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# The resazurin reduction test provides an assessment of sperm activity

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**Objective:** The objective of the study was to determine if reduction of the dye resazurin by semen could be correlated with the concentration of motile sperm.

**Design:** After assessment of sperm count and motility, specimens were incubated for 1 hour with resazurin (25 μg/mL of semen) and visual color changes indicative of dye reduction noted.

**Setting:** Specimens were obtained from men seeking care for infertility at one of four sites: (1) University of California, San Francisco (UCSF) In Vitro Fertilization Program; (2) UCSF Andrology Laboratory; (3) a gynecological practice in Maine; and (4) a private andrology laboratory in Southern California.

**Patients:** Individuals were self-selected by their desire to have a semen analysis in conjunction with the diagnosis or treatment of infertility.

**Interventions:** None.

**Main Outcome Measure:** The reduction of the dye resazurin and its correlation with motile sperm density.

**Results:** When the motile sperm concentration was $\geq 20 \times 10^6$/mL, 86% of specimens produced a positive color change. Conversely, 86% of specimens with a motile sperm concentration of $<20 \times 10^6$/mL either did not change color or changed only over a narrow range.

**Conclusion:** Reduction of resazurin offers an assessment of the active sperm in a specimen without the need to do a sperm count or evaluation of motility. Fertil Steril 56:743, 1991

Semen analysis remains the basic tool for assessing male fertility, despite the introduction of newer technologies. However, dissatisfaction with the prognostic value of the standard semen analysis has prompted a continuing search for alternatives.[1] One weakness of the semen analysis has been an inability to establish absolute guidelines for categorizing males as fertile or infertile. Different values for the lower limit of a normal sperm count have been presented and these range from 5 to $60 \times 10^6$/mL.[2–4] The most widely accepted figure is $20 \times 10^6$/mL, but it too does not readily differentiate fertile from infertile. Approximately 20% to 25% of females whose partners have counts below this figure will achieve a pregnancy without treatment of the male.[5] Sperm motility also can impact on the fertility potential of a specimen, and males often are categorized as normal or abnormal on the basis of sperm motility independent of the sperm count. Attempts to combine the information from the sperm count and the sperm motility have led to results being expressed as total motile sperm (total sperm in the ejaculate × motility) or motile sperm per mL (sperm/mL × motility).[2] This combining of count and motility allows determination of the numbers or concentration of active sperm and may provide a better means of evaluating

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fertility potential than assessing count and motility independently.

In bulls, active sperm can be assessed quantitatively by a dye reduction test that obviates the need for doing sperm counts and sperm motility determinations.[6] The dye, resazurin (7-hydroxy-3H-phenoxazin-3-one 10-oxide), is reduced to resorufin and then to hydroresorufin by motile sperm, and this reaction is manifested by a visual change in color of the dye from purple to pink to white. In the current study, the reduction of resazurin by human sperm was correlated with the number of motile sperm per mL in the specimen. The results indicate that this test could serve as a simple method for assessing the active sperm in a specimen without the need for a microscope or other standard laboratory equipment.

## MATERIALS AND METHODS

Three hundred eighty-three semen samples were obtained from four sites: (1) the In Vitro Fertilization Program (IVF) of the University of California-San Francisco (UCSF). These were screening samples and not the samples used for oocyte insemination (n = 193). Nine additional samples were obtained from two donors, and these are listed in this category for convenience; (2) the Andrology Laboratory at UCSF (n = 122); (3) a private gynecological practice in Lewiston, Maine (n = 31); and (4) an andrology laboratory with units in Santa Ana and San Diego, California (n = 28). Permission to use the specimens that were to be discarded from the UCSF IVF Program and Andrology Laboratory was obtained from the Committee on Human Research UCSF.

