Resazurin Reduction Assay for Ram Sperm Metabolic Activity Measured by Spectrophotometry (44223)

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Abstract. **Resazurin (7-hydroxy-3H-phenoxazin-3-one 10-oxide) is a redox dye that can be reduced by metabolically active spermatozoa to resorufin and manifested as visual color change from blue (resazurin) to pink (resorufin). This study combined the resazurin reduction test with spectrophotometric methods and investigated the correlation between metabolic activity and fertilization potential of spermatozoa, using ram semen as a model. The absorbance at specific wavelengths of resazurin and resorufin was determined by scanning photo spectrometer (600 nm for resazurin and 570 nm for resorufin, respectively). The absorbance at wavelengths of 600 nm (A600) and 570 nm (A570) was measured by spectrophotometry and used to evaluate sperm metabolic activity. A600 decreased and A570 increased in relationship to the increased concentrations of motile spermatozoa and increased resazurin reaction times. We observed, upon using a retrospective experimental design, that fertile rams had greater relative absorbance values than rams with lower fertility. Also, we observed a wide range variation of absorbance between the fertile rams, which is highly correlated to the sperm motility. We conclude that the spectrophotometric measurement of resazurin reduction for sperm activity might be a good assay for ram fertility.**

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Semen evaluation is indispensable in assessing male fertility. Various analytical techniques have been developed to evaluate sperm quality such as sperm concentration, motility, and morphology (1–4). However, there is no single semen assay that provides complete information for predicting fertility. Some alternative methods such as those used to evaluate metabolic status of spermatozoa have been developed (2, 5). Because the metabolism is a basic process of cellular function and biological activities, and because reproductive performance depends on metabolic processes, the assessment of metabolic rates of spermatozoa could provide pertinent information for predicting sperm fertilizing capacity (1, 2, 5).

One of the methods to evaluate metabolic status of spermatozoa is the resazurin test. Resazurin (7-hydroxy-3H-phenoxazin-3-one 10-oxide) is a non-nutrient, nontoxic redox dye that is used as an indicator of dehydrogenase activity (6, 7). When activated by metabolically viable cells such as spermatozoa, resazurin is first reduced to resorufin and then to dihydroresorufin (7, 8). The reduction reaction is manifested by visual changes in dye color from blue (resazurin) to pink (resorufin) to white (dihydroresorufin). Color changes correlate significantly with the concentration of motile spermatozoa (5, 7, 9). The resazurin dye assay of spermatozoa has been used to predict fertility in human males (7) and in the males of several species of domestic animals (6–8, 10, 11).

Most studies using the resazurin assay to assess semen quality have involved the unaided eye and therefore, have been quite subjective because the detection of color change varies between evaluators. We hypothesized that the rate and level of resazurin reduction can be measured with spectrophotometric methods and thereby used as a test to measure sperm metabolic activity quantitatively. The objective

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of this study combined the resazurin reduction test with spectrophotometric methods and investigated the correlation between metabolic activity and fertilization potential of spermatozoa, using ram semen as a model.

Materials and Methods

Semen Concentration. The concentration of motile ram spermatozoa was determined using a hemocytometer (Bright-Line, Cambridge Instruments Inc., Buffalo, NY). Part of a semen sample was diluted 400× with phosphate-buffered saline (PBS), pH 7.4. The number of nonmotile spermatozoa (X_n) in a sample was counted. Another part from the same semen sample was diluted 400× with water to kill all of the spermatozoa. The total number of spermatozoa (X_t) was then determined. The number of motile spermatozoa (X_m) was calculated by $X_m = X_t - X_n$. We did not assess morphologic characteristics.

