Effect of seminal oxidative stress on fertility after vasectomy reversal

Peter N. Kolettis, M.D., Rakesh K. Sharma, Ph.D., Fabio F. Pasqualotto, M.D., David Nelson, M.S., Anthony J. Thomas, Jr., M.D., and Ashok Agarwal, Ph.D.

Andrology Research and Clinical Laboratories, Department of Urology, Cleveland Clinic Foundation, Cleveland, Ohio

Objective: To evaluate seminal oxidative stress in men after vasectomy reversal and to determine whether seminal oxidative stress could predict fertility after vasectomy reversal.

Design: Measurement of seminal reactive oxygen species (ROS) and total antioxidant capacity (TAC) in normal donors, men who were fertile after vasectomy reversal, and men who were infertile after vasectomy reversal.

Setting: A male infertility clinic of a tertiary care center.

Patient(s): Thirty men who underwent vasectomy reversal and 17 normal donors.

Intervention(s): None.

Main Outcome Measure(s): Semen characteristics, seminal ROS, and TAC were measured with chemiluminescence assays in samples from donors and reversal patients.

Result(s): Mean adjusted seminal ROS (log [ROS + 1]) was higher in infertile reversal patients (2.38 ± 0.25) than in normal donors (1.30 ± 0.14). Seminal ROS was also higher in all (fertile and infertile reversal combined) reversal patients than in donors. Total antioxidant capacity did not differ between groups. The ROS-TAC score, a composite index of seminal oxidative stress, was a significant predictor of fertility. A ROS-TAC score of 45 or greater had a positive predictive value of 73% in predicting fertility.

Conclusion(s): Seminal oxidative stress is associated with vasectomy reversal. The ROS-TAC score is a possible predictor of infertility after vasectomy reversal. (Fertil Steril 1999;71:249–55. ©1999 by American Society for Reproductive Medicine.)

Key Words: Vasectomy reversal, male infertility, reactive oxygen species, total antioxidant capacity, oxidative stress

After sterilization by vasectomy, a large number of men request a reversal of the procedure to restore their fertility. Advances in surgical technique have resulted in excellent patency rates, with success dependent partly on the elapsed time since vasectomy (obstructive interval) (1, 2). However, despite patency rates of 71%–97%, there is a 26%–72% chance of persistent infertility. Not all the reasons for persistent infertility are known, but they include partial obstruction of the vas deferens, epididymal dysfunction or obstruction, anti-sperm antibodies, and female infertility (1, 3, 4).

Oxidative stress has been shown to be an important cause of male infertility (5–7). Iwasaki and Gagnon (7) found elevated levels of seminal reactive oxygen species (ROS) in 40% of the infertile men in their study. Reactive oxygen species are highly unstable free oxygen radicals, including hydrogen peroxide, hydroxyl radical, and superoxide anion. These radicals are produced in small amounts by normal spermatozoa and are implicated in signal transduction. Elevated seminal ROS, however, can cause sperm dysfunction through lipid peroxidation of the polyunsaturated fatty acids in the sperm membrane (5–7).

Both sperm and seminal plasma possess antioxidant systems capable of counteracting the harmful effects of ROS, and it is possible to measure the total antioxidant capacity (TAC) of semen (8–10). Studies have demonstrated that infertile men are more likely than fertile ones to have depressed TAC and lower levels of individual antioxidants (9, 11, 12). Further-
more, studies of infertile men empirically treated with anti-
oxidants have demonstrated improved semen characteristics,
Improved fertilization in vitro, and in one study, a higher
pregnancy rate in the treatment group (13–15). Thus, anti-
oxidants also appear to play a role in sperm function and
male infertility.

Given that oxidative stress is a known cause of infertility,
we hypothesized that it might be linked with persistent
infertility after vasectomy reversal. We sought to determine
if oxidative stress was found after vasectomy reversal and if
a measure of oxidative stress could predict infertility after
such procedures.

We compared three groups of men: those who were fertile
after vasectomy reversal; those who were infertile after va-
sectomy reversal; and healthy controls. We measured sper-
matozoal ROS and seminal TAC and derived a novel indi-
cator of oxidative stress, the ROS-TAC score. Because
antisperm antibodies are also a possible cause of infertility,
we compared this variable as well. In addition, we compared
the semen quality in the three patient groups on 10 com-
monly rated semen characteristics and sought other possible
causes of infertility, including the wife’s age and fertility, the
obstructive interval, and the interval between the reversal
and the study.

