rinse Buffer (51-6550AZ) (Becton- Dickinson, Franklin Lakes, NJ) was added to each tube followed by centrifugation 300 × g for 5 minutes. The supernatant was then removed by aspiration. The assay was run on the flow cytometer equipped with a 488-nm argon laser as the light source. Two dual parameter and two single parameter displays were created with the flow cytometer data acquisition software. Accordingly, the gating display was determined as the standard dual parameter DNA doublet discrimination display with the DNA area signal on the Y-axis and the DNA width on the X-axis.

**Serum Hormone Determinations**

On the same morning and before semen collection, blood samples were obtained for measurement of anti Müllerian hormone (AMH), FSH, LH, PRL, T, E2, free androgen index (FAI) calculated as the total T level divided by the sex hormone-binding globulin (SHBG) level × 100, and SHBG. After centrifugation at 100 × g sera were collected and kept at -20°C until analysis. Hormone levels were quantified by chemiluminescent immunometric assays, using the SIEMENS Immulite system (Los Angeles, CA). Interassay and intra-assay coefficients of variation (CV) for all hormones were <5%.

**Statistical Analysis**

Data were analyzed using the SPSS software (Chicago, IL), and results are presented as mean ± SD. Group comparisons were made using Student’s t-test or Kruskal-Wallis test, as appropriate. One-way analysis of variance (ANOVA) test was used assuming a two-tail hypothesis with an α value of 0.05. Correlations were performed using Pearson’s rank correlation test (Spearman’s rho) and simple linear regression, for this purpose age was considered as a continuous variable.

**TABLE 1**

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<td>Rapid progressive</td>
<td>14.8 ± 11.6</td>
<td>8.2 ± 4.5</td>
<td>10.8 ± 7.2</td>
<td>4.5 ± 3.3</td>
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<td>Concentration (×10^6)/mL</td>
<td>101.0 ± 48</td>
<td>41.4 ± 22</td>
<td>85.8 ± 37</td>
<td>41.3 ± 30</td>
<td>18.7 ± 19</td>
<td>.03</td>
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<td>9.2 ± 3.1</td>
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<td>Annexin-V (%)</td>
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<td>8.9 ± 4.6</td>
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<td>28.4 ± 18.7</td>
<td>34.0 ± 25.7</td>
<td>.02</td>
</tr>
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<td>TUNEL (%)</td>
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<td>21.0 ± 19.2</td>
<td>26.9 ± 20.7</td>
<td>26.5 ± 14.3</td>
<td>26.7 ± 19.5</td>
<td>NS</td>
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Note: TUNEL = terminal deoxyribonucleotidyl transferase-mediated dUTP nick end labeling; NS = not significant.

RESULTS

Basic Sperm Parameters

Table 1 presents the results of the basic semen parameters according to male age. Only sperm concentration was significantly different among groups (P = .03), with an obvious decline with advancing age. Although differences in progressive motility and morphology were not significant, there was also a trend for diminished values with age (P = .05 and .08, respectively). The observed relationships were further supported by significant and negative correlations between age and sperm concentration (r = -0.52, P < .01), age and progressive motility (r = -0.67, P < .01), and age and sperm morphology (r = -0.47, P < .05).

Annexin-V Binding and TUNEL Assays

The Annexin-V binding assay results stratified by male age are also presented in Table 1. The percentage of live sperm cells with plasma membrane translocation of PS (Annexin-V⁺/IP⁻) were significantly higher with advancing age (ANOVA, P < .01). Figure 1 presents the gating strategy for flow cytometry studies, the diagrammatic representation of the effect of age on PS translocation (Annexin-V⁺/IP⁻ cells) according to stratified age groups (ANOVA, P = .01), and the linear regression analysis of the relationship between age and membrane PS translocation (r = 0.50, P < .008).

The TUNEL assay results stratified by male age are presented in Table 1. Although there was a trend for increased levels of DNA fragmentation with advancing age these results were not statistically significant (ANOVA, P > .5, r = 0.51). Figure 2 presents the gating strategy for flow cytometry and a diagrammatic representation of the effect of age on DNA fragmentation according to stratified age groups (P = not significant).

The distribution of the Annexin-V binding and TUNEL data demonstrated a natural break at approximately 40 years of age, which

![Figure 1](image_url)

Detection of sperm phosphatidylserine (PS) translocation (early apoptotic marker) according to man’s age. (A) Representative plot and histogram showing the gating strategy and parameters used for the flow cytometry analysis. Annexin-V fluorescein isothiocyanate conjugate (FL1-H [530/30 filter - height of fluorescence intensity])/IP uptake (FL2-H [585/42 filter - height of fluorescence intensity]). (Right) 65.9% represents viable cells [Annexin-V(-)/IP(-)], 29.33% represents cells in early apoptosis [Annexin-V(+)/IP(-)], 4.67% represents necrotic cells [Annexin-V(+)/IP(+)] and 0.1% represents necrotic old cells [Annexin(-)/IP(+)]. (Left) Histogram represents all the positive cells by Annexin-V, necrotic and apoptotic cells. (B) Diagrammatic representation of the effect of age on PS translocation (Annexin-V⁺/IP⁻ cells) according to stratified age groups (*P = .01 by ANOVA). (C) Positive correlation was demonstrated between male age and PS translocation (Annexin-V⁺/IP⁻ cells) (r = 0.50, P < .01).

appeared to represent a threshold of significant age impact. As such, the levels of plasma membrane PS translocation between groups of <40 years and ≥40 years were significantly different, with Annexin-V binding values (Annexin-V⁺/IP⁺) of 9.8% ± 11.7% and 30.7% ± 18%, respectively (P = .003). For TUNEL, this difference did not attain significance. There were no significant correlations between the apoptotic biomarkers (Annexin-V or TUNEL) and the basic semen parameters or hormonal levels (data not shown).

