Enhanced Chemiluminescence Assay vs Colorimetric Assay for Measurement of the Total Antioxidant Capacity of Human Seminal Plasma

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ABSTRACT: Although the enhanced chemiluminescence assay is commonly used to measure the nonenzymatic total antioxidant capacity (TAC) of the human seminal plasma, it is cumbersome, expensive, and time-consuming. We describe herein an alternate method to measure TAC that is based on the ability of antioxidants in seminal plasma to interfere with a reaction between 2,2′-azino-di-[3-ethylbenzthiazoline sulphonate] and metmyoglobin with H_2O_2. This reaction produces a relatively stable blue-green color with absorbance maxima at 600 nm. We compared this colorimetric assay with our established chemiluminescence method and assessed quality control parameters (ie, intra-assay and interassay variabilities) in addition to intraobserver and interobserver differences. Our results show that the colorimetric assay was fairly predictive of an-

tioxidant capacity similar to the chemiluminescence assay ($P <$ .001). Furthermore, there was a high level of agreement between the duplicate measures by the same observer (intraobserver) and intra-assay variability, with a concordance correlation coefficient of 0.99. The interassay coefficient of variation was 4.7% (overall). The mean $\pm$ SD of the difference between the 2 observers was 2.98% $\pm$ 4.1%. In conclusion, we found that the colorimetric assay is a reliable and accurate method to evaluate seminal TAC, and it could be used as a simpler, rapid, and cheaper alternative to the chemiluminescence assay.

Key words: Luminometer, semen, spectrophotometer, Trolox, reactive oxygen species.

J Androl 2003;24:676–680

Antioxidants present in the seminal plasma are the most important form of protection available to spermatozoa against reactive oxygen species (ROS) (Sikka et al, 1995; Sharma and Agarwal, 1996; Aitken, 1999). They provide defense mechanisms through 3 levels of protection: prevention, interception, and repair (Sies, 1993). A growing body of evidence suggests that low seminal total antioxidant capacity (TAC) is related to male infertility (Smith et al, 1996; Lewis et al, 1997; Sharma et al, 1999; Sikka, 2001). Thus, it is important to ensure that any measurement of seminal TAC is accurate and reliable and yet easy to use as a diagnostic tool in the evaluation and follow-up of male infertility.

Several methods have been developed to measure TAC in biological fluids, such as the oxygen radical absorbance

capacity (Cao and Prior, 1998), the ferric reducing ability (Benzie and Strain, 1996), and the phycoerythrin fluorescence-based assay (Glazer, 1990). The enhanced chemiluminescence assay, however, is the most commonly used method for measuring TAC in seminal fluid (Lewis et al, 1995; Kolettis et al, 1999; Sharma et al, 1999; Kobayashi et al, 2001; Pasqualotto et al, 2001; Sharma et al, 2001). The principle behind the enhanced chemiluminescent assay for TAC measurement is best described in the work by Whitehead et al (1992).

To perform the enhanced chemiluminescence assay, a signal reagent (luminol plus para-iodophenol), which is a source of chemiluminescence, is mixed with horseradish peroxidase (HRP)–linked immunoglobulin to produce ROS, which in turn is mixed with a substrate, hydrogen peroxide (H_2O_2). The power of the antioxidants in the seminal plasma to reduce the chemiluminescence of the signal reagent is compared with that of Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a water-soluble tocopherol analogue, and is measured as molar Trolox equivalents. Although accurate, this method is cumbersome and time-consuming, because fresh signaling reagent solution must be prepared each time the assay is performed and then standardized with Trolox.

Moreover, the signal reagent may reduce in intensity, adding another technical problem. Finally, expensive instrumentation (eg, luminometer) is needed to measure the chemiluminescence, which means that this assay is often not readily available in a physician's office.