MATERIALS AND METHODS

Subjects

The study was approved by the Institutional Review
Board of The Cleveland Clinic Foundation, and written
informed consent was obtained from all subjects.

Three groups of men were recruited through the Male
Infertility Clinic and Andrology Laboratory of the Depart-
ment of Urology at our tertiary care hospital: [1] men who
were infertile after vasectomy reversal (no pregnancy re-
sulted from intercourse within 1 year after surgery); [2] men
who were fertile after vasectomy reversal (successful estab-
lishment of a pregnancy through intercourse within 18
months after surgery); and [3] normal donors with no history
of infertility and normal semen analyses. Azoospermic men,
those men who did not actively attempt a pregnancy, and
men with leukocytospermia (>10⁶ white blood cells/mL)
were excluded from the study. Vasectomy reversal patients
were questioned about the time between vasectomy and
reversal, the wife’s age, and any possible female factors.

Semen Analysis

Semen specimens were obtained by masturbation after a
minimum of 2 days of sexual abstinence. Computer-assisted
semen analysis was performed on all specimens, with use of
a Motion Analysis VP-50 semen analyzer (Motion Analysis
Corporation, Santa Rosa, CA). All counts were verified man-
ually. Ten semen characteristics, including volume, sperm
concentration, percent motility, and sperm morphology, were
assessed according to the criteria defined by the World
Health Organization, and by Kruger’s strict criteria (16, 17).
The Endtz test (myeloperoxidase staining) was performed on
all specimens to measure the concentration of granulocytes
(18).

Reactive Oxygen Species Measurement

Aliquots of liquefied semen were centrifuged at 300 × g
for 7 minutes. Seminal plasma was aliquoted and frozen at
−20°C for later measurement of total antioxidant levels. The
sperm pellet was washed twice with phosphate-buffered
saline (PBS), pH 7.4, and resuspended in the same media at
a concentration of 15–20 × 10⁶ sperm/mL. Levels of ROS
were determined by the chemiluminescence method, using
luminol as the probe (18). Ten microliters of 5 mM luminol
(5-amino-2,3-dihydro-1,4 phthalazinedione; Sigma Chemi-
cal Co., St. Louis, MO) prepared in dimethyl sulfoxide
(Sigma Chemical Co.) was added to 400 μL of the washed
sperm suspension, which was then vortexed. Ten microliters
of 5 mM luminol added to 400 μL of PBS served as a
negative control.

Levels of ROS were determined by measuring chemilu-
minescence with a luminometer (model LKB 953; Wallac
Inc., Gaithersburg, MD) in the integrated mode for 15 min-
utes and were expressed as 10⁶ counted photons per minute
(cpm) per 20 × 10⁶ sperm. On the basis of the analysis of
controls, negative controls, and interassay variability, vari-
ance components indicate that intraassay measurement reli-
ability is approximately 98%.

Total Antioxidant Capacity

Total antioxidant activity was measured in the seminal
plasma using the enhanced chemiluminescent assay (10).
Aliquots of the seminal plasma that had been stored at
−20°C were thawed at room temperature and immediately
assessed for their antioxidant capacity as follows. Seminal
plasma was diluted 1:10 with deionized water (dH₂O) and
filtered through a 0.20-


mm Millipore filter (Allegiance
Healthcare Corporation, McGaw Park, IL). Signal reagent
was prepared with use of a chemiluminescence kit (Amer-
sham Life Science, Buckinghamshire, United Kingdom).
Twenty microliters of horseradish peroxidase-linked immu-
noglobulin (HRP-linked Ig; Amersham Life Science) was
added to 4.98 mL of dH₂O. This was further diluted to 1:1 to
give a working solution with the desired luminescence out-
put (3 × 10⁴ cpm).

Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carbox-
ylic acid), a water-soluble tocopherol analogue, was added
as the standard at concentrations between 50 and 150 μM.
The antioxidant capacity of the seminal plasma then was
expressed in molar Trolox equivalents.

With the luminometer in the kinetic mode, 100 μL of
signal reagent and 100 μL of HRP were added to 700 μL of
distilled water and mixed. The solution was equilibrated to
the desired level of chemiluminescence output (between 2
and 3 × 10⁴ cpm) for 100 seconds. One hundred microliters
of the prepared seminal plasma was added immediately to the signal reagent and HRP, and the chemiluminescence was measured. Suppression of chemiluminescence and the time from the addition of seminal plasma to 10% recovery of the initial chemiluminescence were recorded. Antioxidant capacity was expressed as molar Trolox equivalents.