Hormone Levels
Table 2 presents endocrine results according to male age. The FAI was significantly different among groups (P = .005). Although SHBG, FSH, and LH levels increased with age these changes were not significant. The AMH, T, E₂, and PRL levels were unaffected by age. The observed relationships were further supported by significant correlations between age and FAI (r = -0.67, P < .01), age and SHBG (r = 0.63, P < .01), age and FSH (r = 0.59, P < .002), and age and LH (r = 0.51, P < .009).

DISCUSSION
In somatic cells, one of the best markers for programmed cell death (apoptosis) is the presence of elevated caspase activity, the main executor of apoptosis (31). In addition, DNA fragmentation and membrane translocation of PS have been widely used as apoptosis markers (32, 33). During apoptosis, PS normally present on the cytoplasmic face of the plasma membrane is allowed to migrate to...
the outer leaflet, and the exposed PS marks the cells for destruction by phagocytes (34). The presence of apoptosis in ejaculated human sperm has only received considerable attention in the past few years. Ejaculated spermatozoa, particularly in infertile men, have been shown to display morphological and biochemical features that are typical of an apoptotic phenotype in somatic cells (35, 36). Deregulation of apoptosis is known to play roles in a number of disease processes, and it has been postulated that exacerbated or aberrant apoptosis may affect sperm dysfunction.

In subfertile men we have previously demonstrated the presence of immunoactive inactive procaspase-3 and active caspase-3 (by immunoblotting and immunofluorescence), caspase enzymatic activity (using a fluorometric assay), expression of procaspase-3, procaspase-7, and procaspase-9, low levels of active caspase, caspase-7, and caspase-9 (identified by immunoblot analysis), and expression of apoptosis-inducing factor (29, 30). Other investigators have confirmed many of these findings (36, 37). Furthermore, we have shown the presence of DNA fragmentation, as well as PS translocation in ejaculated sperm (29, 30, 35, 38). The latter phenomenon, under certain conditions, has also been related to positive functional changes in sperm such as capacitation (39–41).

In the present study we have demonstrated that advancing male age per se is associated with a significantly increased expression of the early apoptotic biomarker (i.e., plasma membrane PS translocation) in ejaculated spermatozoa of healthy and proven fertile men. It appears that a threshold at approximately 40 years of age discriminates “normal” versus “enhanced” expression of this biomarker. We have also previously reported on a positive correlation of plasma membrane PS translocation with the loss of mitochondrial membrane potential in ejaculated sperm of subfertile men, confirming the expression of an early apoptosis phenotype, as is typically observed in somatic cells (42). In addition, the generation of reactive oxygen species by sperm mitochondria has been related to apoptotic dysfunctional changes (43), but the exact mechanisms related to advancing age remains to be unveiled.

In the present study we also observed a similar trend, albeit not significant, for enhanced presence of DNA fragmentation as the men’s age increased. The TUNEL assay is estimated to measure actual single-stranded DNA and double-stranded DNA (dsDNA) damage in human sperm, without the use of previous DNA denaturation steps, and as such is probably recommended as a test that measures “real” DNA damage (44, 45). The groups of older men showed a large variation in the proportion of spermatozoa with DNA damage, as represented by the observed wide SDs. This fact, together with the limited sample size analyzed, may explain the lack of significant association of this apoptotic marker and age, which was not the case for PS translocation.

A relationship between DNA damage and oxidative stress has been reported (38, 46), which may be one of the mechanisms involved in male aging. Vagnini et al. (15), also using TUNEL, showed that sperm DNA integrity was compromised with advancing age in an unselected group of couples attending infertility investigation. Spanò et al. (16) used the SCSA, which measures the susceptibility of sperm DNA to in situ acid-induced denaturation, and found that the SCSA results can be influenced by the age of the donor. Similar trends were published by other investigators (18, 47, 48).

The present study also confirmed that advancing male age is associated with subtle hormonal changes, such as increased FSH and LH levels, a significant increase in FAI, as well as a decline in sperm concentration, with a similar trend observed for motility and morphology (4, 5, 13, 14). Interestingly, our results demonstrated no changes of the somatic Sertoli cell marker (i.e., AMH) with age, and no relationship between AMH levels and semen parameters or sperm apoptotic biomarkers.

The age-related presence of apoptosis in ejaculated spermatozoa can be the result of various types of injuries, occurring at testicular and/or ductal levels, or even after ejaculation (10, 35, 49). But, irrespective of the insult, spermatozoa undergoing early apoptosis and unrecognized by currently used methodologies in the standard clinical laboratory may be dysfunctional, and as such, may be unable to achieve fertilization. But more dramatically, they may pose the risk of carrying damaged DNA into the egg resulting in poor embryo development, miscarriage, or other untoward negative effects (10, 19, 50–52).

In conclusion, this prospective and cross sectional clinical study revealed that in a healthy population of men with proven fertility, advancing male age was associated with the expression of early apoptotic markers as evidenced by significantly increased plasma membrane translocation of PS, as well as with a more subtle proportion of sperm carrying DNA fragmentation. There was a relatively large intraindividual variation of these biomarkers in the groups with more advanced age. More and larger studies are needed to corroborate these findings and to determine whether the observed changes are associated with sperm dysfunctions leading to decreased reproductive capacity.

REFERENCES


