

Diagnostic value of the total antioxidant capacity (TAC) in human seminal plasma

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Objective: To establish cutoff value, sensitivity, specificity and intra- and interobserver variability of total antioxidant capacity (TAC) in seminal plasma from healthy donors (controls) and infertile patients.

Design: Seminal plasma from proven fertile donors (n = 55), nonproven fertile donors (n = 45), and infertile patients (n = 42) were examined for TAC level.

Setting: Reproductive research center in a tertiary care hospital.

Patient(s): Infertile patients from male infertility clinic of various diagnoses.

Intervention(s): Seminal plasma TAC measurement by a colorimetric assay using the TAC assay kit, receiver operating characteristics curve.

Main Outcome Measure(s): Seminal plasma TAC levels, cutoff value, sensitivity, and specificity.

Result(s): Proven fertile donors showed higher TAC values (median and range): 1700 (1440–2290 μM); compared with the infertile patients: 1310 (1040–1600 μM). The best cutoff to distinguish between fertile controls and infertile men was 1420 μM . At this threshold, specificity was 64% and sensitivity 76%.

Conclusion(s): Total antioxidant capacity of the seminal plasma as measured by the colorimetric assay is a reliable and simple test for the diagnosis and management of male infertility. (Fertil Steril® 2009;91:805–11. ©2009 by American Society for Reproductive Medicine.)

Key Words: Seminal plasma, male infertility, oxidative stress, receiver operating characteristic curve, total antioxidant capacity

Male factor infertility accounts for 30% to 50% of the total infertile couples seeking for infertility management (1). Among the couples seen for infertility, the most common cause of male infertility is defective spermatozoal function. It may result from testicular pathologies, genetic disorders, and exposure to drugs, toxins, or irradiations, or because of oxidative stress damage (2, 3). The mechanism of action for loss of sperm function may be because of elevated levels of reactive oxygen species (ROS) beyond the available total antioxidant capacity in the semen (3–9).

Seminal plasma has a very effective antioxidant systems that can provide the spermatozoa with a protective environment against oxidative stress (10). This protection compensates for the loss of cytoplasmic sperm enzymes that occurs during maturation and transportation processes, which in turn, diminishes the spermatozoa's endogenous enzymatic and repair defenses (7, 8, 11–15). Indeed, the total antioxidant capacity of seminal plasma is because of the sum of enzymatic (e.g., superoxide dismutase, catalase, and glutathione peroxidase) and nonenzymatic (e.g., ascorbate, urate, vi-

tamin E, pyruvate, glutathione, taurine, and hypotaurine) antioxidants (7, 16–21).

Low level of seminal total antioxidant capacity (TAC) has a key role in male infertility (8, 22, 23). 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. Total antioxidant capacity levels have been measured by the enhanced chemiluminescence method earlier in patients with clinical diagnosis such as varicocele, varicocele with infection, vasectomy reversal, as well as idiopathic infertility (9, 24, 25). In addition, using the ROS and TAC results, a novel score called the ROS-TAC score was also described earlier by us in subsets of patients with various clinical diagnosis (1, 16, 25–27). This score was described as a better predictor of oxidative stress in patients with various clinical diagnosis compared with ROS or the TAC values alone. Seminal TAC can be measured as the total available antioxidant protection in the seminal plasma, which is more practical and easy to perform by an enhanced chemiluminescence or the colorimetric assay. In contrast, measuring specific antioxidant assays are expensive and cumbersome in performance (high-pressure liquid chromatography, and so forth) and provide limited information about the assessed antioxidants. Earlier we compared the enhanced chemiluminescence and colorimetric method to assess TAC. We reported that the colorimetric TAC measurement is simple, rapid, cheaper, and accurate (28).

Increase in levels of ROS without a concomitant rise in antioxidant defenses leads to oxidative stress. Oxidative stress

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is involved in the pathogenesis of male and female reproduction. Oxidative stress causes damage to the spermatozoa, oocyte, and embryos. Several reports relate low seminal plasma TAC levels to male infertility, as well as in embryo culture media from the oocytes, cumulus cell mass, and spermatozoa used for insemination in conventional IVF. The potential cellular sources of TAC in an intracytoplasmic sperm injection setting are the spermatozoa and the injected oocytes (8, 22, 23, 29–36). However, a definite cutoff or reference range in infertile patients or proven fertile subjects for a possible use as a diagnostic tool for infertility identification is lacking.