Antisperm Antibody Measurement

Seminal plasma antisperm antibodies were measured in vasectomy reversal patients using the indirect immunobead assay (19).

Statistical Analysis

Data analysis compared three classifications of men: men who were fertile after vasectomy reversal, men who were infertile after vasectomy reversal, and normal donors. Wilcoxon’s rank sum tests were used to compare groups for continuous variables. In addition, Spearman correlations were used to determine significance of correlations between variables. Logistic regression and receiver operating characteristic (ROC) curves were used to evaluate significance and diagnostic ability to predict fertility among the vasectomy reversal patients.

Fertility (defined as pregnancy resulting from intercourse within 1 year after vasectomy reversal) was used as the positive result, and infertility (defined as no pregnancy from intercourse during the year after vasectomy reversal) was the negative result for computations of sensitivity, specificity, positive predictive value, and negative predictive value, and their 95% confidence intervals (CI).

The ROS and TAC values from the normal donors were used to create a scale of these two variables that uses the donor values as reference points. The log of (ROS + 1) was used in calculations so that both values were normalized to the same distribution. First, both TAC and log (ROS + 1) were standardized to z scores (mean [±SD] 0 ± 1) so that both would have the same variability. These standardized scores were calculated by subtracting the mean value of the donors from the patient (fertile and infertile men) value and dividing by the standard deviation of the donor population.

For log (ROS + 1): Standardized ROS

\[ \text{Standardized ROS} = \frac{\log (ROS + 1) - 1.223}{0.5222} \]

For TAC: Standardized TAC

\[ \text{Standardized TAC} = \frac{TAC - 1619.22}{505.29} \]

These two standardized variables then were analyzed with principal components analysis, which provided linear combinations (or weighted sums) that account for the most variability among correlated variables. The first principal component provided the following linear equation:

\[ \text{Principal component} = (-0.707 \times \text{standardized ROS}) + (0.707 \times \text{standardized TAC}) \]

To ensure that the distribution of the ROS-TAC score would have a mean (±SD) of 50 ± 10 in normal donors, the ROS-TAC score was transformed as:

\[ \text{ROS-TAC score} = 50 + \text{Principal component} \times 8.97 \]

Receiver operating characteristic curves were used to examine the diagnostic ability of the ROS-TAC score to predict fertility. The ROC curves illustrate the sensitivity and specificity over the entire range of the ROS-TAC score. The area under the curve can range from 50% to 100% with diagnostic tests that approach 100%, indicating a perfect predictor and 50%, indicating random chance or no predictive ability. Statistical significance among the three groups was assessed with two-tailed tests at the \( P < .05 \) level. Statistical tests were performed using SAS version 6.12 (SAS Institute, Cary, NC).

RESULTS

The mean (±SD) age of the infertile vasectomy reversal patients was 42.7 ± 1.5 years, of the fertile vasectomy reversal patients was 44 ± 1.8 years, and of the healthy donors was 31.1 ± 2.1 years. The healthy donors were significantly younger than both groups of vasectomy reversal patients (\( P < .001 \)). Four patients underwent unilateral vasoepididymostomy, 18 bilateral vasoepididymostomy, and 8 underwent vasoepididymostomy with contralateral vasoepididymostomy (Table 1).

