

·Original Article·

Determination of the diagnostic value of the resazurin reduction assay for evaluating boar semen by receiver operating characteristic analysis

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Abstract

Aim: To assess that metabolic status of spermatozoa could provide a useful tool for evaluation of semen quality.

Methods: The accuracy of the spectrophotometric application of the resazurin reduction assay was assessed using receiver operating characteristic (ROC) analysis. **Results:** Areas under ROC curves (AUC) for motile sperm concentration and sperm index (SI) (sperm concentration multiplied by the square root of percentage sperm motility multiplied by the percentage normal sperm morphology) were 0.922. The best discrimination between poor and good semen samples according to the SI was achieved at a cut-off point of $A_{610} = 0.209$, where high sensitivity (94.1%) and specificity (91.7%) were calculated. The assay was less accurate when motile sperm concentration was used as the criterion value, yielding sensitivity of 88.2% and specificity of 87.5%, respectively. Likelihood ratios (LR) indicate that absorbances lower than 0.209 were at least 11.3 times as likely to be found in good semen samples than those in poor according to the SI, whereas in the case of motile sperm concentration, the LR was calculated to be 7.06. **Conclusion:** These results show that the resazurin reduction assay combined with spectrophotometry is an accurate method of assessing the quality of boar semen. (*Asian J Androl* 2006 May; 8: 343–348)

Keywords: resazurin reduction assay; semen; porcine; receiver operating characteristic analysis; cut-off point selection

1 Introduction

Various laboratory techniques are used to evaluate sperm quality, such as sperm concentration, motility, viability and morphology. However, there is no single semen assay that provides complete information on se-

men quality [1–3]. The resazurin reduction assay measures the metabolic activity of spermatozoa and could be used for evaluating semen quality. Resazurin, an indicator of dehydrogenase activity, is first reduced to resorufin and then to dihydroresorufin [4, 5]. The level of resazurin reduction can be measured with spectrophotometry and used as a test to quantitatively measure sperm metabolic activity. The assay has been applied to evaluate semen quality in humans [6–9], rams [10] and boars [11].

In the present study, receiver operating characteristics (ROC) were used to determine the optimal cut-off value and diagnostic accuracy of the resazurin reduction assay by using boar semen. A complete picture of test

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accuracy is presented by the ROC plot, which provides a view of the whole spectrum of sensitivities and specificities as functions of selected cut-off values [12]. A global summary statistic of the diagnostic accuracy of the assay is quantified by the areas under ROC curves (AUC). Likelihood ratios (LR) are used to revise the probability of the semen status in individual samples [13].

2 Materials and methods

2.1 Semen samples and analysis

Forty-one semen samples from eight 12–24-month-old boars of various breeds were included. Semen was collected with a glove hand using a clean semen collecting flask that filters out gel, dust and bristles, while the boar mounted a dummy sow. Semen was kept at the temperature collected and analyzed within 1 h. Sperm concentration and motility characteristics were determined by computer-assisted semen analysis (Hamilton Thorne IVOS 10.2; Hamilton Thorne Research, MA, USA) with a Makler counting chamber (Sefi Medical Instruments, Haifa, Israel). Sperm morphology was examined on Giemsa-stained samples [14]. Sperm index (SI) was calculated by multiplying sperm concentration by the square root of percentage sperm motility multiplied by the percentage of normal sperm morphology [6].

2.2 Resazurin reduction assay

The resazurin reduction assay was performed as previously described [11] within 1 h after semen collection. Briefly, 30 μ L of 1.8 mmol/L resazurin (Sigma, Steinheim, Germany) diluted in physiological saline was added to 3 mL of semen sample diluted 1:2 with Beltsville thawing solution semen extender (Beltsville Thawing Solution, Truadeco, the Netherlands) and incubated at 37°C in a water bath for 10 min. After incubation, two sub-samples of 1 mL were added to 1.5 mL of butanol (Merc, Germany). After rapid vortexing, samples were centrifuged at $3\,000 \times g$ for 10 min. Absorbance in the clear upper layer of butanol was measured at 610 nm (UV/VIS Spectrometer Lambda 12; Perkin Elmer Corp., Analytical Instruments, Norwalk, CT, USA). The within-run coefficient of variation, calculated as $(7.79 \pm 4.06)\%$, confirmed satisfactory repeatability of the assay [11].