The objectives of our study were to [1] identify a cutoff value for seminal plasma TAC level that can differentiate infertile patients from fertile donors, [2] establish the sensitivity and specificity of the test, and [3] examine the intra- and interobserver variability of the assay to establish the variability between various individuals who may perform the assay in a clinical laboratory setting.

MATERIALS AND METHODS

Subject Selection

This study was approved by the institutional review board of our hospital. Infertile patients ($n = 42$) as well as normal healthy men ($n = 100$) were screened and selected on the basis of normal semen analysis according to the World Health Organization (WHO) (37) guidelines.

Semen Collection and Preparation

Semen specimens were collected by masturbation after 48 to 72 hours of sexual abstinence. The specimens underwent complete liquefaction at 37°C for 20 minutes, and 5 μL of each specimen was loaded on a 20- μL Microcell chamber (Conception Technologies, San Diego, CA) where it was analyzed for sperm concentration and motility. Samples were classified according to the results of semen analysis as per the WHO (1999) (37) criteria and history of establishing a pregnancy in the past 2 years.

Donors or controls ($n = 55$) were further classified as proven fertile, that is, who had initiated a pregnancy within the past 2 years and had normal semen analysis results according to WHO criteria. Unproven fertile ($n = 45$) men were those who had normal semen analysis results but had not established a pregnancy in the past 2 years. The infertile patient group ($n = 42$) consisted of men who had abnormal semen analysis and were presenting for infertility treatment and/or undergoing investigation for various male infertility factors. All samples were centrifuged at $1000 \times g$ for 10 minutes at 4°C. Clear seminal plasma was aliquoted and frozen at -70°C until the time of TAC assay. A total of 142 TAC measures were conducted: 42 for patients, 55 for fertile donors and 45 for donors with unknown fertility status.

Total Antioxidant Assay

Seminal plasma total antioxidant measurement was done using the antioxidant assay kit (Cat #709001; Cayman Chemi-

cal, Ann Arbor, MI). The Cayman Chemical antioxidant assay was used to measure the antioxidant capacity of the seminal fluid samples. The principal of the assay is the ability of aqueous and lipid antioxidants in the seminal plasma specimens to inhibit the oxidation of the 2,2'-Azino-di-[3-ethylbenzthiazoline sulphonate] (ABTS) to ABTS^+ . Under the reaction conditions used, the antioxidants in the seminal plasma cause suppression of the absorbance at 750 nm to a degree that is proportional to their concentration. The capacity of the antioxidants present in the sample to prevent ABTS oxidation was compared with that of standard Trolox, a water-soluble tocopherol analogue. Results are reported as micromoles of Trolox equivalent. This assay measures the combined antioxidant activities of all its constituents including vitamins, proteins, lipids, glutathione, uric acid, and so forth.

The technique for TAC assay used in our study has been described before (38, 39). All seminal plasma samples were diluted 1:10 with the assay buffer before assaying to avoid variability because of interference by the plasma proteins or sample dilution. All reagents and samples were equilibrated to room temperature before beginning the assay. Samples as well as Trolox standards were assayed in duplicate. Trolox standards and reagent were prepared as per the manufacturer's instructions at the time of the assay. After the plate configuration, 10 μL of Trolox standard and samples were loaded on to the corresponding wells of a 96-well plate. Then 10 μL of metmyoglobin and 150 μL of chromogen were added to all standard/sample wells. The reaction was initiated by adding 40 μL of hydrogen peroxide as quickly as possible. The plate was covered and incubated for 5 minutes on a shaker at room temperature. Absorbance was monitored at 750 nm using ELx800 Absorbance Microplate Reader (BioTek Instruments, Inc., Winooski, VT).

Calculation of Assay Result

Determination of the reaction rate was done by calculating the average absorbance of each standard and sample. The average absorbance of the standards as a function of the final Trolox concentration (μM) was plotted for the standards curve in each run, from which the unknown samples were determined (Fig. 1). The total antioxidant concentration of each sample was calculated using the equation obtained from the linear regression of the standard curve by substituting the average absorbance values for each sample into the equation:

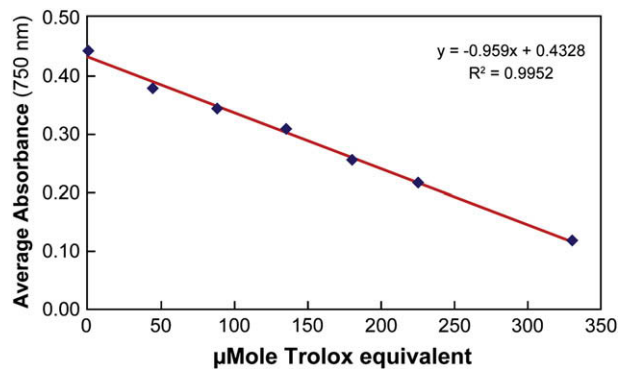
$$\begin{aligned} &\text{Antioxidant } (\mu\text{M}) \\ &= \frac{\text{Unknown average absorbance} - \text{Y-intercept}}{\text{Slope}} \\ &\quad \times \text{dilution} \times 1000 \end{aligned}$$

Precision and Sensitivity of the TAC Assay Kit

According to the manufacturer, the assay precision was: interassay coefficient of variation 3% ($n = 20$) and intraassay coefficient of variation 3.4% ($n = 84$). The assay kit could

FIGURE 1

Example of a standard curve for TAC measurement. Standard Trolox concentrations are represented on the x-axis and the absorbance at the y-axis.



Mahfouz. Seminal plasma TAC reference values. *Fertil Steril* 2009.

measure samples containing antioxidants ranging between 44 and 330 μM without any further dilution.

Interobserver and Intraobserver Variation

For each subject, one to two measurements were recorded. Two observers (R.M. and R.S.) conducted these measurements both from the same subjects as well as from different subjects.

The inter- and intraobserver variability illustrated in Figure 1 was evaluated using a total of 60 TAC measurements. Two observers evaluated eight donors and seven patients in duplicate ($n = 30$ each). The interobserver variation was obtained by analyzing the differences in the results produced by the two observers. The intraobserver variation was obtained by analyzing the differences between measurements within each observer.

Statistical Analysis

The difference in distributions of TAC levels between infertile patients and proven fertile donors was assessed using the Wilcoxon rank sum test. Summaries of the distributions include mean, standard deviation, median, and interquartile range (IQR). A receiver operating characteristic curve was used to display estimated sensitivity and specificity over a range of possible cutoff points for TAC as a predictor of fertility. A cutoff value was chosen to maximize the sum of sensitivity and specificity. A secondary analysis was performed in a similar fashion that added donors with unproven fertility to the group of donors with proven fertility for comparison to the infertile patients.

Inter- and intraobserver reliability of TAC measurement was assessed between two selected observers. This was calculated in a set consisting of both donors and patients using each observer's measurement in duplicate. For intraobserver

reliability, the absolute difference for each sample between the two TAC measurements, divided by the average of the two (denoted as "d/m"), was used as a measure of relative nearness of the measurements. The frequency in which the relative nearness was within set levels was used to describe the intraobserver reliability for each of the two observers. For interobserver reliability, the two observer's average measurements for each subject were treated as a pair. Differences (not in absolute value), divided by the averages (denoted as "d/m"), and within the pairs were computed as measures of relative nearness of the two observers' TAC measurements. Frequencies within 20% were then used to describe interobserver reliability. A one-sample *t* test was used to determine if the mean relative nearness of the two observers' TAC measurements was significantly different from zero, and a 95% confidence interval (CI) for the mean was computed. For both intra- and interobserver reliability, tests based on a Spearman correlation coefficient were used to confirm no evidence of the association between the relative nearness and the measurement magnitudes. All analyses were performed using R version 2.3.1 (www.R-project.org) (40).

RESULTS

Receiver Operator Characteristic Curve Analysis

Proven fertile donors versus infertile patients We have examined the association between TAC levels and the status of a subject, as either infertile patient or a fertile donor. We first considered only proven fertile donors who had normal semen analysis results and had fathered a child in the last 2 years. The distribution of TAC levels within the proven fertile and donors of unproven fertility is shown in Figure 2 and 3.