Sperm counts and progressive motilities were assessed using a Makler chamber (Sefi Medical Industries, Haifa, Israel) (sites 1, 2, and 3) and by hemocytometer and slide at site 4. Morphology was not taken into consideration for this study. Testing was performed within 2 hours of specimen collection. Semen was placed in conical 15-mL test tubes and the volume noted. The sodium salt of resazurin was obtained in dry form from Sigma Chemical Company (St. Louis, MO) and 50 mg diluted in 100 mL of normal saline. It was stored at room temperature. One drop (25 $\mu$g) of dye, which was dark blue in color, was added for each mL of semen. If the semen volume was one half or more between two whole numbers, the number of drops comparable with the higher number was added. For example, for 2.7 mL 3 drops were added. If the volume was less than half between two whole numbers, the lower number was used to determine the number of drops to be added. For example, for 2.4 mL 2 drops were added. Specimens of <1.0 mL were not used. The test tube was shaken to thoroughly mix the dye with the semen, and the test tube placed upright in an ordinary water glass that was $\frac{2}{3}$ to $\frac{3}{4}$ filled with hot tap water at 46° to 48°C. The temperature was monitored with a strip thermometer. The outside of the glass was covered with a thin insulating jacket of the type used to maintain the temperature in aluminum cans. The test tube remained undisturbed, partially floating in the water, with the upper portion resting on the upper edge of the glass for 1 hour, during which time the temperature gradually decreased to 32° to 34°C. At 1 hour, the test tube was shaken to facilitate mixing of any sedimented material, and the color in the test tube was matched with a color chart under an incandescent lamp. The color chart,* which was devised for this study, contained 11 colors ranging from dark purple to pink. Six colors ranging from pink to red-violet represented the positive or normal range, whereas five colors ranging from dark purple to dark burgundy represented the negative or abnormal range. A majority of specimens with counts $\geq 20 \times 10^6$ motile sperm/mL fell within the normal color range, whereas the majority of specimens with a count $< 20 \times 10^6$ motile sperm/mL produced colors in the abnormal range. Thus, $20 \times 10^6$ motile sperm/mL was used as the cutoff point between normal and abnormal semen specimens. No other concentration of motile sperm provided as good a delineation of color groups.

The $t$ statistic was used to test the hypothesis that the mean number of motile sperm per mL from the two groups (semen samples that tested positive or negative) were equal.[7]

## RESULTS

Initial color changes with the very best semen specimens occurred within 15 to 20 minutes. However, 1 hour of incubation was chosen for the end point because the false-negative rate was too high at 30 minutes. Conversely, waiting 2 hours to read the test produced an increase in false-positive results. In 28 of 41 cases in which specimens were read at both 1 and $1\frac{1}{2}$ hours, there were no color differences. In 11 cases, there was a minor change in color that did not change the categorization of the specimen. In one case, the longer incubation allowed a

* Copies of the color chart used in this article are available from the author upon request.

negative result with an abnormal ($<20 \times 10^6$ motile sperm/mL) specimen to become positive (false-positive). However, one normal ($\geq 20 \times 10^6$ motile sperm/mL) specimen that was negative at 1 hour was positive (true-positive) at $1\frac{1}{2}$ hours. With no clear-cut advantage for a longer incubation, the 1 hour end point was maintained.

During preliminary studies, starting temperatures between 43°C and 45°C produced an unacceptably high rate of false-negative results with normal specimens. Therefore, 46° to 48°C was chosen as the starting temperature for the standardized test. Higher starting temperatures were not investigated.

The sensitivity of the test vis-à-vis the motile sperm concentration was 86%, defining sensitivity as the percentage of specimens with counts $< 20 \times 10^6$ motile sperm/mL that produced a color in the abnormal range in the resazurin test. The specificity of the test was also 86%. This represents the percentage of specimens with $20 \times 10^6$ or greater motile sperm/mL that tested in the normal range of the resazurin test. The results for the individual sites are listed in Table 1.

There was a positive correlation between color and motile sperm density ($r = 0.71$, $P < 0.0001$). The mean motile sperm count/mL was 11.5 ± 1.1 (±SEM) million for specimens with a negative resazurin test compared with 62.2 ± 2.7 million for specimens with a positive resazurin test ($P < 0.0001$).

To evaluate the reproducibility of the test, eight semen specimens were divided into two aliquots, and an additional three specimens were divided into three aliquots. The aliquots from each sample were tested with different batches of dye. The aliquots from each sample tested consistently all positive or all negative.

**Table 1** Comparison of Resazurin Test Results With Concentration of Motile Sperm

| Motile sperm/mL | Positive test | Negative test |
|---|---|---|
| $\geq 20 \times 10^6$ | | |
| 1. Andrology (UCSF) | 24 | 5 |
| 2. IVF/Donor (UCSF) | 125 | 21 |
| 3. Lewiston, ME | 24 | 1 |
| 4. Santa Ana/San Diego, CA | 17 | 4 |
| All sites combined | 190 (86)[a] | 31 (14) |
| $<20 \times 10^6$ | | |
| 1. Andrology (UCSF) | 11 | 82 |
| 2. IVF/Donor (UCSF) | 7 | 49 |
| 3. Lewiston, ME | 4 | 2 |
| 4. Santa Ana/San Diego, CA | 0 | 7 |
| All sites combined | 22 (14) | 140 (86) |

[a] Values in parentheses are percents.