2.3 Statistical analysis

The results of the resazurin reduction assay were correlated with motile sperm concentration and SI. Lin-

ear regression analysis was performed.

Semen samples were divided into satisfactory (SAT) and unsatisfactory (UNSAT) according to various criteria. Criteria considering the concentration of motile sperm included pre-selected minimums of 160, 200 and 240×10^6 sperm/mL. Criteria considering the concentration of motile, normal sperm (SI) included pre-selected minimums of 140, 180 and 220×10^6 sperm/mL.

ROC curves (Analyse-it, General + Clinical Laboratory statistics, version 1.71; Analyse-it Software Ltd., Leeds, UK) were applied to identify optimal test cut-off values. A positive test result (T+) was recorded when spermatozoa in a sample reduced resazurin from blue to pink, resulting in A_{610} below the cut-off value. A negative test result (T-) was recorded when spermatozoa in a sample did not reduce resazurin from blue to pink, resulting in A_{610} above the cut-off value. ROC curves plotted all sensitivity versus 1-specificity for the complete range of cut-off points [12, 15]. Sensitivity and specificity were estimated at 39 cut-off values. A diagonal line in a plot corresponds to a test that is positive or negative just by chance. Sensitivity (Se) and specificity (Sp) for each cut-off value were calculated as the proportion of positive test results (T+) for SAT samples and negative test results (T-) for UNSAT samples. All possible combinations of sensitivity and specificity that can be achieved by changing the test's cut-off value were summarized by a single parameter; that is, AUC [12]. The slope of the ROC curve represents the LR for a diagnostic test, and the tangent at a point on the ROC curve corresponds to the LR ($LR = Se/[1 - Sp]$) for a single test value represented by that point [16]. The optimal cut-off values were selected based on the best balance of sensitivity, specificity and Youden index ($J = Se + Sp - 1$) along with larger increases in LR for each criterion value [17].

3 Results

3.1 Relationship between the reduction of resazurin and concentration of motile sperm and SI

There were correlations ($P < 0.001$) between the reduction of resazurin and motile sperm concentration ($r = 0.81$) and SI ($r = 0.82$). Scatter-plots and linear regression equations are shown in Figures 1 and 2.

3.2 Selection of optimal cut-off values according to different criteria to define a satisfactory sample

The selection of cut-off values of absorbance at

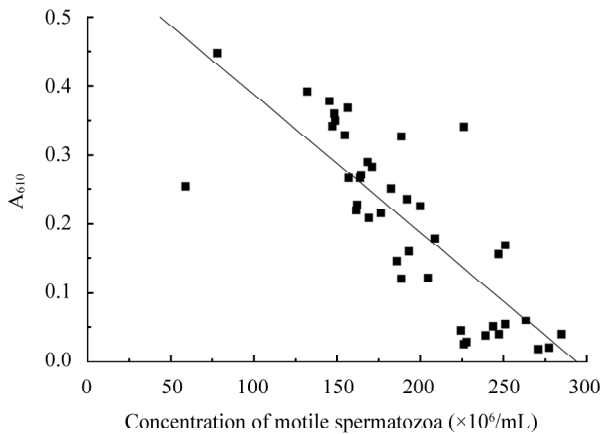


Figure 1. Relationship between the reduction of resazurin expressed as absorbance and the concentration of motile sperm. Regression equation: Concentration of motile sperm = $258.345 - 0.325 \times A_{610}$.

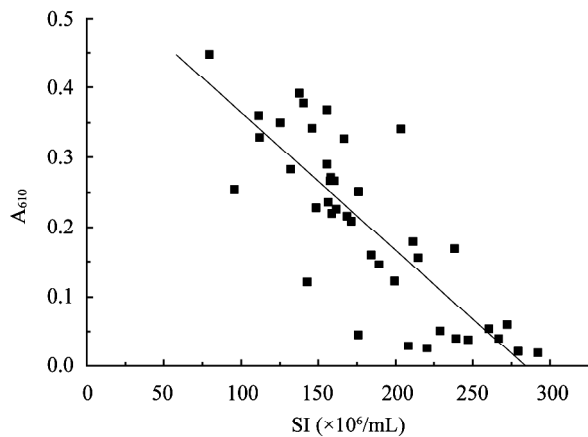


Figure 2. Relationship between the reduction of resazurin expressed as absorbance and sperm index (SI). Regression equation: $SI = 250.546 - 0.339 \times A_{610}$.

