Original Paper

Urologia Internationalis

Urol Int 2007;79(3):231-4

Received: August 10, 2006
Accepted after revision: October 27, 2006

Male Age and Sperm Necrosis in Assisted Reproductive Technologies

Sam Siddighi Christopher A. Chan William C. Patton John D. Jacobson Philip J. Chan

Departments of Gynecology and Obstetrics, Physiology and Pharmacology, Loma Linda University School of Medicine, Loma Linda, Calif., USA

Key Words
Male age · Spermatozoa · Apoptosis and necrosis · Necrozoospermia

Abstract
Introduction: Sperm apoptosis is well characterized but studies on the effect of male age and necrozoospermia are lacking. The objectives were: (a) to analyze percentages of apoptotic and necrotic sperm in ejaculates, and (b) to compare the results between younger and older age groups. ***Materials and Methods:*** Routine semen analyses were carried out (n = 189 males) and sperm cells were analyzed by dual fluorescence assay Hoechst 33342 and propidium iodide, and the acridine orange test. ***Results:*** The percentage of necrotic sperm in the ejaculate increased by 22% for males aged over 35. There was a positive correlation between age and necrosis (R = 0.30). Sperm apoptosis increased by 17% in males aged 45 and older. The population of DNA intact sperm declined in males aged 40 and over (R = −0.21). There were no age-related changes in strict normal morphology, sperm concentration and semen volume. A decrease in rapid progressive motility was correlated (R = −0.24) with male age and was significant after age 35. ***Conclusions:*** The study demonstrated increased necrosis, DNA damage and apoptosis while rapid progression and total motility declined with advancing age in the male beginning as early as age 35. The order of the observed changes was sequential, suggesting the involvement of different pathways in sperm necrosis after age 40.

Portions of the study were presented at the 18th Meeting of the International Federation of Gynecology and Obstetrics, Kuala Lumpur, Malaysia, November 5–10, 2006.

Introduction

The effect of male age on basic sperm parameters has been reported [1–3]. However, male age-related studies on sperm apoptosis and necrosis are lacking [4]. These studies are important because of age-related reproductive disorders. Abnormal sperm is eliminated by a series of events starting with apoptosis, a process regulated by death receptors, p53, p21, Bcl-2, Bcl-Xs, release of mitochondrial cytochrome-c, and activation of caspase enzymes. The characteristics of apoptosis include: development of a semi-permeant membrane; blebbing (zeiosis); membrane translocation of phosphatidylserine; binding of annexin V; fixed-length DNA fragmentation, and Fas positivity [5, 6]. The apoptotic sperm can be detected by terminal deoxynucleotidyl transferase-mediated dUDP nick-end labeling, comet assay, annexin V, and the dual-stain fluorescence assay [4, 6–8].

KARGER
Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

0042–1138/07/0000–0000$23.50/0

Accessible online at:
www.karger.com/uin

Philip J. Chan, PhD
Loma Linda University Center For Fertility and IVF
11370 Anderson Street, Suite 3950
Loma Linda, CA 92354 (USA)
Tel. ■■■, Fax +1 909 558 2450, E-Mail pchann@yahoo.com

The necrotic sperm can be detected using electron microscopy, comet assay, live-dead stains, and the dual-stain fluorescence assay [4, 8, 9]. Using the latter assay, the influence of paternal age and sperm necrosis in males attending a fertility clinic was investigated. The objectives were: (a) to determine the percentages of apoptotic, necrotic and DNA intact sperm in the ejaculates, and (b) to compare the results between the younger and older age groups.

Materials and Methods

Routine semen analyses were carried out in 189 male patients attending a fertility center. The study was approved by the Institutional Review Board. Cases involving azoospermia, microsurgical epididymal sperm aspiration, and donor sperm were excluded from the study. Sperm DNA integrity was assessed by the acridine orange DNA fragmentation assay method [10]. Strict criteria of normal sperm morphology were evaluated as previously reported [11]. The sperm kinematics and heat-induced hyperactive motility parameters were determined using the Hamilton-Thorn HTM-C sperm motility analyzer [12].

Sperm apoptosis and necrosis were analyzed by the dual fluorescence assay procedure [8]. The procedure involved mixing a drop of semen with a drop of Hoechst 33342 stain (10 μM HO342 or bisbenzimide, Sigma Chemical Co., St. Louis, Mo., USA, dissolved in saline) placed on a glass slide. Immediately, a drop of propidium iodide stain (32 μM, Sigma, dissolved in saline) was added and the stained sperm analyzed after a minute using an ultraviolet epi-fluorescent microscope. Three types of sperm could be distinguished: intact DNA sperm (clear or slightly blue-stained at the postacrosomal region); apoptotic sperm (completely blue), and necrotic sperm (red-pink).

