

Age-Related Increase of Reactive Oxygen Species in Neat Semen in Healthy Fertile Men

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OBJECTIVES

The effects of advancing paternal age on the male reproductive system are well known, but its effects on fecundity remain controversial. Although oxidative stress is associated with poor semen quality and function, a relationship with advancing male age has not been established. The objective of this study was to analyze the relationship between male age and seminal reactive oxygen species (ROS) levels in men presenting for voluntary sterilization.

METHODS

We prospectively evaluated 98 fertile men who were candidates for vasectomy. These were divided into 2 age groups: less than 40 years ($n = 78$) and 40 or more years ($n = 20$). We used 46 infertile patients as positive controls. Standard semen analysis, seminal leukocyte count and ROS levels were measured in all samples. Fertile men with leukocytospermia were excluded.

RESULTS

The mean age of the men was 35.1 ± 5.6 years. Men 40 years and older had significantly higher ROS levels compared with younger men ($P < 0.001$). We observed a positive correlation between seminal ROS levels and age ($r = 0.20$; $P = 0.040$). In addition, ROS was negatively correlated with sperm concentration ($r = -0.48$; $P < 0.001$) and motility ($r = -0.21$; $P = 0.030$).

CONCLUSIONS

Reactive oxygen species levels are significant higher in seminal ejaculates of healthy fertile men older than 40 years. ROS levels in whole ejaculate are significantly correlated to age among fertile men. Because ROS are clearly implicated in the pathogenesis of male infertility, these data suggest that delayed fatherhood may reduce the chances of pregnancy as men become progressively less fertile with age. UROLOGY 71: 490–494, 2008. © 2008 Elsevier Inc.

The considerable decline in fertility and higher reproductive risks associated with advancing maternal age prompt the question whether advanced paternal age is also associated with compromised fertility and increased risks of paternity. Changes in human reproductive behavior including prolonged life expectancy and improvements in assisted reproductive techniques have led to an increase in average paternal age.¹ Although based on a small number of cases, studies indicate that men start to contribute to a decline in the couple's fertility in their late thirties and to a decrease in fecundity in early forties.² Although the affect of male age is less prominent than of the female, this becomes especially significant when the female partner is also of advanced age.³

Standard seminal parameters are normally used as an indirect measure of male fertility. During the past decades

several reports have suggested a decrease in seminal quality with increasing age. Recently, there has been interest in the use of seminal reactive oxygen species (ROS) levels as a marker of male infertility.⁴ A positive correlation between excessive levels of ROS and abnormal sperm concentrations, motility, and morphology has been demonstrated.⁵

Research shows DNA damage in the male germ line is associated with poor semen quality, low fertilization rates, impaired preimplantation development, increased abortion, and an elevated incidence of disease in the offspring, including childhood cancer.^{6,7} The causes of this DNA damage are still uncertain but the major candidates are oxidative stress and aberrant apoptosis.

Currently, controversy exists whether high seminal ROS levels have a negative impact on IVF outcomes. Although studies have been unable to identify significant relationships between ROS levels and fertilization in vitro,^{8,9} some studies found that men with elevated ROS levels in semen have a low potential for in vitro as well as in vivo fertility, with negative effects on embryo growth.^{10,11} To date, the effect of increasing male age on seminal ROS levels has not been evaluated. The objec-

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tive of the study was to analyze the age-related changes in ROS levels in men presenting for voluntary sterilization.

MATERIAL AND METHODS

The Institutional Review Board approved the study and we obtained informed consent from participants. The study population consisted of 98 fertile men who were prospectively evaluated and presented for vasectomy between May 2004 and January 2006. This population was considered a fertile group because the subjects had previously fathered children. They were divided into two age groups: men less than 40 years ($n = 78$) and 40 or more years ($n = 20$). A group of infertile patients ($n = 46$) consisted of couples that had failed to conceive after more than 1 year of regular unprotected intercourse. The infertile patients served as a positive control group. All women were normal based on gynecologist evaluation.

Two male infertility specialists (MC and RP) evaluated all patients. The presence of clinical varicocele was evaluated in all groups. Patients were excluded from the study if there was history of: illicit drug use; exposure to any environmental or occupational toxicants; use of medication with proven toxicity on fertility; exposure to radiation or heat; mumps with orchitis; sexually transmitted or systemic diseases; cryptorchidism regardless of treatment; testicular torsion; genitourinary anomalies; epididymal or vas deferens alterations; and scrotal or inguinal surgery.