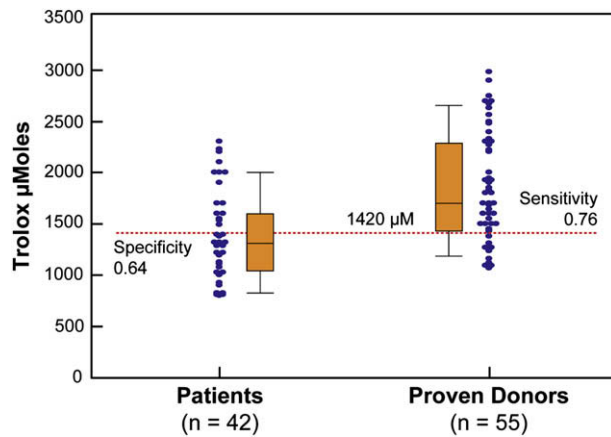
The mean \pm SD and median (IQR) values were as follows: infertile patients: $1380 \pm 430 \mu\text{M}$, and 1310 (1040–1600) μM ; proven fertile donors: $1830 \pm 540 \mu\text{M}$ and 1700 (1440–2290) μM (Fig. 2). Proven fertile donors showed significant higher seminal plasma TAC levels compared with infertile patients groups ($P < .001$). The cutoff that maximized the sum of the sensitivity and specificity when using TAC as a predictor of fertility was 1420 μM seminal plasma TAC level. The receiver operating characteristic curve showed 76% sensitivity and 64% specificity for this optimal cutoff (Fig. 4).

All donors versus infertile patients As a secondary analysis, we compared infertile patients to all donors (whether proven fertile or not). The TAC values in all donors (proven and unproven) was $1740 \pm 510 \mu\text{M}$ and 1670 (1360–2000) μM versus infertile patients: $1380 \pm 430 \mu\text{M}$, and 1310 (1040–1600) μM ; (Fig. 3). The results of this analysis were similar to those that exclude the unproven donors. Significant difference in the TAC values was seen in the two groups when a similar cutoff value of 1420 μM was selected (Fig. 5).

Intra- and interobserver variability The intra- and interobserver differences were calculated using the simple difference rather than a standard deviation. Rather than the CV, we used a ratio of a difference to mean (d/m) to represent

FIGURE 2

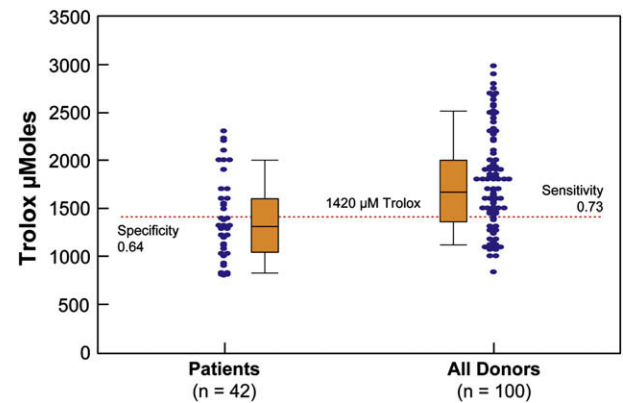
Box plot for seminal plasma TAC values in the infertile patient group versus the proven fertile donors group.



Mahfouz. Seminal plasma TAC reference values. *Fertil Steril* 2009.

FIGURE 4

Box plot for seminal plasma TAC level in the infertile patients versus all donors group.



Mahfouz. Seminal plasma TAC reference values. *Fertil Steril* 2009.

the relative difference of two TAC measurements. Next, we assessed the likelihood that such relative differences would exceed 20% value, which is unacceptable. By using this ratio; the mean intraobserver variability was 13.4% for observer 1 and 15% for observer 2. The mean interobserver variability between the two observers was 19%. All the variability measures were in the acceptable ranges (d/m ratio <20%).

DISCUSSION

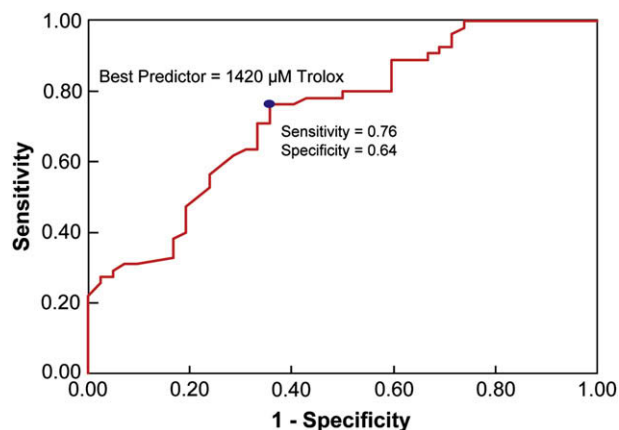
The link between reactive oxygen species and the male infertility has been established. Irrespective of the clinical mani-

festation and the results of semen analysis, the presence of abnormal ROS level plays a major role in the pathogenesis of male infertility (27, 30, 36, 41, 42).