Specific Absorbance Wavelengths of Resazurin and Resorufin. The resazurin test for visual color change followed the procedure of Dart *et al.* (6). Resazurin (R7017, Sigma Chemical Corp., St. Louis, MO) was added to a PBS-diluted ram semen sample (20×) at the final concentration of 5 μg/ml. The PBS (pH 7.4) containing the same concentration of resazurin was used as the control (blue color solution). Samples were then incubated at 37°C. When the semen sample had completely turned pink (approximately 20 min), it was centrifuged and the supernatant collected. The pink solution (resorufin) and the blue solutions (resazurin) were scanned, respectively, in a range from 500- to 650-nm wavelengths of visible light with the integration time of 1 sec, using a scanning spectrophotometer (HP 8452A Diode Array Spectrophotometer, Hewlett-Packard Co., Boise, ID) to find the peak absorbance wavelengths.

Experiment 1: Absorbance and Concentration of Spermatozoa. This experiment investigated the relationship of the absorbance with the concentration of motile spermatozoa. In order to have enough semen for the experimental measurements and average the variation between rams, semen samples were collected from four fertile rams using an artificial vagina and collections were pooled. A serial concentration of motile spermatozoa ranging from 0 to 240×10^6 motile spermatozoa/ml was prepared by stepwise dilution of the semen sample with PBS, with an increase of 12×10^6/dilution. Each concentration of semen was placed into a separate 15-ml centrifuge tube (FisherBrand, Fisher Scientific, Pittsburgh, PA). Resazurin was then added to each tube to a final concentration of 7 μg/ml. All tubes were incubated at 37°C in a water bath for 15 min. Two samples were taken from each tube (600 μl) for repeated measurements. The samples were centrifuged at 13,600*g* (Micro-centrifuge Model 235C, Fisher Scientific, Pittsburgh, PA) for 10 min and the supernatant collected. The supernatant was placed in a semi-micro polystyrene cuvette (Cat. No. 223-9955, Bio-Rad Laboratories, Hercules, CA). The absorbance of the solution at the wavelengths based upon our scanning results (peak absorbance from 500 to 650 nm as described above) was measured using a spectrophotometer (Spectronic 601, Milton Roy Company, Rochester, NY). The relationship of absorbance values with semen concentration was analyzed by simple linear correlation using the statistical program NCSS (12).

Experiment 2: Absorbance of Redox Reaction Over Time. This experiment investigated the relationship of the absorbance with the duration of reaction time. Semen was collected from fertile rams using an artificial vagina and pooled together (the rams were the same as used for semen collection in Experiments 1 and 3, but the time of collection was different). Pooled semen was diluted 20× with PBS, and resazurin was added to a final concentration of 7 μg/ml. The procedure was performed in 50-ml sterile centrifuge tubes (FisherBrand, Fisher Scientific, Pittsburgh, PA). The total volume of the reaction mixture was 45 ml. The tube was incubated at 37°C in a water bath and samples (600 μl) were taken from the reaction mixture at 2-min intervals for 36 min. Two samples were taken at each time to repeat measurements. The reaction mixture was shaken gently before each sampling. Each sample was rapidly cooled in an ice-water bath to stop the reaction. The sample was then centrifuged and measured for absorbance at specific wavelengths determined from scanning results as described above. The relationship of absorbance values with reduction time was analyzed by simple linear correlation using the statistical program NCSS (12).

Experiment 3: Absorbance with Sperm Concentration and Reduction Time. This experiment investigated the relationship of the absorbance with the concentration of motile spermatozoa and with the time of reduction reaction. The semen sample was the same as used in Experiment 1 (i.e., semen was collected from four fertile rams, and collections were pooled). Dilutions of motile spermatozoa from 0 to 240×10^6 motile spermatozoa/ml at increases of 12×10^6 cells/dilution were prepared by stepwise dilution as described in Experiment 1. Each dilution was placed into a 15-ml centrifuge tube, and resazurin was added to a concentration of 7 μg/ml to each reaction tube. All tubes were incubated at 37°C in a water bath. Samples from each tube were taken at 15, 30, and 60 min. Two samples were taken from each tube (600 μl) at each scheduled time for repeated measurements. The samples were centrifuged immediately to separate the spermatozoa. The supernatant was then measured for the specific absorbance at 570- and 600-nm wavelengths, respectively, determined from scanning results as described above.