The results were grouped into subdivisions based on male age: group 1 (<30 vs. >30); group 2 (<35 vs. >35); group 3 (<40 vs. >40), and group 4 (<45 vs. >45). Student's t test was used to compare means while the associations between age and sperm parameters were tested by correlation coefficient statistics. A difference with $p < 0.05$ was considered significant.

Results

The results (table 1) show that the percentage of necrotic sperm in the ejaculate significantly increased by 22% for male patients over age 35 (group 2) when compared with younger patients. This difference was also observed for group 3 (24% increase) and group 4 (33% increase). In addition, there was a positive correlation between age and percent necrotic sperm (correlation coefficient R = 0.30) in the ejaculate. In contrast, a significant increase (17%) in sperm apoptosis was only observed in males aged 45 and older.

Table 1. Male age-related effects on strict normal sperm morphology and DNA parameters (mean ± SEM)

Age group subdivision	n	Strict morphology, %	DNA integrity, %	Apoptosis, %	Necrosis, %
Group 1					
<30	21	7.7 ± 1.7	78.9 ± 4.3	10.7 ± 1.6	26.6 ± 4.1
≥30	168	8.5 ± 0.5	75.5 ± 1.4	10.3 ± 0.7	28.2 ± 1.2
Group 2					
<35	91	8.5 ± 0.7	77.9 ± 1.8	9.7 ± 0.7	25.2 ± 1.4[a]
≥35	98	8.2 ± 0.7	73.9 ± 1.8	10.9 ± 1.0	30.7 ± 1.7
Group 3					
<40	136	8.4 ± 0.6	77.6 ± 1.5[a]	10.0 ± 0.6	26.3 ± 1.2[a]
≥40	53	8.2 ± 0.9	71.4 ± 2.5	11.0 ± 1.5	32.5 ± 2.4
Group 4					
<45	174	8.4 ± 0.5	76.5 ± 1.3[a]	10.2 ± 0.5[a]	27.3 ± 1.1[a]
≥45	15	7.8 ± 1.9	68.1 ± 6.6	11.9 ± 4.6	36.4 ± 4.9

[a] $p < 0.05$.

Table 2. Male age-related effects on basic sperm parameters (mean ± SEM)

Age group subdivision	n	Sperm concentration, 10^6/ml	Total motility, %	Rapid progression, %	Hyperactive motility, %
Group 1					
<30	21	56.4 ± 11.7	59.8 ± 3.3	40.9 ± 3.6	8.2 ± 1.8
≥30	168	52.0 ± 3.5	56.0 ± 1.3	35.1 ± 1.2	7.4 ± 0.5
Group 2					
<35	91	54.5 ± 5.2	58.4 ± 1.7	37.8 ± 1.6[a]	8.1 ± 0.7
≥35	98	50.6 ± 4.3	54.5 ± 1.7	33.7 ± 1.6	6.9 ± 0.7
Group 3					
<40	136	51.7 ± 4.0	58.4 ± 1.3[a]	37.1 ± 1.3[a]	7.5 ± 0.6
≥40	53	54.4 ± 6.0	51.3 ± 2.7	32.1 ± 2.3	7.4 ± 1.0
Group 4					
<45	174	52.5 ± 3.5	57.6 ± 1.2[a]	36.6 ± 1.1[a]	7.4 ± 0.5
≥45	15	52.6 ± 11.6	42.1 ± 5.6	25.4 ± 5.0	8.1 ± 1.8

[a] $p < 0.05$.

Coinciding with the increase in necrotic sperm in older males was the decrease in DNA intact sperm with advancing age (R = –0.21) demonstrated in groups 3 (8% decrease) and 4 (11% decrease). The decrease in DNA intact sperm began in males at age 40 and older. However, there were no age-related changes in the percentage of sperm with strict normal morphology. Similarly, there were no age-related differences noted for sperm concen-

tration and semen volume (table 2). In addition, there were no differences noted for the abstinence period and semen pH in all groups (data not shown).

Interestingly, there was a significant decrease in the percentage of sperm with rapid progressive motility beginning at age 35. The decrease in rapid progression correlated ($R = -0.24$) with advancing age was demonstrated in groups 2 (11% decrease), 3 (13% decrease) and 4 (31% decrease). However, a decrease in total sperm motility was only noted in males aged 40 and older as shown in groups 3 (12% decrease) and 4 (27% decrease).