Total seminal plasma antioxidants are the most protective defensive mechanism available to spermatozoa against ROS. Low seminal total antioxidant capacity has been shown to be related to male infertility (10, 28, 35, 36). Seminal plasma antioxidant capacity may be influenced by a wide range of the factors such as nutrition, vitamin supply, age, infection, and so forth); therefore, it is important to accurately estimate the total antioxidant amount of the seminal plasma (19, 38). From the methods available for measuring TAC, earlier we compared the more commonly used enhanced

FIGURE 3

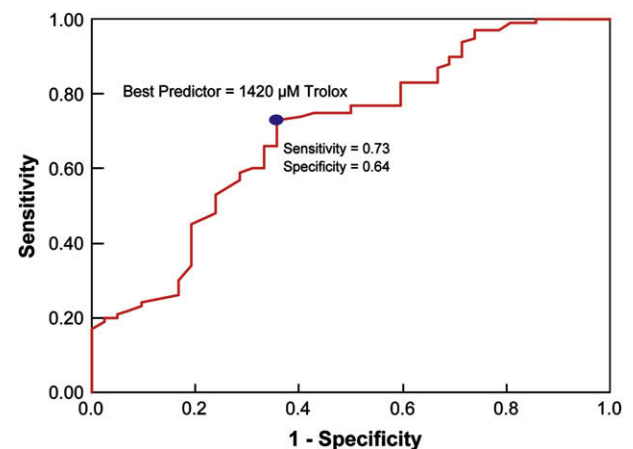
The ROC curve showing best predictor value, sensitivity, and specificity between the infertile patients versus the proven fertile donors with the cutoff threshold value.



Mahfouz. Seminal plasma TAC reference values. *Fertil Steril* 2009.

FIGURE 5

The ROC curve between the infertile patients versus the proven fertile donors along with the cutoff threshold value.



Mahfouz. Seminal plasma TAC reference values. *Fertil Steril* 2009.

chemiluminescence assay versus the colorimetric assay using for measuring TAC levels in seminal plasma.

For the colorimetric assay we used the TAC kit from Randox (Randox Laboratories Ltd, San Francisco, CA). 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 the colorimetric assay. The price of an average luminometer with kinetic setting averages \$30,000 compared with \$6,000 for a simple spectrophotometer. We reported that the colorimetric assay is a reliable, simple, rapid, and accurate assay compared with the chemiluminescence assay (28).

The main objective of our study was to examine the diagnostic value of the seminal plasma TAC assay in infertility clinic in terms of the best cutoff level, assay sensitivity, and specificity using a colorimetric assay. In addition, we examined the inter- and intraobserver variability for this assay to establish the variability between various individuals who may perform the assay in a clinical laboratory setting.

It is important that the technicians are checked off for accuracy especially in routine bench work such as accuracy in pipetting, and so forth. In our study the two observers (R.M. and R.K.S.) were very experienced in routine bench work (>5 years). It is prudent to have more researchers validate our study. The variation is near the acceptable limit. This also depends on the method of calculating the variation. Here we calculated the ratio of the absolute difference to the average value. This is different from the coefficient of variation (CV), which is calculated from the ratio of standard deviation to the average. The d/m ratio is more sensitive to the CV. By using the CV calculations, the inter- and intraobserver variability was around 5%.

In our study, infertile patient group showed lower seminal plasma TAC levels compared with the proven fertile and the overall donor group (Figs. 2 and 3). The proven fertile and all donors groups showed comparable seminal plasma TAC levels. We have established a cutoff value of 1420 μM of Trolox; all infertile men showed TAC levels below this cutoff value, whereas proven fertile men showed TAC levels higher than this cutoff value. Receiver operating characteristic curve showed high sensitivity (76%) and specificity (64%). Interobserver and intraobserver variation were within the acceptable ranges.

Siciliano et al. (21) reported similar total antioxidant status using ABTS-based colorimetric assay in asthenozoospermic ($1240 \pm 200 \mu\text{M}$) and oligoasthenozoospermic patients ($1260 \pm 210 \mu\text{M}$) with normal viscosity compared with normozoospermic healthy donors ($1210 \pm 160 \mu\text{M}$) with proven fertility (21). However significant differences were seen in the TAC levels in these patients with normal viscosity when compared with those that were hyperviscous. They concluded that impaired antioxidants in asthenozoospermic patients may be related to other associated pathologies such as hyperviscosity. Contrary to their findings, low TAC levels

were seen in our infertile patient groups, and these levels were significantly lower than in proven as well as unproven fertile donors. None of the samples in our study was hyperviscous.