Experiment 4: Spectrophotometric Prediction of Fertilizing Capacity. This experiment validated the resazurin reduction test measured by spectrophotometry for the prediction of ram fertilizing capacity using a retrospective study. Based on the results of previous experiments described above, absorbance data were standardized as relative absorbance values and used to measure sperm meta-

bolic activity. Relative absorbance (RA) at 570 and 600 nm was defined as $RA_{570} = (A_1 - A_0)/A_0$ and $RA_{600} = (A_0 - A_t)/A_0$, respectively, where A_0 was the specific absorbance at starting time 0, and A_t was the specific absorbance at specific sampling time. Semen samples were collected from 10 reproductively proven rams for which breeding history was available. Samples also were collected from a ram with epididymitis and a vasectomized ram. Each ram was collected three times, with semen collections separated by approximately 1 week. Rams were collected between December and March. Each sample was microscopically examined for sperm motility as described above. Semen samples from each ram were diluted 20× with PBS, and then each received a final concentration of 7 μg/ml resazurin dye. The mixtures were incubated at 37°C in a water bath. Samples (600 μl) from each mixture were taken after 30 min reaction and centrifuged immediately to separate the spermatozoa. The supernatant was then measured for specific absorbance at 570- and 600-nm wavelengths, respectively. The relative absorbance was used as an indicator of sperm activity. Two samples were taken from each tube (600 μl) for repeated measurements.

Results and Discussion

The resazurin dye solution (blue) had a peak absorbance wavelength at 600 nm. When reduced by metabolically active spermatozoa, another peak absorbance appeared at the wavelength of 570 nm (Fig. 1). The peak absorbance at these wavelengths indicates that the reduction reaction is detectable by spectrophotometry at specific wavelengths of light, which correspond to visible color changes of resazurin dye from blue (resazurin) to pink (resorufin) as reported by Glass *et al.* (7), Fuse *et al.* (13, 14) and Dart *et al.* (6).

Experiment 1. Absorbance at 570 nm and 600 nm was sperm concentration-dependent (Curve T15, Fig. 2; Curve T15, Fig. 3). There was a positive correlation between increased absorbance at 570 nm and concentration of motile spermatozoa ($r = 0.94$, $P < 0.001$), and a negative correlation between absorbance at 600 nm and increased concentration of motile spermatozoa ($r = -0.91$, $P < 0.001$). Studies using visual-eye analysis have shown that color changes in resazurin reduction correlates significantly with the concentration of motile spermatozoa (6, 7, 14). Our results demonstrate that this relationship is measurable quantitatively by spectrophotometry and although not tested in this study the spectrophotometric method will most likely reduce variability between evaluators.

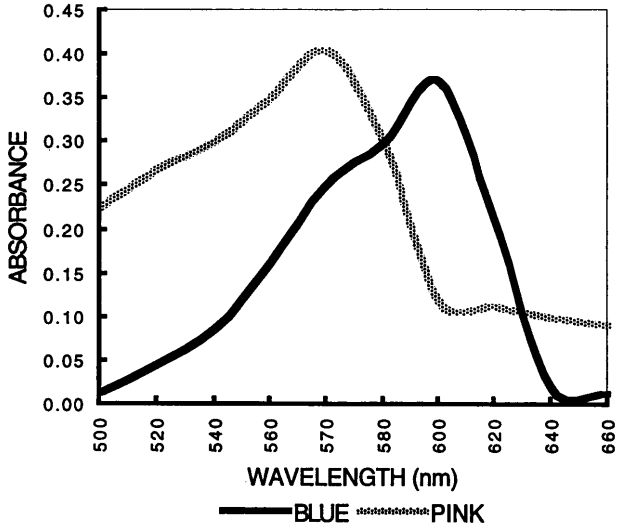

Figure 1. Specific absorbance wavelengths of resazurin solution before (BLUE) and after (PINK) reduction by active spermatozoa. The blue solution (resazurin) has a peak absorbance at 600 nm, and the pink solution (resorufin) has a peak absorbance at 570 nm.