Discussion

The results demonstrate an age-related increase in the percentage of necrotic sperm in the ejaculates of males aged 35 and older. This suggests that the number of quality sperm available for insemination or assisted reproductive procedures would diminish with advancing male age. A study of age-related effects on sperm apoptosis and necrosis is important because aging in males has been associated with hypogonadism, low testosterone production [13], loss of libido [14], loss of DNA integrity [4], chromosomal anomalies in the sperm (nondisjunction, acentric fragmentation and complex radial configuration) [15, 16], and genetic disorders in the offspring (Apert syndrome, polyposis coli, Marfan syndrome, fibrodysplasia ossificans progressiva, achondroplasia, basal cell naevi, and Recklinghausen's neurofibromatosis) [17].

The mechanism involved in increased sperm necrosis with advancing age is unknown but may involve seminal plasma enzymes that regulate sperm death such as aminopeptidase N [18], pyroglutamyl peptidase I, and prolyl endopeptidase [19]. For obvious reasons, an increase in sperm necrosis would be accompanied by a decline in sperm motility [1, 20]. Thus, rapid progressive motility declined in the group aged 35 and older. This parameter is important and is associated with successful pregnancy outcomes after assisted reproduction procedures [21]. The present study suggests that the male also contributes to a reduction in fertility observed after age 35.

Surprisingly, total sperm motility decreased only in the group aged 40 and older. The reason for this was not clear although it was attributed to differences in metabolic energy output from the mitochondria of sperm from the different age groups. In older men, a decrease in zinc removal from the tail outer dense fiber during epididymal maturation was correlated with a decrease in motility. Furthermore, reports have indicated an age-related decrease in biochemical parameters including testosterone, fructose and accessory gland functions resulting in the decline in sperm motility with age [22–24].

The acridine orange DNA integrity test which measured sperm with intact double-stranded DNA versus damaged sperm with single-stranded DNA indicated DNA damage in the sperm of males aged 40 and older. This result is consistent with other studies showing an age-related increase in sperm DNA damage [4]. A possible explanation for the occurrence of DNA-damaged sperm in older males was an age-related failure in converting DNA-damaged sperm into necrotic sperm.

In terms of apoptosis, several studies do not directly link DNA damage with apoptosis, rather sperm DNA damage results either from nuclear remodeling during spermatogenesis [6], from exposure to radiation and chemicals or, more likely, from reactive oxygen species [25]. The results here show significant sperm apoptosis only in males aged 45 and older. In contrast, a recent report suggested an age-related decrease in sperm apoptosis based on the DNA diffusion assay [4]. Although the exact age at which the decrease in apoptosis occurred was not addressed in that study, nevertheless they reported decreased apoptosis in the older age ranges (36–57 and 44–57 years). An explanation for the difference might be due to assay sensitivities. In this study, the dual fluorescence assay detected percentages of apoptotic sperm (9.7–11.9%) that were similar to other reports based on the neutral comet assay (10.0%), terminal deoxynucleotidyl transferase-mediated dUDP nick-end labeling assay (<10%), and the annexin V binding assay (11%) [4, 7].

Not surprisingly, age-related effects on sperm concentration were not observed. This finding is consistent with previous reports [2, 20]. Furthermore, there were no significant age-related changes in strict normal morphology [11]. Although the percentages of strict normal forms were lower in older males when compared with younger males, they were not statistically significant. Some studies have shown a decrease in normal sperm morphology with advancing age [3]. However, the use of different sperm morphology classification systems by different researchers makes comparison between studies tenuous.

In summary, the results showed age-related increases in sperm necrosis, DNA damage and apoptosis while rapid progression and total motility declined with advancing age. The order of the observed changes was sequential and not simultaneous, suggesting the involvement of different molecular pathways in necrotizing DNA-damaged sperm after age 40.