610 nm according to different criteria for motile sperm concentration and SI are presented in Figures 3 and 4, respectively. Values of Youden index peaked at a cut-off point of A_{610} at 0.209 for pre-selected minimum concentration of motile sperm concentration of 200×10^6 sperm/mL (Figure 3B) and SI of 180×10^6 sperm/mL (Figure 4B). This cut-off value yielded estimates of sensitivity of 88.2% and 94.1% with corresponding specificities of 87.5% and 91.7% for motile sperm concentration and SI, respectively. For both criteria, the test is 100% sensitive at A_{610} of 0.342. A cut-off value at A_{610} of 0.121 gives 100% specificity for motile sperm concentration and 95.8% specificity for SI. For pre-selected minimum concentration of motile sperm concentration of

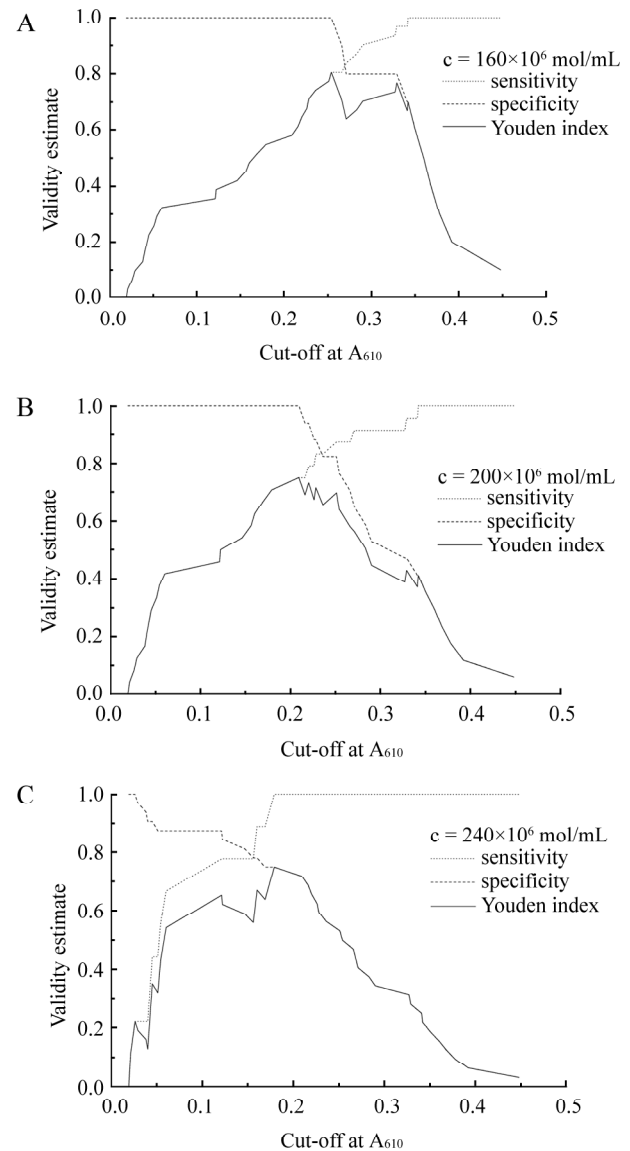


Figure 3. Plot of the diagnostic specificity, sensitivity and Youden index of resazurin reduction assay according to motile sperm concentration (C) as a function of the cut-off value at A_{610} nm.

160×10^6 sperm/mL and SI of 140×10^6 sperm/mL, 100% specificity was obtained at the optimal cut-off value of A_{610} at 0.254, whereas only moderate levels of sensitivity were observed (80.6% and 73.5%, respectively; Figures 3A and 4A). In contrast, at the highest criteria values 100% sensitivity corresponded to only moderate levels of specificity (Figures 3C and 4C).