Fingerova et al. (8) reported seminal plasma TAC level to be about 1.4 times higher than those in the blood serum. They measured the seminal plasma TAC levels by using Randox kit. They found significantly lower values (mean \pm SD $2.15 \pm 0.29 \text{ mM}$) in the infertile patients compared with the control $2.32 \pm 0.33 \text{ mM}$; $P < .02$). When the range and median were compared there appeared to be an overlap between the patients and controls (patients: median and range: 2.11 (1.63–2.64) mM versus control: 2.48 (1.45–2.66) mM). Our results agree with their finding regarding the significantly lower TAC values in patients compared with the controls. However, the difference in our cutoff value between patients and controls is more distinct compared with the values reported in the study by Fingerova et al. (8). This can be attributed to the fact that in their study the patient and control semen analysis results were more or less within normal ranges according to WHO references (37).

Lewis et al. (23) compared the individual antioxidants in serum and in seminal plasma. They reported that in seminal plasma ascorbate, urates, and thiols are the major antioxidant available. However; Donnely et al. (7) reported that single or combined supplementation of ascorbate and tocopherol is not beneficial for sperm motility improvement. Their findings suggest the importance of the TAC assay instead of measuring individual antioxidants that is complex and inaccurate. It may therefore be important to use TAC assay for monitoring and follow up for infertile patients.

Our inter- and intraobserver variability results were in agreement with our earlier work where we reported the actual average difference of 27.6 μM Trolox equivalent between observers, and interobserver variation of 3 % (95% confidence interval [CI]; -3.6% – 9.5%) (28). Benzie et al. (18) reported intraassay CV of $<1.0\%$ and interassay CV of $<3.0\%$ using the ferric reducing ability of plasma as a measure of the antioxidant power in the biologic fluid samples.

Using the enhanced chemiluminescence assay for measuring TAC levels we demonstrated the usefulness of this assay in identifying patients with various clinical diagnosis (9, 24, 25). We also defined a novel ROS-TAC score and its usefulness in differentiating the patients from control healthy men (1, 16, 25, 26, 43). Using the enhanced chemiluminescence assay we reported the TAC values in controls 1650.9 ± 532.22 (1, 16, 27) and 1653.98 ± 115.29 molar trolox equivalents (25). All infertile patients with various clinical diagnoses had significantly lower levels of TAC. However, the enhanced chemiluminescence assay probe is no longer available, and the TAC measurement derived from the luminometer are complex and time consuming. The equipment must be simple, easy to use, and cost effective. Although enhanced chemiluminescence involves the luminometer, many labs also use high-pressure liquid chromatography (HPLC) for

measuring vitamin E and C concentrations. This is again time consuming, requires specific instrumentation, and is expensive.

We therefore sought to look for an alternate, easy, and accurate method for measuring the seminal plasma antioxidant status. We demonstrated the ease of using the colorimetric assay (Randox kit) versus the chemiluminescence method for TAC measurement (28). The accuracy, sensitivity, and the specificity was comparable in the two methods. However, this kit is expensive, uses larger assay volumes, and can be used only with a spectrophotometer. Our goal was to use an easy and simple colorimetric assay where multiple samples can be evaluated in smaller volumes simultaneously, and therefore we looked for alternate assay kits. The colorimetric assay described by us is simple, easy, and requires a simple spectrophotometer, which is easily available in most labs. The assay kit used currently in our study is cost effective, requires a smaller assay and reagent volumes, and more importantly, it can be used in a plate reader for simultaneous measurement of large number of samples unlike the Randox kit. In the current study, we included patients and donor based on their semen analysis and fertility status only. In this study, our primary objective was to establish a diagnostic reference values for seminal plasma TAC levels in infertile patients as well as in healthy donors, and hence the abstinence period required was 2–3 days similar to that defined by WHO for routine semen analysis as recommended by WHO (37). It would be interesting to examine the effect of shorter abstinence period of <48 hours, as this may help decrease the amount of time the spermatozoa are subjected to oxidation while in the male reproductive tract and possibly increase the sensitivity. We recommend further studies to not only reproduce our results, but to establish reference values for different age ranges and also with certain diseases such as varicocele and infection, as well as conditions such as hyperviscosity and varying abstinence period.