We assessed standard semen analysis, seminal leukocyte levels, and levels of ROS in neat semen in all samples. Fertile men with samples containing 1×10^6 or greater leukocytes/mL of semen were excluded to avoid a potential source of ROS generation.

Semen Analysis

Semen was collected by masturbation after 48 to 72 hours of sexual abstinence. After liquefaction all semen analyses were performed manually. We performed the macroscopic and microscopic parameters according to World Health Organization (WHO) guidelines.¹² We assessed semen parameters, including sperm concentration, total motility, grade A sperm, grade B sperm, grade C sperm, total motile sperm, and total sperm according to the WHO and sperm morphology according to Kruger's strict criteria.¹³

Leukocytospermia

We measured leukocyte concentrations in semen by the myeloperoxidase-staining test.¹⁴ We placed a 20- μ L volume of liquefied specimen in a 1.8- μ L microtube and 20 μ L of phosphate-buffered saline (PBS; pH 7.0) with 40 μ L of benzidine solution. We examined the mixture for brown-stained cells indicating that they contained peroxidase and were therefore granulocytes.¹⁴

Measurement of Seminal ROS

We added 10 μ L of 5 mM luminol prepared in dimethylsulfoxide (DMSO) (Sigma Chemical Co., St. Louis, MO) to 400 μ L of the liquefied semen. ROS levels were determined by measuring chemiluminescence with a luminometer MicroBeta Trilux (software version 4.7; PerkinElmer Life Sciences, Turku, Finland) for 15 minutes. Results are expressed as 10^4 counted photons per minute (cpm) per 20×10^6 sperm.

Statistical Analysis

We performed all analyses with MINITAB (version 14.2; Six-Sigma, Austin, TX) and SPSS (version 14.0, SPSS Institute, Inc., Cary, NC). For the normally distributed continuous variables we used the Student *t*-test. We performed pairwise comparisons between groups with Wilcoxon rank-sum tests for continuous, nonnormally distributed data. We performed associations among ROS levels and each category of age using the Chi-square test. We calculated correlations among variables using Spearman's nonparametric method. A *P*-value less than 0.05 was considered statistically significant.

RESULTS

The mean age \pm standard deviation (SD) of the fertile men was 35.1 ± 5.6 years. A total of 78 (79.6%) subjects were under 40 years old and 20 (20.4%) were 40 years or older. There was a significant difference in age \pm SD of men over 40 years (43.5 ± 3.3) compared with younger men (33 ± 3.8) ($P < 0.001$). Clinical varicocele was diagnosed in 32.1% of men under 40 years old, whereas only 10% of men over 40 years had varicocele during physical examination ($P = 0.049$).

Table 1 presents an analysis of standard seminal parameters (sperm concentration, total motility, percentage normal morphology by WHO, and Kruger's criteria), leukocyte levels, and ROS levels in neat semen performed among fertile men 40 years or older, fertile men under 40 years, and infertile patients.

All seminal parameters were significantly lower in infertile men compared with both fertile groups. However, both leukocyte and seminal ROS levels were significantly higher compared with fertile men less than 40 years old and over 40 years old. Although sperm concentration and WHO morphology were higher in men less than 40 years old compared with older men, these differences were not significant. However, men over 40 years of age presented significantly poorer total motility and morphology by Kruger's criteria compared with men less than 40 years old. In addition, men 40 years and older had significantly higher ROS levels compared with younger men (Table 1). No significant difference was seen in leukocyte levels between these groups.

We also compared age and ROS levels in neat semen among fertile and infertile men according to each category of age. Although there was no significant difference in age of fertile and infertile men over 40 years, as expected, ROS levels were significantly higher among the infertile group (Table 2). Infertile men less than 40 years old were significantly younger than fertile men less than 40 years old; however, they still had higher seminal ROS levels (Table 2).