## DISCUSSION

Approximately 40% of infertility has been attributed to a male factor. However, precise categorization of the male undergoing an infertility investigation as potentially fertile or infertile is difficult because conception depends on both partners.[8] Moreover, the routine semen analysis has only limited prognostic value, and many untreated men labeled as oligospermic will achieve a pregnancy with their partners.[1,5]

In an attempt to improve the prognostic value of the sperm count and sperm motility, a few investigators[2,8,9] have combined the two values to obtain the number of motile sperm/mL. Hargreave and Elton[9] reported that males with <24 months of infertility and a motile sperm count of $>10 \times 10^6$/mL had a higher pregnancy rate (PR) than those with $2 \times 10^6$/mL to $10 \times 10^6$/mL motile sperm. The category $> 10 \times 10^6$/mL was not further subdivided. Smith et al.[2] found that even with motile sperm counts $< 5.1 \times 10^6$/mL the PR was 37.5%. However, with counts of $60.1 \times 10^6$/mL to $100 \times 10^6$/mL the PR rose to 78.6%. In a study of males whose partners were judged to be normal, those with motile sperm counts $< 20 \times 10^6$/mL had a 50% conception rate compared with the 90% found when the motile sperm count was $>60 \times 10^6$/mL.[8]

Albertsen et al.[10] found that 92% of specimens from fertile males were correctly identified when $15 \times 10^6$ motile sperm/mL was used as the lower limit of normal. In a study of the relationship of semen analysis to success with the gamete intrafallopian transfer procedure, it was shown that the clinical PR was 25.7% when the motile sperm count was $>40 \times 10^6$/mL, 13.4% when the motile sperm count was $10 \times 10^6$/mL to $40 \times 10^6$/mL, and 12.5% when the motile sperm count was $<10 \times 10^6$/mL.[11] In our study, a cutoff of $20 \times 10^6$ motile sperm/mL was used to distinguish normal from abnormal. This cutoff can be supported in general by the foremention articles. In addition, this point allowed a good division of the two groups of colors. Using $20 \times 10^6$ motile sperm/mL as the dividing point provided a sensitivity of 86% and specificity of 86%. Despite our categorization of normal and abnormal, a specimen with $<20 \times 10^6$ motile sperm/mL can be compatible with fertility and one with $>20 \times 10^6$ motile sperm/mL may be unable to accomplish conceptions. Thus, neither this value nor any other value of the semen analysis is a perfect prognosticator of fertility.

A few of the discrepancies (false-positive and false-negative tests) between the resazurin test and semen analysis could reflect the inaccuracies of the semen analysis. Jequier and Ukombe[12] asked 29 technicians to do a semen analysis on the same pooled semen specimen using a modified Neubauer counting chamber. The mean sperm concentration obtained was $46.7 \times 10^6$/mL, with a range of values lying between $10 \times 10^6$/mL and $98 \times 10^6$/mL and a coefficient of variation of 44.3%. Similarly, Freund and Carol[13] showed that as much as 20% intraoperator error can occur on the same specimen. Discrepancies in the sperm count with the Makler chamber have also been reported, depending to some extent on the volume used to fill the chamber.[14,15]

Resazurin is reduced by bacteria as well as by metabolically active sperm, and the dye has been used to estimate the bacterial content of food.[16] Thus if an abnormal semen specimen was contaminated by bacteria, a false-positive result might occur. Specimens that contained excess white blood cells (WBC) (>10 WBCs/high power field) were not included in this study. However, further evidence of bacterial contamination was not sought, and it is remotely possible that a few of the false-positive tests could have resulted from infection that was not heralded by an increase in WBCs.

The requirements for the resazurin test are simple, namely a test tube, a water glass with an insulating jacket, hot water, a thermometer, the color chart, and the dye. Because the test result is read with the naked eye, neither a microscope nor an instrument for reading color (spectrophotometer or luminometer) is necessary. Therefore, the test can provide an assessment of metabolically active sperm without the need for an equipped laboratory. Future tests based on monoclonal antibodies or deoxyribonucleic acid probes might measure only numbers of sperm without regard to motility, and this will not be clinically useful. Tests that measure semen adenosine triphosphate or sperm creatine kinase and that may differentiate fertile from subfertile specimens are dependent on more complex chemical reactions and fluorometric methods.[17,18] For the moment, this will limit their widespread use.

Whether the resazurin test assessment of actively metabolizing sperm provides prognostic information beyond that provided by the routine semen analysis was not investigated in the current study. Now that correlation of the test to sperm activity has been established, the relationship of the test to subsequent fertility and to other tests, such as the hamster penetration assay, is worthy of investigation.

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