Experiment 2. Absorbance at 570 nm and 600 nm was reduction time-dependent. There was a positive correlation between absorbance at 570 nm and duration of the resazurin reaction ($r = 0.92$, $P < 0.001$), and a negative correlation between absorbance at 600 nm and the reduction time ($r = -0.96$, $P < 0.001$).

Experiment 3. Absorbance at 500 and 600 nm was both reaction time- and sperm concentration–dependent. Figures 2 and 3 show that the absorbance rate increases along with the duration of reducing time as indicated by the shift of the curves to the left. As mentioned above, the resazurin dye reduction has been used to predict fertility in humans (7), cattle (6) and horses (10). These studies showed that the higher the concentration of motile sperm, the higher overall metabolic activity and the faster the color change. Based upon the fact that resazurin reaction depends on both reduction time and concentration of motile spermatozoa, the

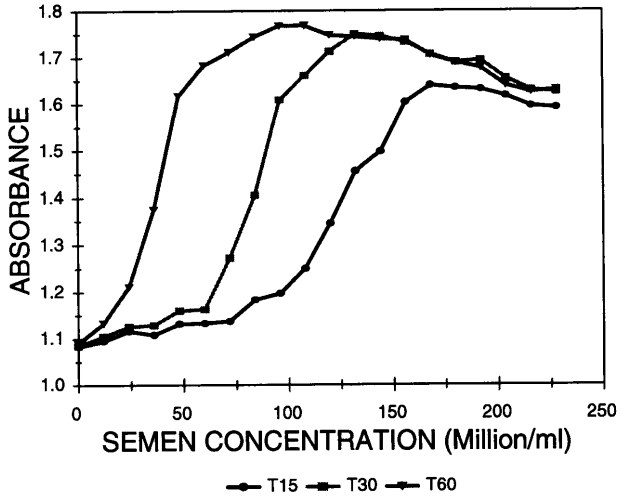

Figure 2. Curves showing absorbance at 570 nm is time- and concentration-dependent. Mixtures of resazurin reaction from each concentration of spermatozoa were sampled at 15 min (T15), 30 min (T30) and 60 min (T60) reduction, respectively. Absorbance at 570 nm of resazurin dye mixed (incremental increases of 12 million spermatozoa) with motile spermatozoa concentrations from 0 to 240 million spermatozoa/ml. Note that under a fixed reduction time (here, 15 min) the absorbance did not increase when the number of motile spermatozoa was less than 72 million spermatozoa/ml whereas the absorbance reached the maximum value when the number of motile spermatozoa was more than 168 million spermatozoa/ml. There was an approximately linear relationship between the absorbance and number of motile spermatozoa within this range of concentration.