3.3 ROC plots

The AUC was the same for criteria of 200×10^6 motile

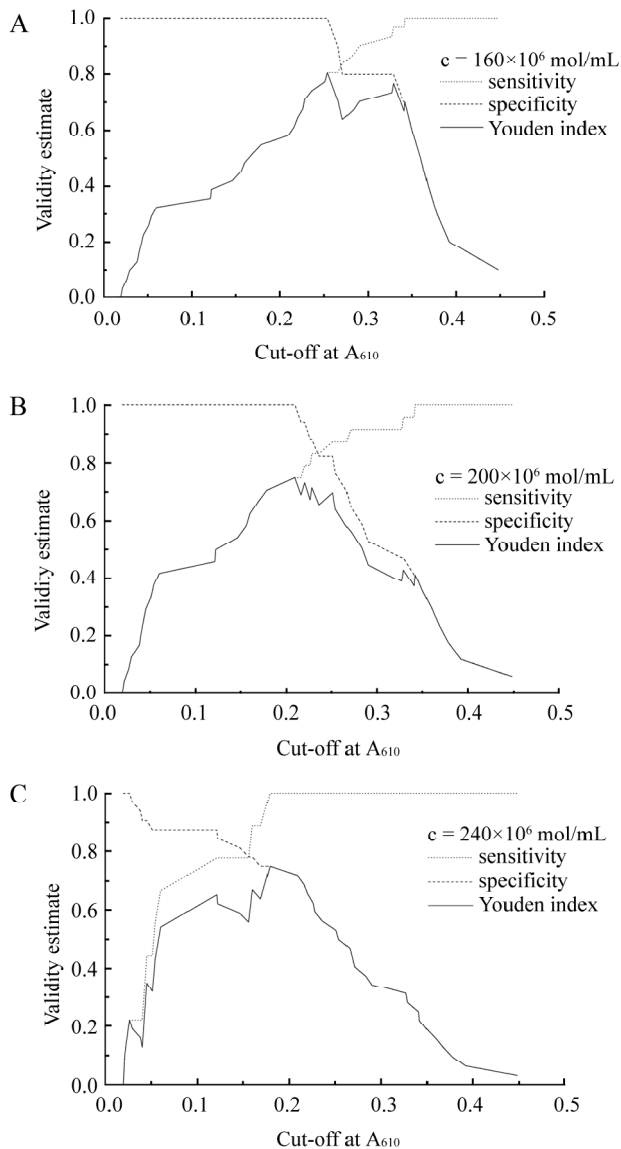


Figure 4. Plot of the diagnostic specificity, sensitivity and Youden index of resazurin reduction assay according to sperm index (SI) as a function of the cut-off value at A_{610} nm.

sperm/mL and 180×10^6 motile, normal sperm/mL (AUC = 0.92; standard error for ROC curve [SE] = 0.047 and 0.048, respectively; $P < 0.0001$; Figure 5), and the LR were 7.1 and 11.3, respectively.

4 Discussion

It has been reported that resazurin reduction correlates significantly with concentration of motile spermatozoa in human and ovine semen samples [5,18]. Moreover,