In conclusion, the simple colorimetric seminal plasma TAC assay used in our study can discriminate proven fertile from infertile patients. It may be used as a diagnostic and prognostic tool in the male infertility clinic. The best cutoff value of seminal plasma TAC level is 1420 μM , with high sensitivity and specificity as seen in this study. Therefore, we can recommend seminal plasma TAC measurement as a quick in-office test for the evaluation of patients with male infertility.

REFERENCES

1. Sharma RK, Pasqualotto FF, Nelson DR, Thomas AJ Jr, Agarwal A. The reactive oxygen species-total antioxidant capacity score is a new measure of oxidative stress to predict male infertility. *Hum Reprod* 1999;14:2801–7.
2. Hull MG, Glazener CM, Kelly NJ, Conway DI, Foster PA, Hinton RA, et al. Population study of causes, treatment, and outcome of infertility. *Br Med J* 1985;291:1693–7.
3. Fleming R, McQueen D, Yates RW, Coutts JR. Spontaneous follicular and luteal function in infertile women with oligomenorrhoea: role of luteinizing hormone. *Clin Endocrinol* 1995;43:735–9.
4. Aitken RJ, Clarkson JS. Cellular basis of defective sperm function and its association with the genesis of reactive oxygen species by human spermatozoa. *J Reprod Fertil* 1987;81:459–69.
5. Agarwal A, Saleh RA. Role of oxidants in male infertility: rationale, significance, and treatment. *Urol Clin North Am* 2002;29:817–27.
6. Agarwal A, Saleh RA, Bedaiwy MA. Role of reactive oxygen species in the pathophysiology of human reproduction. *Fertil Steril* 2003;79:829–43.
7. Donnelly ET, McClure N, Lewis SE. Antioxidant supplementation in vitro does not improve human sperm motility. *Fertil Steril* 1999;72:484–95.
8. Fingerova H, Novotny J, Barborik J, Brezinova J, Svobodova M, Krskova M, et al. Antioxidant capacity of seminal plasma measured by TAS Randox(R). *Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub* 2007;151:37–40.
9. Kolettis PN, Sharma RK, Pasqualotto FF, Nelson D, Thomas AJ Jr, Agarwal A. Effect of seminal oxidative stress on fertility after vasectomy reversal. *Fertil Steril* 1999;71:249–55.
10. Aitken RJ. The Amoroso lecture. The human spermatozoon—a cell in crisis? *J Reprod Fertil* 1999;115:1–7.
11. Aitken RJ. A free radical theory of male infertility. *Reprod Fertil Dev* 1994;6:19–23.
12. Gagnon C, Iwasaki A, De Lamirande E, Kovalski N. Reactive oxygen species and human spermatozoa. *Ann N Y Acad Sci* 1991;637:436–44.
13. Iwasaki A, Gagnon C. Formation of reactive oxygen species in spermatozoa of infertile patients. *Fertil Steril* 1992;57:409–16.
14. Mortimer D, Pandya JJ, Sawers RS. Relationship between human sperm motility characteristics and sperm penetration into human cervical mucus in vitro. *J Reprod Fertil* 1986;78:93–102.
15. O'Neill DA, McVicar CM, McClure N, Maxwell P, Cooke I, Pogue KM, et al. Reduced sperm yield from testicular biopsies of vasectomized men is due to increased apoptosis. *Fertil Steril* 2007;87:834–41.
16. Saleh RA, Agarwal A. Oxidative stress and male infertility: from research bench to clinical practice. *J Androl* 2002;23:737–52.
17. Alvarez JG, Storey BT. Role of glutathione peroxidase in protecting mammalian spermatozoa from loss of motility caused by spontaneous lipid peroxidation. *Gamete Res* 1989;23:77–90.
18. Benzie IF, Strain JJ. The ferric reducing ability of plasma (FRAP) as a measure of “antioxidant power”: the FRAP assay. *Anal Biochem* 1996;239:70–6.
19. Kampa M, Nistikaki A, Tsaousis V, Maliaraki N, Notas G, Castanas E. A new automated method for the determination of the Total Antioxidant Capacity (TAC) of human plasma, based on the crocin bleaching assay. *BMC Clin Pathol* 2002;2:3.
20. Sharma RK, Agarwal A. Role of reactive oxygen species in male infertility. *Urology* 1996;48:835–50.
21. Siciliano L, Tarantino P, Longobardi F, Rago V, De Stefano C, Carpino A. Impaired seminal antioxidant capacity in human semen with hyperviscosity or oligo-asthenozoospermia. *J Androl* 2001;22:798–803.
22. Smith R, Vantman D, Ponce J, Escobar J, Lissi E. Total antioxidant capacity of human seminal plasma. *Hum Reprod* 1996;11:1655–60.
23. Lewis SE, Sterling ES, Young IS, Thompson W. Comparison of individual antioxidants of sperm and seminal plasma in fertile and infertile men. *Fertil Steril* 1997;67:142–7.
24. Hendin BN, Kolettis PN, Sharma RK, Thomas AJ Jr, Agarwal A. Varicocele is associated with elevated spermatozoal reactive oxygen species production and diminished seminal plasma antioxidant capacity. *J Urol* 1999;161:1831–4.
25. Pasqualotto FF, Sharma RK, Nelson DR, Thomas AJ, Agarwal A. Relationship between oxidative stress, semen characteristics, and clinical diagnosis in men undergoing infertility investigation. *Fertil Steril* 2000;73:459–64.
26. Pasqualotto FF, Sharma RK, Kobayashi H, Nelson DR, Thomas AJ Jr, Agarwal A. Oxidative stress in normospermic men undergoing infertility evaluation. *J Androl* 2001;22:316–22.
27. Saleh RA, Agarwal A, Nada EA, El-Tonsy MH, Sharma RK, Meyer A, et al. Negative effects of increased sperm DNA damage in relation to