Semen Characteristics

There was no statistical significance in any semen characteristic between fertile and infertile reversal patients. Semen quality was significantly better in healthy donors than in infertile reversal patients, except in linearity. Donor semen was also significantly better than that of fertile reversal patients, except in the amplitude of lateral head displacement. When reversal patients (fertile and infertile combined)
Comparison of age and semen characteristics between normal donors and men after vasectomy reversal.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Study group</th>
<th>P values*</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Normal donors (n = 17)</td>
<td>Fertile after reversal (n = 11)</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>Patient age (y)</td>
<td>31.1 ± 2.1</td>
<td>44 ± 1.78</td>
</tr>
<tr>
<td>Semen characteristics</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Volume (mL)</td>
<td>2.7 ± 0.8</td>
<td>3.4 ± 0.5</td>
</tr>
<tr>
<td>Concentration (× 10^9/mL)</td>
<td>69.4 ± 9.1</td>
<td>48.4 ± 9.5</td>
</tr>
<tr>
<td>Percent motility</td>
<td>55.5 ± 4.9</td>
<td>33 ± 4.9</td>
</tr>
<tr>
<td>VCL (μm/s)</td>
<td>35.15 ± 2.05</td>
<td>25.57 ± 2.1</td>
</tr>
<tr>
<td>VSL (μm/s)</td>
<td>13.89 ± 0.82</td>
<td>10.45 ± 1.13</td>
</tr>
<tr>
<td>VAP (μm/s)</td>
<td>23.52 ± 1.32</td>
<td>13.87 ± 1.64</td>
</tr>
<tr>
<td>Linearity (%)</td>
<td>39.45 ± 0.98</td>
<td>40.53 ± 1.29</td>
</tr>
<tr>
<td>ALH (μm)</td>
<td>2.24 ± 0.13</td>
<td>1.79 ± 0.13</td>
</tr>
<tr>
<td>WHO: normal sperm forms (%)</td>
<td>39.79 ± 2.5</td>
<td>35.80 ± 2.85</td>
</tr>
<tr>
<td>Kruger: normal sperm forms (%)</td>
<td>12.07 ± 0.86</td>
<td>11 ± 1.21</td>
</tr>
</tbody>
</table>

Note: All values are means ± SE. A = overall three groups; B = normal donors versus fertile vasectomy reversal; C = normal donors versus fertile vasectomy reversal; D = normal donors versus all vasectomy reversal; E = fertile vasectomy reversal versus infertile vasectomy reversal. ALH = amplitude of lateral head displacement; VAP = average path velocity; VCL = curvilinear velocity; VSL = straight line velocity; WHO = World Health Organization. *P < .05 was considered statistically significant.

were compared as a group with the healthy donors, all semen characteristics were significantly different except linearity and percent normal forms by Kruger’s strict morphology. Semen volume did not differ between groups (Table 2).

Reactive Oxygen Species

Mean (±SE) adjusted ROS levels (log [ROS + 1]) were 1.30 ± 0.14 for the donors, 1.80 ± 0.27 for the fertile reversal patients, and 2.38 ± 0.25 for the infertile reversal patients (Table 3). These differences were statistically significant across all three groups (P = .005) and when comparing donors with infertile reversal (P = .001) and donors with all (fertile and infertile combined) reversal patients (P = .004). However, there was no difference in ROS levels between fertile reversal patients and donors or fertile and infertile reversal patients.

Seminal Total Antioxidant Capacity

Mean (±SE) seminal plasma TAC levels were 1,654 ± 121 for the donors, 1,955 ± 222 for the fertile reversal patients, and 1,527 ± 170 for the infertile reversal patients (Table 3). The differences in TAC were not statistically significant among the groups. Based on the sample sizes of fertile and infertile patients, this study had 90% power to detect a difference of 925 Trolox equivalents between the two groups.

Comparison of reactive oxygen species and total antioxidant capacity between normal donors and men after vasectomy reversal.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Study group</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal donors (n = 17)</td>
<td>Fertile after reversal (n = 11)</td>
</tr>
<tr>
<td>log (ROS + 1)</td>
<td>1.30 ± 0.14</td>
<td>1.80 ± 0.27</td>
</tr>
<tr>
<td>TAC</td>
<td>1,654 ± 121</td>
<td>1,955 ± 222</td>
</tr>
<tr>
<td>ROS-TAC score</td>
<td>50 ± 2.60</td>
<td>47.1 ± 3.9</td>
</tr>
</tbody>
</table>

Note: All values are means ± SE. ROS = reactive oxygen species; TAC = Total antioxidant capacity; A = overall three groups; B = normal donors versus infertile vasectomy reversal; C = normal donors versus fertile vasectomy reversal; D = normal donors versus all vasectomy reversal; E = fertile vasectomy reversal versus infertile vasectomy reversal. *P < .05 was considered statistically significant.
ROS-TAC Score

The ROS-TAC score, an overall index of seminal oxidative stress, varied significantly across the three groups ($P = .01$). The mean ROS-TAC score was significantly different between donors and infertile reversal patients ($P = .004$) and between donors and all (fertile and infertile combined) reversal patients ($P = .03$). Most importantly, this index was significantly different between fertile and infertile reversal patients ($P = .048$).