A significant positive correlation was seen between ROS and age when ROS was used as the sole predictor in each model. We performed correlations between seminal ROS in neat semen, age, seminal parameters, and seminal leukocyte level (Table 3). In addition, ROS were significantly negatively correlated with sperm concentration and motility. Although we found a negative corre-

Table 1. Seminal parameters, leukocyte levels, and reactive oxygen species (ROS) levels in neat semen among fertile men over 40 years old, fertile men under 40 years old, and control patients

Parameters	Men \leq 40 Years	Men < 40 Years	Controls	A	B	C
	Old (n = 20)	Old (n = 78)				
Sperm concentration ($\times 10^6$ /mL)	73.6 (41, 143)	95.2 (59, 144)	29 (17, 78)	0.28	<0.0001*	<0.0001*
Sperm motility (%)	61.5 (52, 71)	69 (61, 74)	49 (35, 59)	0.02*	0.0012*	<0.0001*
Grade A motility	8 (4, 11)	9 (1, 15)	0 (0, 4)	0.66	<0.0001*	<0.0001*
Grade B motility	35 (24, 39)	36 (28, 42)	21 (11, 28)	0.45	0.0008*	<0.0001*
Grade C motility	16 (13, 21)	28 (19, 45)	21 (15, 32)	0.10	0.07	0.72
WHO morphology (%)	15 (11, 31)	21 (13, 28)	10 (5, 22)	0.42	0.02*	<0.0001*
Kruger's morphology (%)	4 (3, 8)	5.5 (3, 9)	2 (0, 5)	0.09*	0.01*	<0.0001*
Leukocytes ($\times 10^6$ /mL)	0 (0, 0.2)	0 (0, 0)	0.4 (0, 1.6)	0.22	<0.0014*	<0.0001*
ROS in neat semen ($\times 10^4$ /cpm)	0.68 (0.43, 0.9)	0.29 (0.18, 0.58)	1.51 (0.69, 4.26)	0.0009*	0.006*	<0.0001*

WHO, World Health Organization. A = *P*-value between fertile men 40 or more years old and fertile men less than 40 years old; B = *P*-value between fertile men 40 or more years old and controls; C = *P*-value between fertile men less than 40 years old and controls. Values are median and interquartile range (25%, 75%). We used Wilcoxon rank sum test for the analysis and considered * *P* < 0.05 statistically significant.

Table 2. Age and ROS levels in neat semen among fertile and infertile men according to each age category

Parameters	Fertile Men \geq 40	Infertile Men \geq 40	A	Fertile Men < 40	Infertile Men < 40	B
	Years Old (n = 20)	Years Old (n = 11)		Years Old (n = 78)	Years Old (n = 35)	
Age	43.5 \pm 3.3	42.65 \pm 3.1	0.909	335 \pm 3.8	27.95 \pm 6.9	<0.001*
ROS in neat semen ($\times 10^4$ /cpm)	0.63 (0.22, 9.51)	3.25 (0.29, 106.76)	0.014*	0.30 (0.04, 1.66)	1.34 (0.10, 386.53)	<0.001*

Abbreviation as in Table 1. A = *P*-value between fertile men 40 or years old and infertile men 40 or more years old; B = *P*-value between fertile men less than 40 years old and infertile men less than 40 years old. Age values are mean and standard deviation. ROS values are median and interquartile range (25%, 75%). We used Wilcoxon rank sum test for ROS levels analysis and considered * *P* < 0.05 statistically significant.

Table 3. Correlation of ROS levels in semen with sperm parameters and age in fertile men (n = 98)

Variable	ROS Levels	
	<i>r</i>	<i>P</i> -Value
Sperm concentration	-0.48	<0.001*
Sperm motility	-0.21	0.030*
Grade A motility	-0.20	0.043*
Grade B motility	-0.25	0.010*
Grade C motility	0.06	0.510
WHO morphology	-0.15	0.120
Kruger's morphology	-0.15	0.120
Seminal leukocytes	0.32	<0.001*
Age	0.20	0.043

Abbreviations as in previous tables.

* *P* < 0.05 was considered statistically significant (Spearman's Rho test).

lation between seminal ROS levels and morphology by WHO and Kruger's criteria, these findings were not statistically significant. ROS levels were positively correlated with leukocyte concentrations (Table 3).

COMMENT

Delayed childbearing is a common phenomenon in industrialized countries. Therefore, age-related changes in

the male reproductive system are becoming commonly recognized.² Although men at any age can establish pregnancy in a woman, we still do not know how safe and wise it is to attain pregnancy through assisted reproductive techniques at advanced age.

There is evidence now that birth defects, especially those arising from new autosomal mutations, increase with paternal age.¹⁵ In addition, advanced paternal age is associated with new mutations in paternal genome and increased risk of aneuploidy in the fetus. These findings suggest that genetic risks associated to increased paternal age should be of high interest to andrologists counseling older men who wish to father a child. The American Society of Reproductive Medicine in their revised guidelines (2006) recommended 40 years as the upper age limit for semen donors owing to a progressive increase in the prevalence of aneuploid sperm.¹⁶ The results of the present study also substantiate these recommendations.