Miller et al (1993) described another technique for TAC measurement based on colorimetry. This assay is based on the principle that when 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) is incubated with a peroxidase (such as metmyoglobin) and H_2O_2, a relatively stable radical cation, $ABTS^+$, is formed (see equation below). The formation of $ABTS^+$ on interaction with ferryl myoglobin produces a relatively stable blue-green color, measured at 600 nm. Antioxidants in the fluid sample suppress this color production to a degree that is proportional to their concentrations.

$$HX–Fe^{III} + H_2O_2 \rightarrow X - [Fe^{IV} = 0] + H_2O$$

$$ABTS + X - [Fe^{IV} = 0] \rightarrow ABTS + HX - Fe^{III}$$
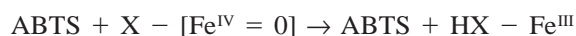
In this equation, $HX–Fe^{III}$ = metmyoglobin, $X - [Fe^{IV} = 0]$ = ferrylmyoglobin, ABTS = 2,2′-azino-di-[3-ethyl-bensthiazoline sulphonate].

The main objective of our study was to determine whether the colorimetric method could be used in place of the enhanced chemiluminescence assay for measuring TAC in seminal fluid. We compared the values obtained with both assays and assessed the quality control parameters of the colorimetric method (ie, intra-assay and interassay variability in addition to intraobserver and interobserver differences).

Materials and Methods

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

Sample Collection and Preparation

The study consisted of 17 semen samples collected from normal healthy donors. All the samples had normal semen parameters, ie, sperm concentration of more than 20 million/mL, motility of more than 50%, and more than 30% normal sperm morphology without any indication of leukocytospermia (white blood cell count of $<1 \times 10^6$/mL) according to World Health Organization (1999) guidelines. The samples were collected by masturbation after a period of sexual abstinence of 48 to 72 hours. After liquefaction, the raw semen specimens were centrifuged at $300 \times g$ for 7 minutes. The supernatant was aspirated and recentrifuged at $300 \times g$ for 10 minutes. The seminal plasma was frozen at $-70°C$ until further use.

The frozen seminal plasma was thawed by placing the vials in an incubator at 37°C for 20 minutes and immediately assessed for its antioxidant capacity. To assess the relationship between the chemiluminescence and colorimetric assay, specimens from
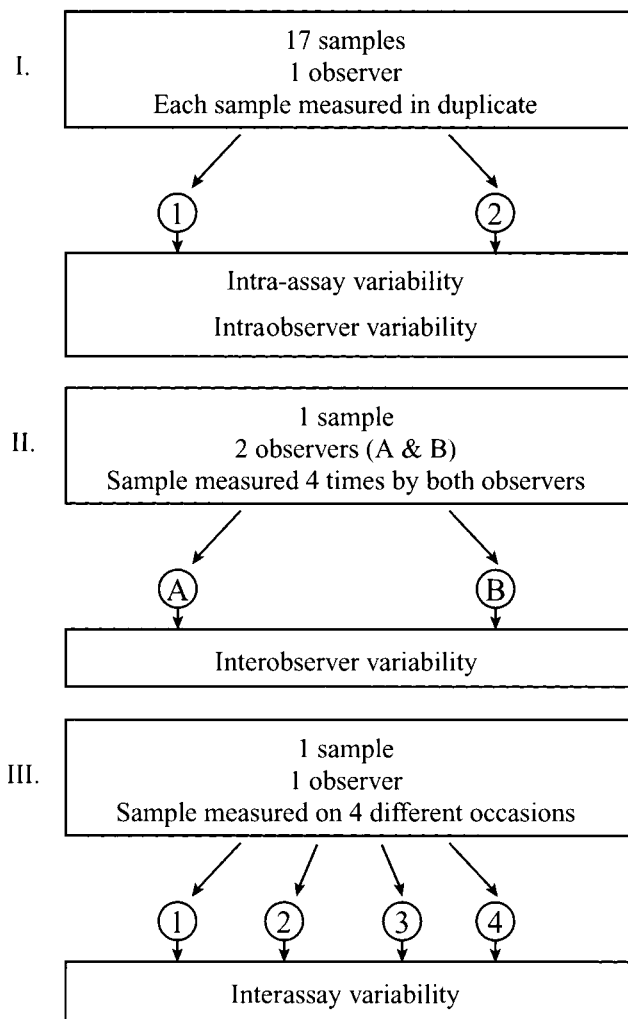

Figure 1. Experimental design for quality control of the new colorimetric method: **I)** intra-assay variability/intraobserver variability, **II)** interobserver variability, and **III)** interassay variability.