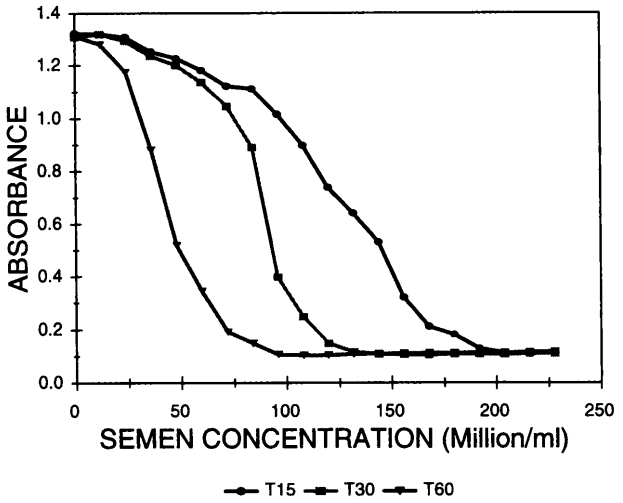

Figure 3. Curves showing absorbance at 600 nm is time- and concentration-dependent. Mixtures of resazurin reaction from each concentration of spermatozoa were sampled at 15 min (T15), 30 min (T30) and 60 min (T60) reduction, respectively. Absorbance at 600 nm of resazurin dye mixed (incremental increases of 12 million spermatozoa) with motile spermatozoa concentrations from 0 to 240 million spermatozoa/ml. Note that under a fixed reduction time (here, 15 min) the absorbance did not increase when the number of motile spermatozoa was less than 72 million spermatozoa/ml whereas the absorbance reached the maximum value when the number of motile spermatozoa was more than 168 million spermatozoa/ml. There was an approximately linear relationship between the absorbance and number of motile spermatozoa within this range of concentration.

reducing time can therefore be used as an indicator of semen fertility (9). The results of this study confirm this relationship in assessing fertility.

The sigmoid absorbance curves in Figures 2 and 3 indicate that the sensitivity and specificity of the resazurin test are affected by the number of motile sperm. Herein the sensitivity is defined as the ability to detect color change of the dye (absorbance differences at specific wavelengths) reduced by the lowest limit concentration of motile sperm in a sample, and the specificity is defined as the ability to differentiate the reduction of the dye in a sample with a highest limit concentration of motile sperm (6, 7, 10). Under a fixed reducing time (Curve T15, Fig. 2), for example, there was no significant absorbance when the concentration of motile sperm was lower than 72 $\times$ 10^6/ml. The absorbance was quantitatively indistinguishable when the sample concentration was higher than 168 $\times$ 10^6/ml. There was a linear relationship between the absorbance and number of motile spermatozoa within the range of 72 $\times$ 10^6/ml to 168 $\times$ 10^6/ml (Fig. 2). Beyond these concentrations, sensitivity decreased upon greater dilution, and specificity increased with less dilution. This rationale can be applied to the data in Figure 3, except absorbance values at 600 nm decrease with increasing reduction times and sperm concentration, rather than increase in absorbance at 570 nm as in Figure 2. Based upon these observations, semen dilutions of 20× were selected to optimize the sensitivity and specificity during resazurin analysis of ram semen samples.

The curves further show that absorbance increases over time with a constant concentration of motile spermatozoa (Fig. 2). Considering the fact that there is a strong correlation between absorbance and resazurin reduction time (Experiment 2), the reaction time must be controlled during a test. Glass *et al.* (7) reported that false-negative rates increase with short reaction (incubation) times whereas false-positive rates increased with long reaction times. In this study, the reaction time was set at 30 min for testing the metabolic activity of ram sperm so as to lessen the likelihood of false-negatives and false-positives.

Table I. Comparison of Relative Absorbance at 570 nm and 600 nm of Resazurin Reduction Test with Spermatozoa Concentration of Semen Collected from Different Rams

Ram ID number	Sperm concentration ($\times$ 10^9/ml)	Number of motile sperm ($\times$ 10^9/ml)	Sperm motility (%)	Relative absorbance at:	
				570 nm	600 nm
Fertile Rams					
N49	7.05	4.71	66.79	0.61	0.92
N48	6.52	3.82	57.62	0.59	0.92
U84	6.57	3.71	56.21	0.46	0.62
N75	6.39	3.41	54.03	0.67	0.91
U82	4.77	3.40	69.15	0.56	0.77
N42	4.21	2.88	64.27	0.47	0.67
U85	4.14	2.18	54.71	0.36	0.45
U87	2.66	2.06	76.58	0.41	0.51
U90	3.58	2.01	56.80	0.33	0.48
U88	2.33	1.70	72.28	0.43	0.64
Negative Control Rams					
D81	0.49	0.20	1.67	0.00	0.03
N51	0.00	0.00	0.00	0.00	0.00

Note. Semen samples were collected from 10 rams with known breeding history and proven fertility. Samples were also collected from one ram with epididymitis (D81) and one vasectomized teaser ram (N51) and used as negative controls. Each ram was collected three times, and the data listed in the table represent the means of the three collections.