- seminal oxidative stress in men with idiopathic and male factor infertility. *Fertil Steril* 2003;79:1597–605.
28. Said TM, Kattal N, Sharma RK, Sikka SC, Thomas AJ Jr, Mascha E, et al. Enhanced chemiluminescence assay vs colorimetric assay for measurement of the total antioxidant capacity of human seminal plasma. *J Androl* 2003;24:676–80.
 29. Bedaiwy M, Agarwal A, Said TM, Goldberg JM, Sharma RK, Worley S, et al. Role of total antioxidant capacity in the differential growth of human embryos in vitro. *Fertil Steril* 2006;86:304–9.
 30. Pasqualotto FF, Sundaram A, Sharma RK, Borges E Jr, Pasqualotto EB, Agarwal A. Semen quality and oxidative stress scores in fertile and infertile patients with varicocele. *Fertil Steril* 2007 [Epub ahead of print].
 31. Sies H. Strategies of antioxidant defense. *Eur J Biochem* 1993;215:213–9.
 32. Sikka SC, Rajasekaran M, Hellstrom WJ. Role of oxidative stress and antioxidants in male infertility. *J Androl* 1995;16:464–8.
 33. Thiele JJ, Friesleben HJ, Fuchs J, Ochsendorf FR. Ascorbic acid and urate in human seminal plasma: determination and interrelationships with chemiluminescence in washed semen. *Hum Reprod* 1995;10:110–5.
 34. Zini A, Garrels K, Phang D. Antioxidant activity in the semen of fertile and infertile men. *Urology* 2000;55:922–6.
 35. Agarwal A, Prabakaran S, Allamaneni SS. Relationship between oxidative stress, varicocele and infertility: a meta-analysis. *Reprod Biomed Online* 2006;12:630–3.
 36. Agarwal A, Prabakaran SA. Mechanism, measurement, and prevention of oxidative stress in male reproductive physiology. *Indian J Exp Biol* 2005;43:963–74.
 37. World Health Organization. WHO laboratory manual for the examination of human semen and sperm-cervical mucus interaction. 4th ed. Cambridge: Cambridge University Press, 1999.
 38. Miller NJ, Rice-Evans CA. Factors influencing the antioxidant activity determined by the ABTS+ radical cation assay. *Free Radic Res* 1997;26:195–9.
 39. Rice-Evans C, Miller NJ. Total antioxidant status in plasma and body fluids. *Methods Enzymol* 1994;234:279–93.
 40. R-Development-Core-Team. R: a language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing, 2006.
 41. Kobayashi H, Gil-Guzman E, Mahran AM, Rakesh, Nelson DR, Thomas AJ Jr, et al. Quality control of reactive oxygen species measurement by luminol-dependent chemiluminescence assay. *J Androl* 2001;22:568–74.
 42. Agarwal A, Saleh RA. Utility of oxidative stress test in the male infertility clinic. *Zhonghua nan ke xue (Nat J Androl)* 2002;8:1–9.
 43. Sharma RK, Pasqualotto AE, Nelson DR, Thomas AJ Jr, Agarwal A. Relationship between seminal white blood cell counts and oxidative stress in men treated at an infertility clinic. *J Androl* 2001;22:575–83.