References

1 Spira A: The decline of fecundity with age. Maturitas 1988(suppl 1):15–22.
2 Krause W, Habermann B: No change with age in semen volume, sperm count and sperm motility in individual men consulting an infertility clinic. Urol Int 2000;64:139–142.
3 Jung A, Schuppe HC, Schill WB: Comparison of semen quality in older and younger men attending an andrology clinic. Andrologia 2002;34:116–122.
4 Singh NP, Muller CH, Berger RE: Effects of age on DNA double-strand breaks and apoptosis in human sperm. Fertil Steril 2003;80:1420–1430.
5 Darzynkiewicz Z, Bruno S, Del Bino G, Gorczyca W, Hotz MA, Lassota P, Traganos F: Features of apoptotic cells measured by flow cytometry. Cytometry 1992;13:795–808.
6 Sakkas D, Moffatt O, Manicardi GC, Marietho E, Tarozzi N, Bizzaro D: Nature of DNA damage in ejaculated human spermatozoa and the possible involvement of apoptosis. Biol Reprod 2002;66:1061–1067.
7 Barroso G, Morshedi M, Oehninger S: Analysis of DNA fragmentation, plasma membrane translocation of phosphatidylserine and oxidative stress in human spermatozoa. Hum Reprod 2000;15:1338–1344.
8 Rowland SC, Jacobson JD, Patton WC, King A, Chan PJ: Dual fluorescence analysis of DNA apoptosis in sperm. Am J Obstet Gynecol 2003;188:1156–1157.
9 Dive C, Gregory CD, Phipps DJ, Evans DL, Milner AE, Wyllie AH: Analysis and discrimination of necrosis and apoptosis (programmed cell death) by multiparameter flow cytometry. Biochem Biophys Acta 1992;1133:275–285.
10 Tejada RI, Mitchell JC, Norman A, Marik JJ, Friedman S: A test for the practical evaluation of male fertility by acridine orange (AO) fluorescence. Fertil Steril 1984;42:87–91.
11 Kruger TF, Acosta AA, Simmons KF, Swanson RJ, Matta JF, Veeck LL, Morshedi M, Brugo S: New method of evaluating sperm morphology with predictive value for human in vitro fertilization. Urology 1987;30:248–251.
12 Chan PJ, Tredway DR, Su BC, Corselli J, Davidson BJ, Sakugawa M: Sperm hyperactivation as quality control for sperm penetration assay. Urology 1992;39:63–66.
13 Seidman SN: The aging male: androgens, erectile dysfunction, and depression. J Clin Psychiatry 2003;64(suppl 10):31–37.
14 Mulligan T, Godschalk M: Male menopause. Drugs Today (Barc) 1998;34:455–461.
15 Asada H, Sueoka K, Hashiba T, Kuroshima M, Kobayashi N, Yoshimura Y: The effects of age and abnormal sperm count on the non-disjunction of spermatozoa. J Assist Reprod Genet 2000;17:51–59.
16 Sartorelli EM, Mazzucatto LF, de Pina-Neto JM: Effect of paternal age on human sperm chromosomes. Fertil Steril 2001;76:1119–1123.
17 Rolf C, Nieschlag E: Reproductive functions, fertility and genetic risks of ageing men. Exp Clin Endocrinol Diabetes 2001;109:68–74.
18 Irazusta J, Valdivia A, Fernandez D, Agirregoitia E, Ochoa C, Casis L: Enkephalin-degrading enzymes in normal and subfertile human semen. J Androl 2004;25:733–739.
19 Valdivia A, Irazusta J, Fernandez D, Mugica J, Ochoa C, Casis L: Pyroglutamyl peptidase I and prolyl endopeptidase in human semen: increased activity in necrozoospermia. Regul Pept 2004;122:79–84.
20 Luetjens CM, Rolf C, Gassner P, Werny JE, Nieschlag E: Sperm aneuploidy rates in younger and older men. Hum Reprod 2002;17:1826–1832.
21 Sills ES, Fryman JT, Perloe M, Michels KB, Tucker MJ: Chromatin fluorescence characteristics and standard semen analysis parameters: correlations observed in andrology testing among 136 males referred for infertility evaluation. J Obstet Gynaecol 2004;24:74–77.
22 Henkel R, Maass G, Schuppe HC, Jung A, Schubert J, Schill WB: Molecular aspects of declining sperm motility in older men. Fertil Steril 2005;84:1430–1437.
23 Foldesy RG, Leathem JH: Age-related changes in the metabolism of [3H] testosterone in vitro by the epididymis of the immature rat. J Endocrinol 1981;91:43–51.
24 Jung A, Schuppe HC, Schill WB: Comparison of semen quality in older and younger men attending an andrology clinic. Andrologia 2002;34:116–122.
25 Aitken RJ, Gordon E, Harkiss D, Twigg JP, Milne P, Jennings Z, Irvine DS: Relative impact of oxidative stress on the functional competence and genomic integrity of human spermatozoa. Biol Reprod 1998;59:1037–1046.