Logistic regression indicated that the ROS-TAC score was related significantly to fertility among the vasectomy reversal patients ($P = .046$). The area under the ROC curve was 73%. A ROS-TAC score of 45 achieved a sensitivity of 73% (95% CI: 39%–94%) and a specificity of 82% (95% CI: 57%–96%) in predicting fertility. In addition, the positive predictive value (the probability that individuals with a score 45 or higher were fertile) was 73% (95% CI: 39%–94%), and the negative predictive value (the probability that individuals with a score lower than 45 were infertile) was 82% (95% CI: 57%–96%) (Fig. 1).

Correlation Between ROS and TAC With Other Variables

There was no correlation between ROS and TAC in the study population ($P = .86$). The ROS did not correlate with the obstructive interval ($P = .48$) or with the time between reversal and ROS measurement ($P = .18$). The ROS correlated with the percent of sperm bound by IgM ($P = .04$), but not by IgA or IgG; ROS did not correlate with the type of surgery performed ($P = .35$).

Total antioxidant capacity correlated with the time interval between reversal and the study ($P = .04$), but not with the obstructive interval ($P = .74$). The TAC showed no correlation with the percent of sperm bound by any class of antibody or the type of surgery performed ($P = .81$).
tisperm antibodies, and female factors are possible contributing causes (1, 3, 4).

Complete epididymal obstruction was an unlikely factor in our study because azoospermic men were excluded. The infertile reversal group did have a higher percentage of sperm bound by IgA antibodies than the fertile group. However, the antibody screen results and percentage of sperm bound by the antibody classes other than IgA did not differ significantly between the two groups. These data suggest that IgA binding could impair fertility in these individuals, although it should be noted that 3 of the 11 fertile men had 20% or more sperm bound by IgA. Also, sperm motility did not differ significantly between the fertile and infertile groups.

Because fertility depends on both members of a couple, female infertility certainly also could impede a pregnancy. However, in our patients, the same proportion of couples in the fertile and infertile groups had possible contributing female factors, and the wives’ ages were not significantly different. The obstructive interval, also related to fertility in other studies (1), was not different between the fertile and infertile groups. Finally, conventional semen characteristics were unable to differentiate between fertile and infertile men in our study; none of the characteristics were significantly different between the fertile and infertile groups.

Elevated levels of seminal ROS have been demonstrated in 40%–88% of infertile men (11). Elevated seminal ROS can damage the sperm membrane through lipid peroxidation, causing lower fertilization rates in vitro (20). Depressed seminal antioxidant capacity also has been implicated in male infertility. Both TAC and individual antioxidant levels have been shown to be lower in the semen of infertile men (11, 12).

Our study found a significant difference in seminal ROS levels between normal donors and all vasectomy reversal patients, but not between the fertile and infertile groups. In addition, the TAC did not differ between any two groups. This suggests a possible relationship between oxidative stress and vasectomy reversal, but not between oxidative stress and fertility in this population of men. It is possible that our sample size was insufficient to detect a clinically meaningful difference between the fertile and infertile groups. It is also possible that neither variable alone adequately quantifies seminal oxidative stress. The combination of these two variables may be better than either alone at quantifying the overall oxidative stress in the seminal plasma.

The ROS and TAC have different degrees of variability. Our composite ROS-TAC score combines these two measurements to compensate for this differing variability. Indeed, the ROS-TAC score did correlate with fertility in our study. A ROS-TAC score below 45 had a sensitivity for infertility of 73% and a specificity of 82%. This index requires further validation in other populations of infertile men.

Empiric trials of antioxidant supplementation in infertile men have shown encouraging results. Improved semen characteristics and higher rates of fertilization in vitro have resulted from intramuscular glutathione and oral vitamin E therapy, respectively (13, 14). Furthermore, a trial of oral vitamin E in men with asthenospermia resulted in a higher pregnancy rate in the treatment group (15). Our findings demonstrate oxidative stress in men after vasectomy reversal and suggests a rationale for empiric antioxidant supplementation in this group of men as well.

In conclusion, seminal oxidative stress, a known cause of infertility, is associated with vasectomy reversal. Neither conventional semen characteristics nor conventional oxidative stress measures (ROS and TAC) can distinguish between fertile and infertile men after vasectomy reversal. However, the ROS-TAC score, a novel measure of oxidative stress, appears to be a good predictor of infertility in these men. This link between oxidative stress and infertility suggests the possibility that antioxidant supplements might improve fertility in these men.

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