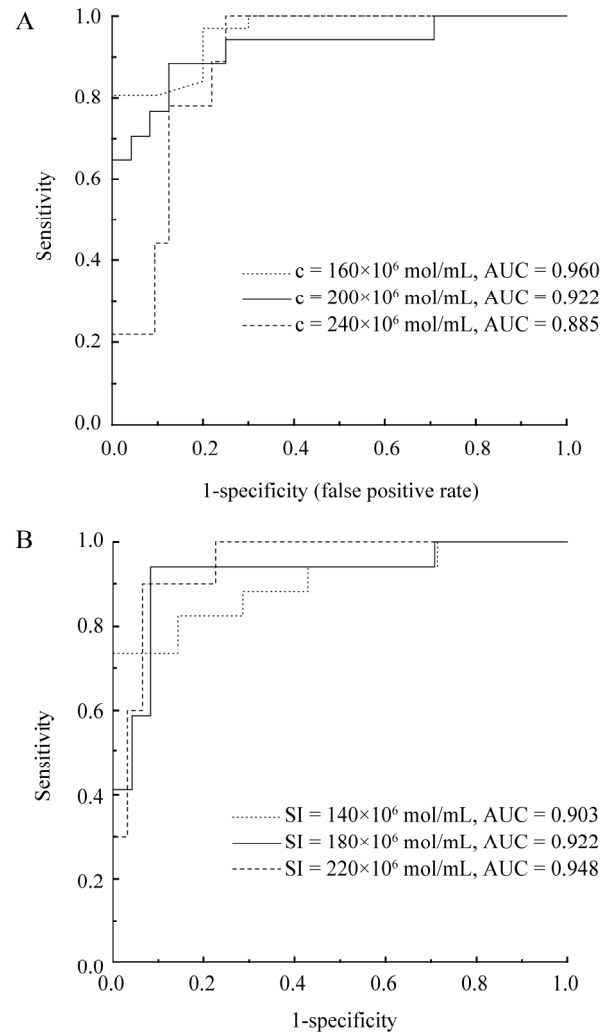


Figure 5. Receiver operating characteristics (ROC) plots of resazurin reduction assay for identifying semen samples with pre-selected minimum concentration of motile sperm concentration (C; A) and motile and normal sperm (sperm index [SI]; B).

because of the correlations of the extent of resazurin reduction with various sperm characteristics it was found that a resazurin test could also detect the oxidative stress of spermatozoa and enzymatic function of the prostate [8].

The use of the resazurin reduction test for semen has been described. We evaluated the resazurin reduction assay for boar semen according to motile sperm concentration and concentration of motile and normal sperm (SI). Combining concentration, motility and morphology in SI allows the concentration of functional spermatozoa to be determined, which might provide a better parameter for

evaluating semen quality than assessing the mentioned characteristics independently. In our quantitative test, the maximum overall accuracy of 92.9% confirmed the high discrimination power for boar semen according to a criterion value of SI at 180×10^6 sperm/mL. Our results also indicate the maximum sensitivity and specificity at different cut-off points.

The performance of diagnostic tests is usually described in terms of sensitivity and specificity [19]. ROC curves constructed on the basis of a few sensitivity/specificity pairs provide only a partial assessment of a test's performance. However, a complete ROC analysis, including AUC based on a plot of all sensitivity versus 1-specificity for the complete range of cut-off points, provides an index of accuracy by demonstrating the limits of a test's ability to discriminate between different semen status values [20].

The diagnostic potential of resazurin reduction assay according to motile sperm concentration and SI was not different on the basis of AUC. On the basis of LR, absorbance lower than or equal to the optimal cut-off point were 11.3 and 7.1 times as likely to be found in positive as in negative semen samples according to SI and motile sperm concentration, respectively.

A plot of sensitivity, specificity and Youden index as a function of the cut-off value provides a useful visualisation [12] and is helpful in selecting optimal cut-off values of the assay. The optimal cut-off value at A_{610} of 0.209 provided the best discrimination power according to both motile sperm concentration and SI. At this point, maximum overall accuracy was achieved for both cases. However, in clinical use of the test, it is often important to 100% correctly identify satisfactory or unsatisfactory samples. Therefore, a cut-off value of A_{610} at 0.342 was selected to enable 100% correct identification of unsatisfactory semen samples. In contrast, semen samples with A_{610} below 0.121 in the resazurin reduction assay were 100% and 95.8% correctly identified as satisfactory according to the criteria of 200×10^6 motile sperm/mL or 180×10^6 motile, normal sperm/mL, respectively.

A high degree of agreement is obtained between the results of the resazurin reduction assay and SI. Because reproductive performance depends on metabolic processes, the assessment of metabolic rates of spermatozoa could provide even better or more complete information about semen quality than other tests. A more valid approach to determining which is superior is to assess the accuracy of both methods against the "true"

value. Expressing the latter in semen evaluation is complex, although fertility results from insemination with evaluated semen could provide a gold standard of fertilizing capacity. Additional research is required for relevant and valid information about replacing or updating the methodology of semen evaluation.

The resazurin reduction assay was shown to be a reliable, easy-to-perform test that requires no sophisticated equipment. It was demonstrated that the results of the assay can be used to select semen samples with minimum requirements of sperm concentration, motility and normal morphology. The major potential application of the test is as an indicator of semen dose to be used for insemination.

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