Reactive oxygen species are involved in the modulation of an entire spectrum of physiological reproductive functions. However, oxidative stress characterized by an excess of ROS is well recognized to be one of the major factors leading to infertility. One consequence of high ROS production is peroxidative damage to the plasma membrane. Aitken *et al.*¹⁷ showed that plasma membrane

damage leads to an impairment of sperm function as well as decreased spontaneous pregnancy rates and the functional impairment of in vitro fertilization.

It is known that with advancing age an organism is under greater oxidative stress as the result of impairment of the function of the mitochondrial respiratory chain.¹⁸ One of the reasons that spermatozoa are susceptible to the damage induced by excessive ROS is the fact that their cytoplasm contains low concentrations of antioxidant scavenging enzymes.¹⁹ Although the effects aging have on the antioxidative abilities of the reproductive system have not been reported in men, an experimental study indicated that the male reproductive system undergoes age-related changes in antioxidant enzyme activities.²⁰ Antioxidants are the most important defense against oxidative stress induced by free radicals. Currently, the literature supports the use of systemic antioxidants for the management of selective cases of male infertility as well as in vitro supplements during different sperm preparation technique.²¹

Kobayashi *et al.*²² showed that luminol-dependent chemiluminescence assay for ROS measurement is both accurate and reliable when the sperm concentration is greater than 1×10^5 /mL and the samples are analyzed within the first hour after specimen collection. Also the authors reported no statistically significant interobserver, intraobserver, or interassay variation using this technique. The assessment of ROS levels in semen can be performed both in seminal ejaculates without further processing (neat) or after preparation such as the wash and resuspend technique.^{23,24} Although both measurements reflect with reliability the oxidative stress condition, ROS levels in neat semen seems to be more coherent with the real status owing to the maintenance of seminal plasma and antioxidant capacity.²⁵ However, still there is no consensus concerning the inclusion of ROS measurement in clinical settings as part of the diagnostic workup of infertile men, primarily as a result of the lack of standardization of normal ROS levels in semen. Recently, our center reported a median (IQR) seminal ROS levels in neat samples of $0.4 (0.2, 0.9) \times 10^4$ cpm/ 20×10^6 sperm in a group of fertile men. In the present study we found 90% of men over 40 years old had seminal ROS levels higher than 0.4×10^4 cpm/ 20×10^6 sperm, whereas 42.3% of men less than 40 years old had ROS levels higher than 0.4×10^4 cpm/ 20×10^6 sperm ($P < 0.001$).

Seminal oxidative stress causes impairment of semen quality by multiple mechanisms including damage of sperm DNA integrity. Recently, a significant age-related increase in DNA fragmentation was reported. In addition, one study showed that paternal age over 40 years significantly increases the risk of miscarriage in couples particularly if the woman is over 35 years old.³ These findings may be attributed to the greater DNA fragmentation found in older men, possibly the result of a less efficient apoptotic mechanism.²⁶

The current study shows that age is one likely factor in the increase of seminal ROS levels. However, there are many other factors that could be involved in decreasing semen quality, independent of age. The gradual trend of decreasing semen quality and fertility may be associated with environmental pollution, lifestyle risk factors (smoking, caffeine intake, or alcohol), and occupational exposure to industrial agents and heavy metals.²⁷ Reports suggest a decline in semen quality over the past decade among fertile men that is independent of the age of the men.^{28,29} In accordance with our findings, significant age-related seminal parameter changes include a decrease in motility and morphology. However, no consistent data confirm that sperm concentrations also decline with advancing age.³⁰ The observation that seminal ROS levels are significantly correlated with age is of particular concern. Older men tend to reproduce with women of advancing age, which increases the chance of requiring assisted reproductive technology. These couples are at an increased risk of unsuccessful pregnancy outcomes.

CONCLUSIONS

Seminal ROS levels are significantly higher in healthy fertile men older than 40 years. These levels are significantly correlated to age among fertile men. Because high ROS levels have been associated in the pathogenesis of male infertility, our data suggest that delayed fatherhood may reduce the chances of pregnancy as men become progressively less fertile. Treatment with antioxidants may be beneficial in men older than 40 years who are attempting to father a child.

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