13 samples (of the total 17) were each divided in 2 aliquots. In aliquot 1, TAC was measured with the enhanced chemiluminescence assay (Saleh and Agarwal, 2002). In aliquot 2, TAC was measured with the colorimetric method using the Randox total antioxidant status kit (Randox Laboratories Ltd, San Francisco, Calif).

To establish the quality control parameters, the colorimetric assay was performed in all 17 samples in duplicate by the same observer (Figure 1, step I). The difference in duplicate measurements was used to calculate the intra-assay and the intraobserver variability. Subsequently, 16 aliquots of the seminal plasma from the same sample were assessed for TAC by 2 observers (4 times by each observer; 2 aliquots were used for each run of the spectrophotometer) (Figure 1, step II). The average of the duplicate readings was calculated, and the difference in the 4 averages for each observer was used to calculate the interobserver variability. The variability of the 4 averages for each observer was used to assess the interassay variability (Figure 1, step III).

Enhanced Chemiluminescence Method for TAC Measurement of the Seminal Plasma

Seminal plasma from aliquot 1 was diluted 1:10 with deionized water (dH$_2$O) and filtered through a 0.20-μm millipore filter (Allegiance Healthcare Corporation, McGraw Park, Ill). Signal reagent was prepared by adding 30 μL of H$_2$O$_2$ (8.8 mol/L), 110 μL of luminol stock solution (3.1 mM), and 10 μL of para-iodophenol stock solution (41.72 μM) to 10 mL of Tris buffer (0.1 M, pH 7.0) (Saleh and Agarwal, 2002). The HRP working solution was prepared from HRP stock solution by making a dilution of 1:1 with dH$_2$O to give the desired luminescence output (3 $\times$ 10^7 cpm).

Trolox (6-hydroxyl-2,5,7,8-tetramethylchroman-2-carboxylic acid), a water-soluble tocopherol analogue, was added as the standard at concentrations between 12.5 and 75 μM for TAC calibration. The antioxidant capacity of the seminal plasma was expressed in molar Trolox equivalents.

With the luminometer set in the kinetic mode, 100 μL of signal reagent and 100 μL of HRP working solution were added to 700 μL of dH$_2$O and mixed. The solution was equilibrated to the desired level of chemiluminescent output (between 2 and 3 $\times$ 10^7 cpm) for 100 seconds. One hundred microliters of the seminal plasma was immediately added to the signal reagent and HRP, and the chemiluminescence was measured. Suppression of luminescence and the time from the addition of seminal plasma to 10% recovery of the initial chemiluminescence were recorded. The time of recovery was recorded and compared with the standard. The total antioxidant capacity was expressed as micromolar (μM) of Trolox equivalents.

Colorimetric Assay for TAC Measurement of the Seminal Plasma

Seminal plasma from aliquot 2 was used to measure TAC. Twenty microliters of seminal plasma was added to 1 mL of the reconstituted chromogen, ABTS-metmyoglobin (10-mL vial with 10 mL of phosphate-buffered saline buffer). Twenty microliters of Trolox (6-hydroxyl-2,5,7,8-tetramethylchroman-2-carboxylic acid) at a concentration of 1.73 mmol/L was used as the standard, whereas 20 μL of deionized water was used as a blank. One milliliter of chromogen was added to the standard, blank, and sample. With spectrophotometer adjusted at a wavelength of 600 nm and with the temperature at 37°C, the initial absorbance (A$_1$) was measured. Two hundred microliters of H$_2$O$_2$ (250 μmol/L) was then added to all tubes (standard, blank, and sample), and absorbance (A$_2$) was measured exactly after 3 minutes. The difference between A$_2$ and A$_1$ (ΔA) was calculated. The TAC of the sample, in terms of Trolox equivalents, was then calculated by the following formula: TAC = Concentration of the Standard $\times$ (ΔA Blank $-$ ΔA Sample)/(ΔA Blank $-$ ΔA Standard). The results were expressed as micromolar (μM) of Trolox equivalents.