Data from Experiment 4 (Table I) show that fertile rams have higher relative absorbance values. The relative absorbance at 570 nm of semen from fertile rams had values $\geq$ 0.33 and at 600 nm, had values $\geq$ 0.48. Rams with either a low number of motile sperm attributed to epididymitis (D81) or no sperm (vasectomized-N51) had nearly 0 absorbance values at both wavelengths, indicating the ability of this assay to distinguish between fertile and infertile rams.

The data in Table I also show a wide range in variation of absorbance between fertile rams. The relative absorbance at 570 nm, for example, was as high as 0.67 in some rams whereas it was only about 0.33 in other rams. The same was observed for the relative absorbance at 600 nm, which ranged from 0.48 to 0.92 in fertile rams. Semen samples in Experiment 4 were collected from reproductively proven rams. The variation in absorbance might represent the varying degrees of fertility between rams as detected by resazurin reduction. Furthermore, the results from Experiment 4 indicate that the relative absorbance values were significantly ($P < 0.01$) correlated with the number of motile sperm of fertile rams. The correlation efficiency (r) between absorbance and number of motile sperm from fertile rams was 0.79 at 570 nm and 0.81 at 600 nm. In conventional semen evaluation, the number of motile sperm is used as an indication of potential fertility, thus distinguishing varying degrees of fertility between males (6, 7). For example, in humans, a cutoff of 20×10^6 motile sperm/ml is used to distinguish normal from abnormal male fertility (7). In cattle, 100×10^6 motile sperm/ml is the cutoff for fertile and infertile bulls (6). Semen samples in Experiment 4 were collected from reproductively proven rams. Due to an insufficiency of direct breeding records, we cannot determine with any degree of accuracy the low, medium, and high fertility conditions between the rams. However, the number of motile spermatozoa from these rams obtained for conventional semen analysis may be viewed as a prognostic parameter to represent the varying fertilizing capacity between rams.

To determine the fertility status of male animals precisely is difficult because both male and female factors affect pregnancy rates (1–3, 4). Furthermore, there is no single assay that provides a complete picture of the sperm's ability to fertilize a mature ovum. The assay reported here is based only on the metabolic activity of spermatozoa and therefore is also subject to certain limitations in predicting potential fertility of spermatozoa. Different characteristics of spermatozoa that do not significantly affect metabolic activity but yet decrease fertility may be present in semen samples. For example, spermatozoa with morphological anomalies such as abnormal acrosome, may exhibit good metabolic activity. Under this condition, high metabolic rates cannot be correlated with fertility. However, good metabolic activity is necessity for fertilizing to occur. We suggest that during semen evaluation, metabolic activity be determined first and then other semen assessments of choice be performed so that the combination of semen assessing methods will override the limitation of any single assay.

Whether or not the resazurin test, which measures spermatozoa metabolic activity, provides better prognostic information over and above the more common methods of routine semen analysis was not investigated in this study. Some investigations have presented strong evidence to substantiate the use of the resazurin test for semen analysis (5, 14, 16). The reaction depends mainly on live sperm concentration rather than sperm motility and morphology (5, 16). The results from the resazurin test are reported to correlate better with male fertility than almost all sperm characteristics used in routine test protocols (1, 2, 5, 14). The resazurin test has been applied to predict the fertility of human (7) and several domestic animals (6, 8, 10, 11) and has proven its simplicity and accuracy. As an indicator of dehydrogenase activity with high sensitivity and reproducibility (6, 7), the resazurin test is a better metabolic assay than measuring ATP (1, 5). The resazurin test can also detect the oxidative stress of spermatozoa. Therefore, it might provide additional information beyond that of sperm concentration and motility (15). The results from this study show that the resazurin reduction reaction is measurable by spectrophotometric method and can be used as a practical method to assess quantitatively the metabolic activity of spermatozoa. This metabolic parameter provides useful information to evaluate the fertilizing capacity of ram semen.

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