Statistical Analysis

Linear regression was used to assess the linear relationship between the colorimetric assay and the chemiluminescence assay. Intraobserver (replicates of the same sample by the same observer at the same point of time) and intra-assay variability (replicates of the same sample measured at the same point of time)
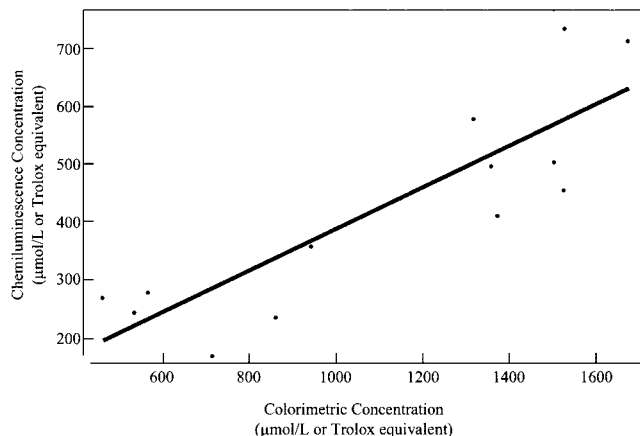

Figure 2. Linear regression analysis to evaluate the relationship between the enhanced chemiluminescence assay and the colorimetric assay. The results are expressed in molar Trolox equivalents (μM).

in the colorimetric measurements were calculated using Lin's concordance correlation coefficient and the corresponding 95% confidence interval (CI). Individual differences (bias) are reported in the form of the mean ($\pm$SD) and 95% CI.

Interassay Variability (Same Sample Observed at Different Times by the Same Observers)—Pairs of colorimetric measures within observer and experiment (8 averages with 4 per observer) were averaged, and the coefficient of variation [(SD/Mean) $\times$ 100] was calculated overall and for each observer.

Interobserver Variability (Multiple Observers on the Same Day With the Same Sample)—We first averaged pairs of colorimetric measures within observer and experiment. The mean difference in measures between observers across the 4 experiments was reported along with its mean ($\pm$SD) and 95% CI.

Two-tailed tests were performed with a significance level of .05 for each hypothesis. Data were analyzed using the SAS statistical software package (version 8.2, SAS Institute Inc, Cary, NC).

Results

The values (mean $\pm$ SD) for the sperm parameters in our study population were 77.89 $\pm$ 65.43 $\times$ 10^6/mL for sperm concentration, 55.18% $\pm$ 23.47% for motility, and 19.11% $\pm$ 9.84% for normal morphology. The colorimetric assay was fairly predictive of the chemiluminescence assay ($P <$ .001). The equation representative of this relationship is Y (chemiluminescence) = 28.48 + 0.36 $\times$ X (colorimetric) (Figure 2). In this equation, 28.84 is the estimated intercept or the value of Y when X = 0; 0.36 is the estimated change in Y for a change of 1.0 in X. The correlation coefficient (R^2 value for the model) is 0.75, with square of the Pearson correlation estimate of 0.87. This indicates that the colorimetric assay explains an estimated 75% of the variability in the chemiluminescence assay. An R^2 of 1.0 would result in perfect prediction, with all of the data sitting on the regression line.

The minimal detection value as observed from our regression line for the assay (Figure 2) was between 50 and 100 μM of Trolox equivalents.

Intraobserver and Intra-assay Variability of the Colorimetric Assay

There was a high degree of agreement between measures by the same observer, with a concordance correlation coefficient of 0.992 and corresponding 95% CI of 0.978 to 0.997. The mean (±SD) and 95% CI of the percent differences (first observation − second observation/first observation) × 100 were −3.2 (±3.6) and −5.1 to −1.3, respectively. The variability within rater on the same experiment was very small relative to the between-experiment variability.

Interassay Variability of the Colorimetric Assay

The coefficient of variation was 4.7% overall, 4.4% for observer A, and 5.5% for observer B. This was calculated on the average for each experiment within rater, so the sample sizes are 8 overall and 4 for each rater.

Interobserver Variability of the Colorimetric Assay

The mean (±SD) and 95% CI of the 4 differences between observers were 27.6 (±38.5) and −10.1 to 65.3, respectively. Again, the 2 measures at each time point were averaged first. The mean (±SD) percent difference [{(Observer A − Observer B)/Observer B} × 100] was 2.98% (±4.1%), with a 95% CI of −3.6 to 9.5.

Discussion

Seminal plasma is well endowed with an array of antioxidants that act as free radical scavengers to protect spermatozoa against oxidative stress (Armstrong et al, 1998; Aitken 1999). This defense mechanism compensates for the loss of sperm cytoplasmic enzymes that occurs when the cytoplasm is extruded during maturation, which in turn, diminishes endogenous repair mechanisms and enzymatic defenses (Aitken, 1994; Donnelly et al, 1999). Seminal plasma contains a number of enzymatic antioxidants such as superoxide dismutase, catalase (Jeulin et al, 1989; Zini et al, 1993) and glutathione peroxidase (Alvarez and Storey, 1989). In addition, it contains a variety of nonenzymatic antioxidants such as ascorbate, urate, α-tocopherol, pyruvate, glutathione, taurine, and hypotaurine (Saleh and Agarwal, 2002).

The measurement of total antioxidant capacity by enhanced chemiluminescent method generally takes approximately 40 to 45 minutes; it requires stringent assay conditions as opposed to 10 to 15 minutes for simple colorimetric approach. Since it is convenient to implement, the colorimetric assay has been widely used to assess TAC

in human serum (Digiesi et al, 1997; Boisseau-Garsaud et al, 2002) and as a reference benchmark for other assays (Korasevic et al, 2001). Although another assay for measuring TAC—the oxygen radical absorbance capacity—has a significantly less reagent cost, it requires a fluorescence detector and takes more than 70 minutes longer to complete than the colorimetric assay (Cao and Prior, 1998). Although the cost of reagents for the enhanced chemiluminescence assay (buffers, signal reagent, peroxidase reagent) is comparable to the cost of a colorimetric assay kit, the price of an average luminometer with kinetic setting averages $30 000 compared with $6000 for a simple spectrophotometer.

The main objective of this study was to determine whether the colorimetry method could be used instead of the enhanced chemiluminescence assay—a common method for measuring TAC in seminal fluid. We found a strong correlation among the values obtained by both the colorimetric and chemiluminescence assays. The average difference of 27.6 μM of Trolox equivalents between observers was low compared with the actual values (range, 400 to 1600 μM Trolox equivalents). The more relevant difference of 2.98% between raters in our study was very small, with a 95% CI of −3.6% to 9.5%. In addition, the small intra-assay, interassay, and interobserver variability supports the reliability of this assay. The estimated interobserver variability, for example, was on average 3% (95% CI, −3.6% to 9.5%). The sensitivity of the colorimetric method in our hands was between 50 and 100 μM of Trolox equivalents (Figure 2).

Another important application of our findings would be the measurement of the normal values of TAC in human seminal plasma. There is a lack of consensus in the literature regarding these values despite the strong correlation between low levels of seminal TAC and male infertility. Therefore, the standardization of this simple and inexpensive assay should be considered the first step to reach such a consensus.

One limitation of our study is the small sample size for the comparison of 2 methods. However, our purpose was to identify, develop, and improve an assay that ultimately can be used as a clinician-office laboratory procedure. We have described our preliminary results in this manuscript with such an objective.

In conclusion, the colorimetric assay is a simple, rapid, relatively inexpensive, and reliable method for measuring seminal TAC. It is less expensive and less time-consuming than the traditional enhanced chemiluminescence assay. We therefore recommend this method to be used as an in-office test for the estimation of the seminal TAC in the evaluation of patients with male infertility.

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